Paul Christou
Roxana Savin
Barry A. Costa-Pierce
Ignacy Misztal
C. Bruce A. Whitelaw
Editors

Sustainable Food Production

Selected entries from the Encyclopedia of Sustainability Science and Technology

Springer Reference

Sustainable Food Production

This volume collects selected topical entries from the *Encyclopedia of Sustainability Science and Technology* (ESST). ESST addresses the grand challenges for science and engineering today. It provides unprecedented, peer-reviewed coverage of sustainability science and technology with contributions from nearly 1,000 of the world's leading scientists and engineers, who write on more than 600 separate topics in 38 sections. ESST establishes a foundation for the research, engineering, and economics supporting the many sustainability and policy evaluations being performed in institutions worldwide.

Paul Christou, Roxana Savin, Barry A. Costa-Pierce, Ignacy Misztal and
C. Bruce A. Whitelaw (Eds.)

Sustainable Food Production

With 375 Figures and 164 Tables

Springer

Editors
Paul Christou
Department de Produccio Vegetal i Ciencia Forestal
Universitat de Lleida/ICREA
Lleida, Spain
and
Institució Catalana de Recerca i Estudis Avançats (ICREA)
Barcelona, Spain

Roxana Savin
Department of Crop and Forest Sciences
University of Lleida
Lleida, Spain

Barry A. Costa-Pierce
University of New England
Marine Science Center
Biddeford, ME, USA

Ignacy Misztal
Department of Animal and Dairy Science
Breeding and Genetics
University of Georgia
Athens, GA, USA

C. Bruce A. Whitelaw
The Roslin Institute and Royal (Dick) School of Veterinary Studies
Division of Developmental Biology, University of Edinburgh
Midlothian, Scotland, UK

ISBN 978-1-4614-5796-1 ISBN 978-1-4614-5797-8 (eBook)
ISBN 978-1-4614-5849-4 (print and electronic bundle)
DOI 10.1007/978-1-4614-5797-8
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2012953402

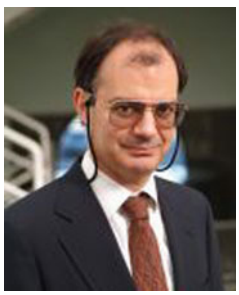
This book consists of selection from the *Encyclopedia of Sustainability Science and Technology* edited by Robert A. Meyers, published by Springer New York in 2012.

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Editors

Crop Science and Technology



Paul Christou
Department de Produccio Vegetal i Ciencia Forestal
Universitat de Lleida/ICREA
Lleida
Spain
and
Institució Catalana de Recerca i Estudis Avançats
(ICREA)
Barcelona
Spain

and

Roxana Savin
Department of Crop and Forest Sciences
University of Lleida
Lleida
Spain

Ocean Farming and Sustainable Aquaculture Science and Technology

Barry A. Costa-Pierce
University of New England
Marine Science Center
Biddeford, ME
USA

Animal Breeding and Genetics

Ignacy Misztal
Department of Animal and Dairy Science
Breeding and Genetics
University of Georgia
Athens, GA
USA

Transgenic Livestock for Food Production

C. Bruce A. Whitelaw
The Roslin Institute and Royal (Dick) School of Veterinary Studies
Division of Developmental Biology, University of Edinburgh
Midlothian, Scotland
UK

Sections

Animal Breeding and Genetics
Editor: Ignacy Misztal

Animal Breeding and Genetics, Introduction
Animal Breeding Methods and Sustainability
Animal Breeding, Foundations of
Animal Breeding, Long-Term Challenges
Animal Breeding, Modeling in
Animal Genetic in Environment Interaction
Animal Molecular Genetics from Major Genes to Genomics
Breeding in Beef Cattle
Breeding in Developing Countries and Tropics
Breeding in Horses
Dairy Cattle Breeding
Pig Breeding for Increased Sustainability
Poultry Breeding
Socially Affected Traits, Inheritance and Genetic Improvement

Crop Science and Technology
Editors: Paul Christou and Roxana Savin

Abiotic Stress Tolerant Crops: Genes, Pathways and Bottlenecks
Agroecological Basis for Managing Biotic Constraints
Agronomic Interactions with CO2 Sequestration
Biomass Crops for Biofuels and Bio-based Products
Biotechnology and Nutritional Improvement of Crops
Commercialisation of GM Crops: Comparison of Regulatory Frameworks
Crop Breeding for Sustainable Agriculture, Genomics Interventions in
Crop Development Related to Temperature and Photoperiod
Crop Diseases, Management and Control of
Crop Plants Transformation Methods
Crop Radiation Capture and Use Efficiency
Crop Responses to Available Soil Water
Crop Responses to Nitrogen
Crop Science and Technology, Introduction
Crop Traits: Gene Isolation
Crop Yields Around the World: Closing the Gap and Raising the Potential
Cropping Systems: Shaping Nature
Fertilizer Science and Technology
Genetic Engineering of Crops for Insect Resistance
Genotype by Environment Interaction and Adaptation

Ocean Farming and Sustainable Aquaculture Science and Technology
Editor: Barry A. Costa-Pierce

Transgenic Livestock for Food Production
Editor: C. Bruce A. Whitelaw

Table of Contents

List of Contributors

Ramon Albajes
Department of Plant Production and Forest Sciences
Universitat de Lleida, Centre UdL-IRTA
Lleida
Spain

Klaus Ammann
Botanical Garden
University of Bern
Bern
Switzerland

Dror L. Angel
Leon Recanati Institute for Maritime Studies
University of Haifa
Mt Carmel, Haifa
Israel

J. F. Angus
CSIRO Plant Industry
Canberra, ACT
Australia

Thorvaldur Árnason
Agricultural University of Iceland
Borgarnes
Iceland
and
International Horse Breeding Consultant
Morgongava
Sweden

Cheryl J. Ashworth
The Roslin Institute and Royal (Dick) School of Veterinary Studies
University of Edinburgh
Scotland
UK

Chao Bai
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Diego Batlla
IFEVA – Cátedra de Cerealicultura/
CONICET-Universidad de Buenos Aires
Buenos Aires
Argentina

Roberto L. Benech-Arnold
IFEVA – Cátedra de Cultivos Industriales/
CONICET-Universidad de Buenos Aires
Buenos Aires
Argentina

Peer Berg
Faculty of Agricultural Sciences
Aarhus University
Tjele
Denmark

Pete M. Berry
Sustainable Crop Management
ADAS UK Ltd
Malton, North Yorkshire
UK

Piter Bijma
Animal Breeding and Genomics Centre
Wageningen University
Wageningen
The Netherlands

Agustin Blasco
Institute of Animal Science and Technology
Universidad Politécnica de Valencia
Valencia
Spain

David Bonnett
CIMMYT Int
Mexico D.F
Mexico

Lucas Borrás
CONICET, National Council of Scientific and Technical Research
Buenos Aires
Argentina
and
Departamento de Producción Vegetal
Universidad Nacional de Rosario
Zavalla, Santa Fe
Argentina

Gisela Borràs-Gelonch
Department of Crop and Forest Sciences
University of Lleida
Lleida
Spain

Graham Brookes
Agricultural Economist, PG Economics Ltd
Dorset
UK

Bela H. Buck
Biological Sciences – Marine Aquaculture
Alfred Wegener Institute for Polar and Marine Research (AWI)
Bremerhaven
Germany
and
Marine Aquaculture for Sustainable Fisheries
Institute for Marine Resources (IMARE)
Bremerhaven
Germany
and
Maritime Technologies – Applied Marine Biology
University for Applied Sciences
Bremerhaven
Germany

Jorge Cabrera Medaglia
Centre for International Sustainable Development Law, Lead Counsel on Biodiversity and Biosafety
Montreal
Canada
and
University of Costa Rica
San Rafael de Heredia
Costa Rica

Keith H. S. Campbell
School of Biosciences
University of Nottingham
Sutton Bonington, Loughborough, Leicestershire
UK

Teresa Capell
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

P. Carvalho
Division of Plant and Crop Sciences, School of Biosciences
University of Nottingham
Loughborough
UK

Manuela M. Chaves
Plant Molecular Ecophysiology Laboratory (LEM)
Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa
Oeiras
Portugal
and
Instituto Superior de Agronomia
Universidade Técnica de Lisboa
Lisbon
Portugal

Thierry Chopin
Canadian Integrated Multi-Trophic Aquaculture Network (CIMTAN), University of New Brunswick
Saint John, NB
Canada

Paul Christou
Department de Produccio Vegetal i Ciencia Forestal
Universitat de Lleida/ICREA
Lleida
Spain
and
Institució Catalana de Recerca i Estudis Avançats (ICREA)
Barcelona
Spain

SWP Cloete
Department of Animal Sciences
University of Stellenbosch
Matieland
South Africa
and
Institute for Animal Production
Elsenburg
South Africa

Udo Conrad
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Phytoantibodies
Gatersleben
Germany

Barry A. Costa-Pierce
University of New England
Marine Science Center
Biddeford, ME
USA

Wendy Craig
Biosafety Unit
International Centre for Genetic Engineering and Biotechnology (ICGEB)
Trieste
Italy

Alan T. Critchley
Acadian Seaplants Limited
Dartmouth, NS
Canada

Stephen F. Cross
Coastal Aquaculture Research & Training (CART) Network, Department of Geography
University of Victoria
Victoria, BC
Canada
and
SEA–Vision Group of Companies
Courtenay, BC
Canada

Svetlana Dashevskaya
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Sylvie De Buck
Department of Plant Biotechnology and Genetics
Ghent University
Ghent
Belgium

Annelies De Paepe
Department of Plant Systems Biology
Flanders Institute for Biotechnology VIB
Ghent
Belgium

Xavier Delannay
Integrated Breeding Platform
Consultative Group on International Agricultural Research, Generation Challenge Programme, c/o CIMMYT
Texcoco
Edo. Mexico

Ann Depicker
UGent Vakgroep Plantenbiotechnologie en Genetica, VIB Plant Systems Biology Department
Ghent University
Ghent
Belgium

Shivakumar P. Devaiah
Arkansas Biosciences Institute
Arkansas State University
Jonesboro, AR
USA

Yann Devos
GMO Unit
European Food Safety Authority (EFSA)
Parma
Italy

F. Xavier Donadeu
The Roslin Institute and Royal (Dick) School of Veterinary Studies
University of Edinburgh
Scotland
UK

M. Fernanda Dreccer
CSIRO Plant Industry Cooper Laboratory
Gatton, QLD
Australia

Martin G. Edwards
School of Biology
Newcastle Institute for Research on Sustainability
Newcastle University
Newcastle upon Tyne
UK

Fred van Eeuwijk
Biometris
Wageningen University and Research Centre
Wageningen
The Netherlands

Yoav Eitan
Department of Genetics, The Silberman Life Sciences Institute
The Hebrew University of Jerusalem
Jerusalem
Israel

Gemma Farre
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Elías Fereres
Institute for Sustainable Agriculture
IAS-CSIC and University of Cordoba
Cordoba
Spain

Joao G. Ferreira
Departamento de Ciências e Engenharia do Ambiente
Faculdade de Ciências e Tecnologia Universidade Nova de Lisboa
Monte Caparica
Portugal

Natalie Ferry
School of Biology
Newcastle Institute for Research on Sustainability
Newcastle University
Newcastle upon Tyne
UK
and
School of Environment and Life Sciences
University of Salford
Salford
UK

Marc Fibla
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

R. A. (Tony) Fischer
CSIRO Plant Industry
Canberra City
Australia

Doreen M. Floss
Institute of Biochemistry and Molecular Biology II
Heinrich-Heine-University Düsseldorf
Düsseldorf
Germany

Cecil W. Forsberg
Department of Molecular and Cellular Biology
University of Guelph
Guelph, ON
Canada

M. J. Foulkes
Division of Plant and Crop Sciences, School of Biosciences
University of Nottingham
Loughborough
UK

Cesare Galli
Avantea, Laboratorio di Tecnologie della Riproduzione
Cremona
Italy
and
Clinical Veterinary Department
University of Bologna
Italy

Margarita García-Vila
Institute for Sustainable Agriculture
IAS-CSIC and University of Cordoba
Cordoba
Spain

Angharad M. R. Gatehouse
School of Biology
Newcastle Institute for Research on Sustainability
Newcastle University
Newcastle upon Tyne
UK

John A. Gatehouse
School of Biological and Biomedical Sciences
Durham University
Durham
UK

Sergio D. German
School of Biosciences
University of Nottingham
Sutton Bonington, Loughborough, Leicestershire
UK

Claudio M. Ghersa
IFEVA-CONICET, Depto. de Recursos Naturales y Ambiente
Universidad de Buenos Aires (UBA)
Buenos Aires
Argentina

Mickey Gjerris
Danish Centre for Bioethics and Risk Assessment
Institute of Food and Resource Economics
University of Copenhagen
Frederiksberg
Denmark

Serguei P. Golovan
Department of Animal and Food Science
University of Delaware
Newark, DE
USA

Sonia Gomez-Galera
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Jon Grant
Department of Oceanography
Dalhousie University
Halifax, NS
Canada

Jonathan Gressel
Plant Sciences Department
Weizmann Institute of Science
Rehovot
Israel

Suvi T. Häkkinen
VTT Technical Research Centre of Finland
Espoo
Finland

Anthony E. Hall
Department of Botany and Plant Sciences
University of California Riverside
Quincy, CA
USA

Eric M. Hallerman
Department of Fisheries and Wildlife Sciences
Virginia Polytechnic Institute and State University
Blacksburg, VA
USA

Patrik J. G. Henriksson
Institute of Environmental Sciences
Leiden University
Leiden
The Netherlands
and
Department of System Ecology
Stockholm University
Stockholm
Sweden

Elizabeth E. Hood
College of Agriculture and Technology
Arkansas State University, AR Biosciences Institute
Jonesboro, AR
USA

Louis-Marie Houdebine
Biologie du Développement et Reproduction
Institut National de le Recherche Agronomique
Jouy en Josas
France

John Howard
Applied Biotechnology Institute
San Luis Obispo, CA
USA

Reinhard Huber
Laboratory Animal Science Group
IBMC – Instituto de Biologia Molecular e Celular
Universidade do Porto
Porto
Portugal

Herman van Keulen
Chairgroup Plant Production Systems
Wageningen University
Wageningen
The Netherlands
and
Business Unit Agrosystems Research, Plant Research International
Wageningen University and Research Centre
Wageningen
The Netherlands

Saifullah Khan
Plant Biotechnology Section
International Center for Chemical and Biological Sciences, HEJ research Institute
University of Karachi
Karachi
Pakistan

Alexander Kind
Chair of Livestock Biotechnology
Technical University of Munich
Freising
Germany

Pieter W. Knap
PIC International Group
Schleswig
Germany

Gesche Krause
Leibniz Center for Tropical Marine Ecology (ZMT)
Bremen
Germany

Pawan L. Kulwal
State Level Biotechnology Centre
Mahatma Phule Agricultural University
Rahuri, Maharashtra
India

Tanguy Lafarge
CIRAD, UMR AGAP
Montpellier
France
and
IRRI, CESD
Los Baños
Philippines

Götz Laible
AgResearch, Ruakura Research Centre
Hamilton
New Zealand

Rattan Lal
OARDC/FAES Kottman Hall
Carbon Management and Sequestration Center
The Ohio State University
Columbus, OH
USA

Thomas Landry
Fisheries and Oceans Canada
Gulf Fisheries Centre
Moncton, NB
Canada

Richard Langan
Atlantic Mariner Aquaculture Center
University of New Hampshire
Durham, NH
USA

Jesper Lassen
Danish Centre for Bioethics and Risk Assessment
Institute of Food and Resource Economics
University of Copenhagen
Frederiksberg
Denmark

Caroline Lassnig
Institute of Animal Breeding and Genetics &
Biomodels Austria
University of Veterinary Medicine Vienna
Vienna
Austria

Giovanna Lazzari
Avantea, Laboratorio di Tecnologie della
Riproduzione
Cremona
Italy

Kenneth M. Leber
Center for Fisheries Enhancement
Mote Marine Laboratory
Sarasota, FL
USA

Gilles Lemaire
INRA
Lusignan
France

Sarah Loker
Department of Animal and Poultry Science
University of Guelph
Guelph, ON
Canada

Luis López-Bellido
Departamento de Ciencias y Recursos Agrícolas y
Forestales
University of Córdoba
Córdoba
Spain

Rafael J. López-Bellido
Departamento de Ciencias y Recursos Agrícolas y
Forestales
University of Córdoba
Córdoba
Spain

Franco Lucchini
Centro Ricerche Biotecnologiche
Università Cattolica del Sacro Cuore
Cremona
Italy

Asko Mäki-Tanila
Biotechnology and Food Research
MTT Agrifood Research Finland
Jokioinen
Finland

Fuchs Marc
Department of Plant Pathology and Plant-Microbe Biology
Cornell University, New York State Agricultural Experiment Station
Geneva, NY
USA

Michael J. McGrew
Avian Germ Cell Laboratory, Division of Developmental Biology
The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh
Roslin, Midlothian
UK

Christopher W. McKindsey
Ocean and Environmental Sciences Division
Fisheries and Oceans Canada
Institut Maurice-Lamontagne Fisheries and Oceans Canada
Mont-Joli, Québec
Canada

Graham McLaren
Integrated Breeding Platform
Consultative Group on International Agricultural Research, Generation Challenge Programme, c/o CIMMYT
Texcoco
Edo. Mexico

Gregory S. McMaster
USDA-ARS, Agricultural Systems Research Unit
Fort Collins, CO
USA

Michael Metzlaff
Bayer BioScience N.V
Ghent
Belgium

Filippo Miglior
Guelph Food Research Centre, Agriculture and Agri–Food Canada
ON
Canada
and
Canadian Dairy Network
Guelph, ON
Canada

Ignacy Misztal
Department of Animal and Dairy Science
Breeding and Genetics
University of Georgia
Athens, GA
USA

Marc Moragues
Department of Soil and Crop Sciences
Colorado State University
Fort Collins, CO
USA

Mathias Müller
Institute of Animal Breeding and Genetics & Biomodels Austria
University of Veterinary Medicine Vienna
Vienna
Austria

Erik Murchie
Division of Plant and Crop Sciences, School of Biosciences
University of Nottingham
Sutton Bonington
UK

Shaista Naqvi
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Martina Newell-McGloughlin
Department of Plant Pathology
University of California Davis
Davis, CA
USA

Francis X. O'Beirn
Marine Institute
Galway
Ireland

Frederick Okono
Integrated Breeding Platform
Consultative Group on International Agricultural Research, Generation Challenge Programme, c/o CIMMYT
Texcoco
Edo. Mexico

K.-M. Oksman-Caldentey
VTT Technical Research Centre of Finland
Espoo
Finland

I Anna S. Olsson
Laboratory Animal Science Group
IBMC – Instituto de Biologia Molecular e Celular
Universidade do Porto
Porto
Portugal

Micheal D. K. Owen
Department of Agronomy
Iowa State University
Ames, IA
USA

Glenn G. Page
SustainaMetrix, LLC
Johns Hopkins University
Baltimore, MD
USA

Stephen H. Page
Ocean Farm Technologies, Inc.
Morrill, ME
USA

Tennant Paula
Department of Life Sciences
University of the West Indies
Mona, Kingston, WI
Jamaica

Nathan L. Pelletier
Dalhousie University
Halifax, Nova Scotia
Canada

Andy Pereira
University of Arkansas
Fayetteville, AR
USA

Rui Pereira
CIMAR/CIIMAR – Centre for Marine and Environmental Research
University of Porto
Porto
Portugal

Andrea Perota
Avantea, Laboratorio di Tecnologie della Riproduzione
Cremona
Italy

Vidya Rajan
Applied Biotechnology Institute
San Luis Obispo, CA
USA

Deborah Vicuna Requesens
Arkansas Biosciences Institute
Arkansas State University
Jonesboro, AR
USA

Matthew Reynolds
International Maize and Wheat Improvement Centre (CIMMYT)
Mexico DF
Mexico

Jean-Marcel Ribaut
Integrated Breeding Platform
Consultative Group on International Agricultural Research, Generation Challenge Programme, c/o CIMMYT
Texcoco
Edo. Mexico

Heiko Rischer
VTT Technical Research Centre of Finland
Espoo
Finland

Anneli Ritala
VTT Technical Research Centre of Finland
Espoo
Finland

M. Verónica Rodriguez
IFEVA – Cátedra de Fisiología Vegetal/
CONICET-Universidad de Buenos Aires
Buenos Aires
Argentina

Eduard Rojas
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Ignacio Romagosa
Department of Crop and Forest Sciences
University of Lleida
Lleida
Spain

Déborah P. Rondanini
Department of Crop Production
University of Buenos Aires
Buenos Aires
Argentina
and
CONICET, National Council of Scientific and Technical Research
Buenos Aires
Argentina

Guilherme J. M. Rosa
Departments of Animal Sciences, Biostatistics & Medical Informatics, Dairy Science
University of Wisconsin
Madison, WI
USA

Stuart J. Roy
Australian Centre for Plant Functional Genomics
University of Adelaide
Adelaide, SA
Australia

Georgina Sanahuja
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Peter Sandøe
Danish Centre for Bioethics and Risk Assessment
Institute of Food and Resource Economics
University of Copenhagen
Frederiksberg
Denmark

Emilio H. Satorre
Cátedra de Cerealicultura
University of Buenos Aires
Buenos Aires
Argentina
and
CONICET (National Research Council)
University of Buenos Aires
Buenos Aires
Argentina
and
AACREA
Buenos Aires
Argentina

Roxana Savin
Department of Crop and Forest Sciences
University of Lleida
Lleida
Spain

Lawrence R. Schaeffer
Department of Animal and Poultry Science
Centre for Genetic Improvement of Livestock
University of Guelph
Guelph, ON
Canada

Joachim Schiemann
Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Biosafety of Genetically Modified Plants
Quedlinburg
Germany

Andreas Schiermeyer
Department of Plant Biotechnology
Fraunhofer IME
Aachen
Germany

Stefan Schillberg
Department of Plant Biotechnology
Fraunhofer IME
Aachen
Germany

Angelika Schnieke
Chair of Livestock Biotechnology
Technical University of Munich
Freising
Germany

Roger D. Shanks
Department of Animal Sciences
University of Illinois
Urbana, IL
USA

Muki Shpigel
Israel Oceanographic and Limnological Research
National Center for Mariculture
Eilat
Israel

Neil Anthony Sims
Kona Blue Water Farms, Inc
Kailua-Kona, HI
USA

Gustavo Slafer
Department of Crop and Forest Sciences
University of Lleida
Lleida
Spain

Morris Soller
Department of Genetics, The Silberman Life Sciences Institute
The Hebrew University of Jerusalem
Jerusalem
Israel

Matthew L. Spangler
Department of Animal Science
University of Nebraska
Lincoln, NE
USA

Robert R. Stickney
Texas Sea Grant College Program
Texas A&M University
College Station, TX
USA

Erling Strandberg
Department of Animal Breeding and Genetics
Swedish University of Agricultural Sciences
Uppsala
Sweden

Nick G. H. Taylor
Cefas Weymouth Laboratory
Weymouth, Dorset
UK

Keat (Thomas) Teoh
Arkansas Biosciences Institute
Arkansas State University
Jonesboro, AR
USA

Mark Tester
Australian Centre for Plant Functional Genomics
University of Adelaide
Adelaide, SA
Australia

Mahendar Thudi
Centre of Excellence in Genomics (CEG)
International Crops Research Institute for the
Semi-Arid Tropics (ICRISAT)
Patancheru, Hyderabad, AP
India

Kurniawan Rudi Trijatmiko
International Rice Research Institute
Manila
Philippines

Max Troell
The Royal Swedish Academy of Sciences
The Beijer Institute of Ecological Economics
Stockholm
Sweden
and
Stockholm Resilience Centre
Stockholm University
Stockholm
Sweden

Roberto Tuberosa
Department of Agroenvironmental Science and
Technology
University of Bologna
Bologna
Italy

Laura Tutusaus Codony
Department of Vegetal Production and Forestry
Science, ETSEA
University of Lleida
Lleida
Spain

Richard M. Twyman
Department of Biological Sciences
University of Warwick
Coventry
UK

Peter H. Tyedmers
Dalhousie University
Halifax, Nova Scotia
Canada

Korneel Vandenbroucke
Bayer BioScience N.V
Ghent
Belgium

Siva Reddy Vanga
ICGEB, Plant Biology: Plant Transformation Group
New Delhi
India

Rajeev K. Varshney
Centre of Excellence in Genomics (CEG)
International Crops Research Institute for the
Semi–Arid Tropics (ICRISAT)
Patancheru, Hyderabad, AP
India
and
Genomics Towards Gene Discovery Sub programme
Generation Challenge Programme (GCP)
Generation Challenge Programme (GCP) c/o CIMMYT
Mexico, DF
Mexico

and
School of Plant Biology, Faculty of Natural and Agricultural Sciences
The University of Western Australia
Crawley, WA
Australia

David W. Verner-Jeffreys
Cefas Weymouth Laboratory
Weymouth, Dorset
UK

Dale R. Walters
Crop & Soil Systems Research Group
Scottish Agricultural College
Edinburgh
UK

C. Bruce A. Whitelaw
The Roslin Institute and Royal (Dick) School of Veterinary Studies, Division of Developmental Biology
University of Edinburgh
Scotland
UK

Charles Yarish
Departments of Ecology & Evolutionary Biology and Marine Sciences
University of Connecticut
Stamford, CT
USA

Dawei Yuan
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

O. Zarrouk
Plant Molecular Ecophysiology Laboratory (LEM)
Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa
Oeiras
Portugal

Changfu Zhu
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Uxue Zorrilla
Department of Vegetal Production and Forestry Science, ETSEA
University of Lleida
Lleida
Spain

Abiotic Stress Tolerant Crops: Genes, Pathways and Bottlenecks

Korneel Vandenbroucke, Michael Metzlaff
Bayer BioScience N.V, Ghent, Belgium

Article Outline

Glossary

Abiotic stress Any negative impact on a living organism caused by a nonliving factor in the environment in which the organism is present.

Biological modeling *In silico* description of a biological process to generate predictions for experimental validation.

Conventional breeding Development of new plant varieties by selection after natural reproduction.

Epigenetics The study of inherited changes in phenotype or gene expression caused by mechanisms other than changes in the DNA sequence.

Genetic engineering Development of a new plant variety through genetic modification by using recombinant DNA technology.

Phenotype Observable trait or characteristic (i.e., appearance) of an organism in a specific environment.

Phenome Collection of phenotypes of an organism in all possible environments.

Phenotyping Process of studying the phenotype.

Plant productivity Ability of a plant to produce a certain amount of biomass, either as green tissue and/or as seeds (yield).

Quantitative trait locus (QTL) Stretches of DNA that are closely linked to the genes that underlie the trait in question.

Regulon biotechnology Genetic engineering by targeting genes encoding proteins with regulatory function, often transcription factors.

Trait Characteristic of an object.

Definition of the Subject

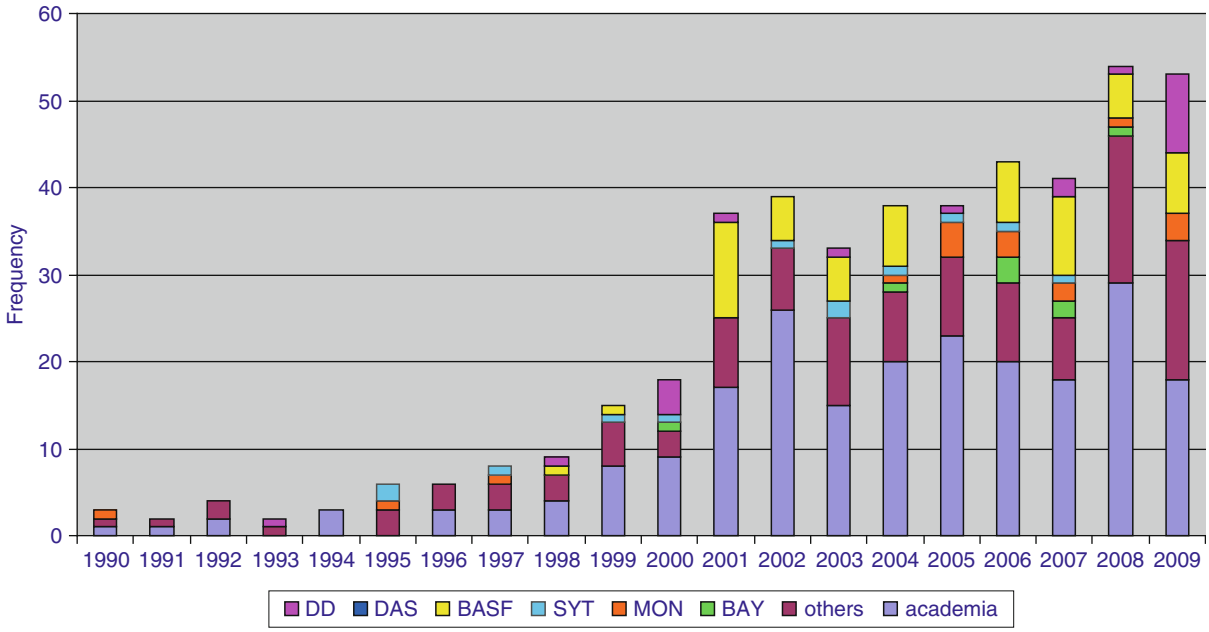
World food and feed security is increasingly dependent on continuous crop improvement and, in particular, the development of crops with increased resistance to abiotic stresses. This economical and social challenge has attracted the global community of plant breeders and scientists and many potential solutions have been put forward. Our understanding of the response of plants to abiotic stress has significantly improved over the last year. However, abiotic stress tolerance is a complex trait that can be affected by many external factors. Abiotic stress tolerance involves many processes that are not yet completely understood and several limitations still need to be overcome. Recent advances in many areas of plant research, including phenotyping, make scientists optimistic that valuable solutions will be found to allow deployment/commercialization of plants better able to tolerate abiotic stresses.

Introduction

A growing world population with increased living standards combined with the urgent need for a more sustainable agriculture demands the development of crop varieties which are able to cope with fluctuating and adverse environmental conditions limiting plant growth and productivity, referred to as abiotic stresses [1]. Further global warming is expected to aggravate the negative impact of abiotic stresses. Low water availability (drought stress), high salinity (salt stress), and high temperatures (heat stress) are considered to be among the most threatening abiotic stresses, which can compromise up to 80% of the attainable yield [2]. New technologies for plant improvement that aim to overcome the negative impact of these stresses therefore need to be urgently identified and implemented. Abiotic stresses and their effects on plant productivity have attracted academic scientists as well as large and small Ag biotech companies. This is reflected by increasingly more publications in peer-reviewed journals (including recent issues in plant physiology

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Abiotic Stress Tolerant Crops: Genes, Pathways and Bottlenecks. Figure 1
Abiotic stress-related publications of international patent applications by academia and agbiotech companies (provided by Rolf Deblaere). *BAY* Bayer, *MON* Monsanto, *SYT* Syngenta, *DD* Dupont, *DAS* Dow AgroSciences

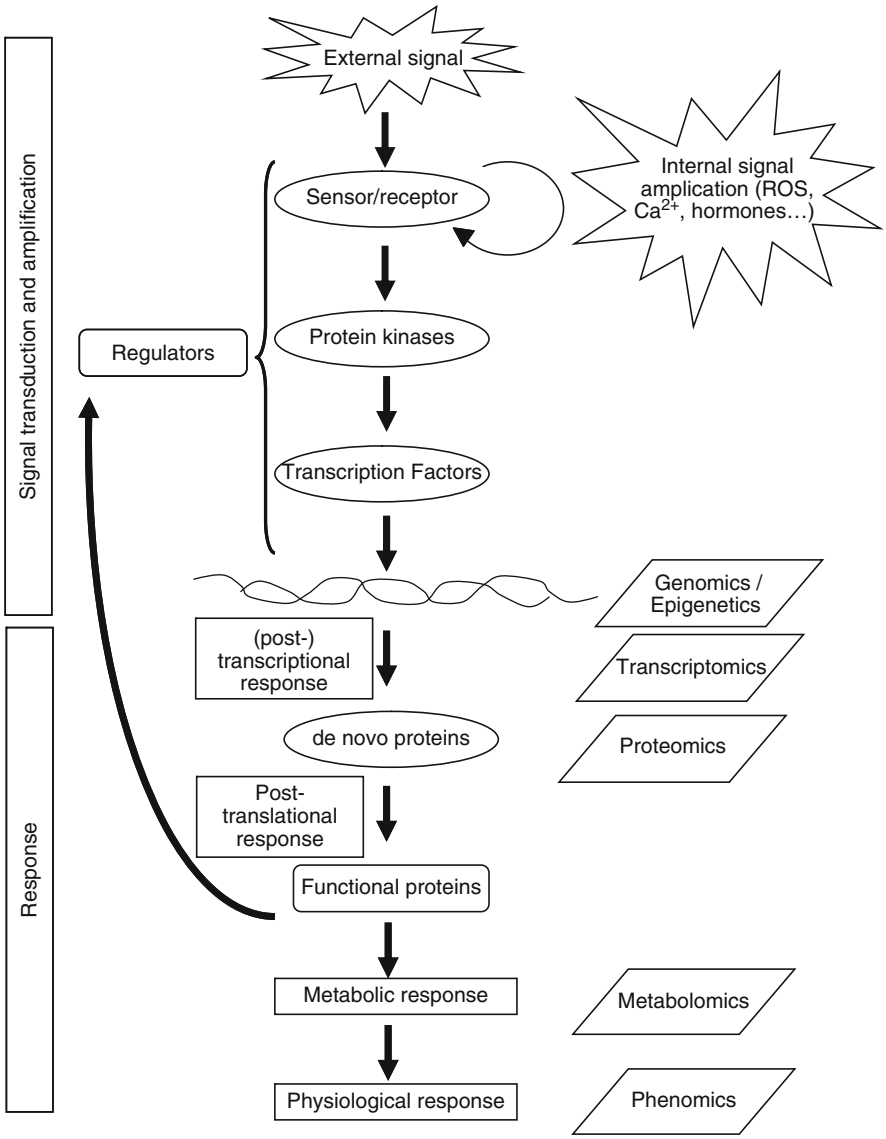
and functional plant biology dedicated to abiotic stress), online centralization of relevant information (www.plantstress.com; www.yieldbooster.org) and numerous patent applications protecting potential new solutions (Fig. 1).

Plants respond to abiotic stresses at different levels in an avoidance-tolerance mode, which includes physiological and molecular changes (Fig. 2). An important interest of plant biologists aims not only to better understand the adaptive response of plants to abiotic stress, but also to exploit this knowledge for the production of varieties that are better protected against the detrimental effects. Before the genomics era, classical or conventional breeding of plants with improved physiological characteristics was the only way to improve crop productivity. In the last 20 years, mapping of quantitative trait loci (QTL), genetic engineering, and their implementation in marker-assistant (molecular) breeding have become increasingly important [3–5]. Such genomics-based approaches rely on the identification of genes (gene discovery) that may be valuable candidates for crop improvement and were empowered by the advent of state-of-the-art molecular tools, such as DNA sequencing and expression profiling [6].

In this entry, some of the recent genomics-based advances in engineering stress-tolerant crops are summarized, existing limitations associated with these approaches are described, and some emerging trends facilitating better evaluation of crop performance in the greenhouse and in the field are outlined.

Abiotic Stress-Tolerant Crops

Plants protect themselves from the detrimental effects of abiotic stresses by increasing the expression of defense or stress tolerance genes. Plant varieties that evolved to have constitutive or high(er) expressed levels of such genes are better adapted to abiotic stress conditions. Genes with potential value for genetic engineering of stress tolerance have been ever more discovered either indirectly by genetic dissection of identified QTLs in stress-tolerant varieties, or directly through their changed expression in plants exposed to stress. Introduction of selected genes seems to be the favored way to improve plants in the future [1]. Plant



Abiotic Stress Tolerant Crops: Genes, Pathways and Bottlenecks. Figure 2
Signal transduction and response of plants to abiotic stress with the corresponding evaluation platforms

biotechnologists have been reporting genetically engineered plants with increased stress tolerance for almost 2 decades. Many genes have already been directly employed to improve stress tolerance of higher plants using a wide variety of approaches [7].

One attractive approach for engineering stress tolerance in crops has been the constitutive overexpression of stress genes from bacteria, yeast, or model plant species, such as *Arabidopsis thaliana (Arabidopsis)*. Such a heterologous gene approach worked particularly well when the genes encode proteins with single biological functions that are often absent or only expressed at low levels in the target plant. Such genes are often referred to as functional genes and can encode molecular protectants, detoxifying proteins, or ion transporters. A second group is comprised of regulatory proteins with often multiple biological functions, including enzymes involved in (phospho)lipid signaling, protein kinases, protein phosphatases, calcium/calmodulin-binding proteins,

and various transcription factors (TFs) [8]. With the emergence of large-scale genome sequencing in crops, several crop orthologues of stress genes could be identified and increasingly be used to engineer stress tolerance in crops. Here some of the approaches for engineering stress tolerance using genes involved in different cellular processes or pathways are summarized, including detoxification, protein stabilization, osmoregulation, transport, lipid metabolism, transcription and signaling, and posttranscriptional, and (post-) translational regulation. However, genetic engineering for stress tolerance in crops is not limited to these gene classes as recently shown for mustard annexin Bj1, a gene encoding a calcium-dependent phospholipid and cytoskeleton binding protein that is involved in golgi-mediated secretion, which resulted in stress tolerance when expressed in cotton [9].

Detoxifying Genes

Most, if not all, abiotic stresses induce the accumulation of reactive oxygen species (ROS), which in turn cause oxidative stress [10, 11]. ROS are extremely reactive, and therefore can undergo uncontrollable and damaging reactions with cellular components, including DNA, lipids, and proteins. This can aggravate the detrimental effects of the initial stress and can even lead to cell death [12, 13]. To protect against oxidative stress, plant cells possess an extensive ROS scavenging network, which involves nonenzymatic antioxidants, including vitamin C, vitamin E, glutathione, carotenoids, and flavonoids, as well as numerous enzymatic mechanisms such as multiple superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione peroxidases (GPX), glutathione-S-transferases (GST), alternative oxidases, and peroxiredoxines [12]. It was hypothesized that alleviation of oxidative damage by the use of ROS scavengers would enhance plant resistance. This was confirmed by a number of transgene transfers using this detoxification strategy. Stress tolerance could be improved by either direct scavenging of ROS or by enhanced removal of oxidative damaged and hazardous components accumulating in the cell. Since the accumulation of ROS and derivatives thereof is a common theme during most, if not all, abiotic stresses, the detoxification strategy enabled the generation of transgenic plants with simultaneous tolerance to multiple stresses [14–22].

In crops, enhanced stress tolerance was achieved by increasing the level of typical scavenging enzymes such as GST, different SOD isoforms, APX, and CAT [23–28]. Overexpression of a GST enzyme in rice resulted in increased protection against salt, low temperature, and oxidative stress [23]. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2). Transgenic rice plants expressing a manganese SOD from pea were more tolerant to drought stress [26]. Similarly, transgenic oilseed plants ectopically expressing a wheat manganese SOD were tolerant to oxidative and heavy metal stress [15]. A copper/zinc SOD was shown to be effective to protect rice plants against drought, salt, and oxidative stress [28]. Excess H_2O_2, produced either as by-product of superoxide detoxification by SOD or directly by metabolic processes, can be removed by APX, which catalyzes the reduction of H_2O_2 to water. Cotton plants with high levels of an APX isolated from pea were tolerant to low temperature stress [24]. Because of their complementary functions (i.e., removal of superoxide and H_2O_2), also the combination of SOD and APX resulted in enhanced stress tolerance [27]. As APX, also CAT protects plants from toxic H_2O_2 molecules. Hence, expression of a wheat CAT enzyme in rice protected these plants against low temperature stress by reducing the levels of H_2O_2 [25].

Engineering tolerance to oxidative stress is not limited to the use of the traditional ROS detoxifying enzymes. Significant improvement of stress tolerance in tobacco plants was achieved by overexpressing a stress-responsive aldehyde dehydrogenase gene from maize [29]. Recently, it was also shown that ectopic expression of the cotton stress-responsive MT3a gene in tobacco increased tolerance to salt, drought, and low temperature [30]. MT3a belongs to the metallothionein family that has numerous cellular functions including the regulation of metal homeostasis and oxidative stress. Another gene that can protect plants from abiotic stress-induced ROS is the mitochondrial alternative oxidase (AOX), the terminal oxidase in the alternative respiratory pathway of plants [31]. For example, tobacco AOX1a is necessary

for survival against oxidative stress when the cytochrome pathway is dysfunctional [32].

Protein Stabilization by Molecular Chaperones

One major detrimental effect of abiotic stresses is that they usually cause protein dysfunction through denaturation and aggregation of nonfunctional or aberrant proteins. Maintaining proteins in their functional conformation is therefore important for cell survival under stress. This can be accomplished, for example, through transcriptional induction of genes encoding heat shock proteins (HSPs, [33]). HSPs control the correct folding and conformation of both structural (e.g., cell membrane) and functional (e.g., enzymes) proteins. This important function has prompted researches to create transgenic lines with increased HSP levels. Studies on HSP proteins in plants have mostly focused on heat stress [34–42]. Although most of the evidence is limited to *Arabidopsis*, the protective capacities of HSP proteins and their potential economical value for crop engineering for heat stress tolerance was proven in tomato [42]. Recently, it was demonstrated that constitutive expression of a cotton HSP, GHSP26, enhanced drought tolerance in transgenic cotton plants [43].

In addition to HSPs, also LEA-type proteins can confer molecular protection of cellular components during abiotic stress [44]. LEA-type proteins are encoded by RD (responsive to dehydration), ERD (early responsive to dehydration), KIN (cold inducible), COR (cold regulated), and RAB (responsive to abscisic acid) genes in different plant species [45, 46]. As HSPs are typically induced by high temperatures, LEA proteins accumulate in response to dehydration (drought, osmotic, and/or cold stress). The actual functions of these proteins remain however largely unknown. Their hydrophilic nature suggest that LEA proteins act as water-binding proteins, but additional functions, including ion sequestration and protein and membrane stability, have also been proposed [47, 48]. Few examples for the use of LEA proteins to engineer stress tolerance in crop exist. Increasing the levels of endogenous LEA3 through genetic engineering of rice made these plants more tolerant to drought stress [47]. Ectopic expression of barley HVA1 in oat resulted in enhanced salt and osmotic stress tolerance [49].

Osmoregulation and Protection by Genes Involved in Metabolite Biosynthesis

Plants respond to drought stress by producing organic compounds to avoid water loss from cells (dehydration) and damage to essential components (osmotic stress). Therefore, one of the earliest approaches for genetic engineering of stress tolerance in plants (reports dating from the early 1990s) consists in enhanced synthesis of such metabolites, called osmoprotectants [50, 51]. Osmoprotectants include sugars and sugar alcohols (e.g., mannitol, trehalose, and galactinol), amines (e.g., polyamines and glycine betaine), and amino acids (e.g., proline) [52, 53]. These molecules normally do not interfere with cellular functions and are therefore often referred to as compatible solutes. Many plant species lack the ability to synthesize the special osmoprotectants that naturally accumulate in stress-tolerant species. Therefore, several transgenic approaches to increase the synthesis of osmoprotectants used bacterial biosynthetic genes, such as CodA and BetA (glycine betaine), MtlD (mannitol), and genes from the ectoine or trehalose biosynthesis operon [54–59]. Alternatively, key biosynthetic genes, including betaine aldehyde dehydrogenase and choline monooxygenase (glycine betaine biosynthesis), and pyrroline carboxylate synthase (proline synthesis), were isolated from specific plant species, such as *Vigna aconitifolia* and *Spinacia oleracea* and used to induce drought and salt tolerance in wheat and rice respectively [60, 61]. Although the accumulation of compatible solutes during stress is mainly important for osmoregulation and for maintaining correct protein structures, this may also be important for reducing or preventing the damaging effects of ROS [62, 63].

Transport Proteins

Ion transport proteins are involved in reestablishing ionic homeostasis after salt stress either by increasing ion storage in the vacuole, or by improving ion excretion from the cells [64]. Different types of ion transporters, depending on their localization and selectivity, have been the target of genetic engineering. These include both vacuolar and membrane Na^+/H^+ antiporters, vacuolar Ca^{2+}/H^+ antiporter, and Mg^{2+}, Na^+/K^+, and Ca^{2+} transporters [43, 64]. Known salt stress tolerance genes encoding ion transporters

isolated from crops include NHX1 (rice), KAT1 (rice), and HKT1 (wheat) [65–67]. Plasma membrane cation/proton antiporters cause alkalinization of the apoplast, thereby changing the activity and conformation of membrane proteins, which might serve as a signal to mediate gene regulation and induce a general stress response [68]. Besides using ion transporters to induce tolerance to salt stress, tolerance to osmotic stresses was engineered by increasing the levels of proteins involved in water transport [69]. Tolerance to heavy metal stress was achieved by constitutive expression of heavy metal transport proteins, including wheat ALMT1 [70–72].

Lipid Metabolism

Adaptation of living cells to low temperatures involves alterations in the membrane lipid composition, for example, by decreasing membrane fluidity through fatty acid unsaturation. It was demonstrated that increasing the number of unsaturated fatty acids by genetic engineering could improve stress tolerance in plants [73–77]. Overexpression of a spinach glycerol-3-phosphate acyltransferase (GPAT) in rice increased tolerance to low temperature whereas downregulation of a fatty acid desaturase in rice increased tolerance to high temperatures [73, 78]. Upon overexpression, tomato GPAT also increased the tolerance of tomato plants to low temperature stress [76].

Stress Sensing and Signal Transduction

The first and probably most important step in the response of plants to abiotic stress is the sensing or detection of the external stimuli by receptors, typically located in the cellular membranes. The identification of important stress receptors has been a difficult challenge and until recently, there was no report on their use in engineering stress tolerance. Overexpression of *Arabidopsis* membrane-bound receptor-like protein kinase 1 and a membrane located calcium/calmodulin-binding receptor-like kinase from soybean in transgenic *Arabidopsis* resulted in enhanced plant tolerance to drought and salt stress, respectively [79, 80]. In rice, overexpression of SIK1 resulted in higher tolerance to salt and drought stresses [81]. Other receptors operate in the cytosol and react to internal stimuli that amplify abiotic stress signals, such as calcium and ROS, as well as various hormones and other small molecules. The best-studied stress hormone is abscisic acid (ABA), which is involved in stomatal closure and plays a crucial role in tolerance against drought stress by preventing transpiration and water loss from stomata [82]. ABA also has an essential role in activating signal transduction pathways involved in tolerance to drought, cold, and salt stress. Recently, an important ABA receptor protein family has been identified [83, 84].

Stress receptors or sensors transduce external and internal signals into an intracellular response, for example, through phosphorylation and dephosphorylation cascades controlled by protein kinases and phosphatases. Genes encoding protein kinases were successfully exploited to engineer stress-tolerant crops. Modification of normal endogenous levels of GSK1, SAPK4, CDPK7, CIPK03, CIPK12, and CIPK15 in rice protected the plants against various abiotic stresses, including drought, salt, and low temperature stress [85–88]. Expression of tobacco mitogen-activated protein kinase, NPK1, in maize resulted in drought and low temperature stress tolerance [89]. Constitutive overexpression of a stress-inducible small GTP-binding protein PgRAB7 from *Pennisetum glaucum* enhances abiotic stress tolerance in transgenic tobacco [90].

Transcriptional Regulation

In the last decade, the most widely used and probably most important strategy for engineering abiotic stress tolerance in plants relied on the expression of genes that are involved in signaling and regulatory pathways [91, 92]. The use of TFs for tailoring stress tolerance is often referred to as regulon biotechnology because it affects the expression of many target genes in parallel [93, 94]. One of the reasons for their popularity is that TFs are believed to mediate durable tolerance to multiple stresses. Most TFs that control stress tolerance in plants belong to (large) protein families based on the presence of common DNA binding motifs and selectivity toward certain cis-regulatory elements in the promoters of target genes. These families include APETALA2/ ethylene responsive element binding proteins (AP2/EREBP) such as ethylene responsive factors (ERF), the DREB/CBF (drought-responsive element binding/cold-responsive element binding factor) proteins, basic domain leucine-zipper (bZIP) proteins

such as ABFs (abscisic acid (ABA)-responsive element binding factor), basic helix-loop-helix proteins (including MYC proteins), NAC (petunia NAM *Arabidopsis* ATAF1/2, and CUC2-domain) proteins, MYB-related proteins, as well as different families of zinc-fingers domain-containing proteins, such as WRKY binding factors, C_3H- and C_2H_2-type TFs. In recent years, many excellent reviews have been published on the role and use of TFs for engineering of stress tolerance in plants [48, 93–96].

Many AP2/EREBP–type TFs, the best studied being DREB/CBF proteins, have been used to engineer stress-tolerant crops. Transgenic rice plants with higher levels of CBF1/DREB1B and CBF3/DREB1A were more resistant to drought, salt, and low temperature stress [97]. A similar approach with CBF15 and CBF17 in oilseed and NF-YB2 in maize resulted in increased tolerance to low temperature and drought stress, respectively [98, 99]. Potato plants with increased levels of endogenous EREBP1 were more tolerant to salt stress and low temperatures [100]. Ectopic expression of the soybean ERF3 gene in transgenic tobacco plants gave tolerance to drought and salt stress [101]. Similarly, ectopic expression of an *Arabidopsis* AP2/ERF TF, HARDY, in rice also induced tolerance to drought and salt [102]. Expression of TERF1 in rice regulates expression of stress-responsive genes and enhances tolerance to drought and high salinity [103].

However, not only the AP2/EREBP-type TFs have been exploited to engineer stress-tolerant crops. For example, in rice, it was shown that constitutive expression of proteins from various other TF types, including bZIP23, zinc-finger protein 245, TIFY11, MYB3R-2, IRO2, NAC6, SNAC1, and PF1 TFs, could induce tolerance to drought, salt, low temperature, and nutrient deficiency [104–111], and the rice dst mutant, DST encoding a novel C_2H_2-type TF, showed increased tolerance to drought and salt [112]. In tomato, ectopic expression of rice MYB4 and pepper PIF1 induced low temperature and drought stress tolerance, respectively [113, 114]. Also in tomato, constitutive expression of SlAREB, a bZIP TF with affinity for ABA-responsive elements, increased tolerance to drought and salt stress [115]. Ectopic expression of cotton ZFP1, encoding a CCCH-type zinc-finger protein, and rice ZFP177, an A20/AN1-type zinc finger, enhanced stress tolerance in tobacco [116, 117].

Posttranscriptional and (Post-)Translational Regulation

Posttranscriptional control of stress gene expression is mediated by proteins that are involved in splicing, export, and degradation of gene transcripts, which contributes to correct function of the encoded proteins. In the last years, it has become evident that nonprotein coding RNA molecules, including microRNA and other small RNA molecules, play a very important role in posttranscriptional regulation of plant stress responses [118]. miRNA-mediated posttranscriptional control of antioxidant gene expression seems very important in plants, as shown for APX during programmed cell death and drought stress, and for Cu/Zn SOD during tolerance against oxidative stress [21, 119, 120]. ROS-induced stabilization of SOS1 mRNA transcripts is essential for SOS1-dependent salt tolerance [68].

Several genes that encode proteins involved in RNA processing were discovered to be involved in stress tolerance processes [121–125]. In addition, also proteins that control translation (deoxyhypusine synthase, DHS), posttranslational modification (peptide methionine sulfoxide reductase, PMSR4), and protein degradation (SDIR1) are interesting candidates for engineering stress tolerance in plants [126–128]. However, the above examples resulted only from *Arabidopsis* research and much less information on the importance of such processes is available for crops. Recently, it was shown that transgenic rice plants with increased levels of methione sulfoxide reductases, MSRA and MSRB, are more tolerant to salt stress [129]. Two other approaches were reported to work in both *Arabidopsis* and oilseeds. Wang and coworkers (2005) reported that loss-of-function of farnesyl transferase (FTA and FTB) increased tolerance to drought stress [130]. Similarly, reduction of poly-ADP-ribose polymerase (PARP) activity improved drought tolerance by increasing energy use efficiency [131]. PARP is involved in the modification of nuclear genes, such as histones. Downregulation of PARP resulted in the deregulation of the expression of genes in response to stress [132]. Processes that control stress tolerance and general fitness of plants in the field, such as energy use efficiency, can even be controlled by an epigenetic component [133]. By starting with an isogenic canola line,

Hauben and coworkers could separate in subsequently selfed generations "good" and "bad" performing plants, which only differed in epigenetic parameters but remained genetically identical.

All these examples show that the plant response to abiotic stress is not only dependent on transcriptional changes, but also on RNA processing, translation, post-translational processes, and epigenetics, which are nearly unexploited until today. The lack of understanding these processes is a limiting factor in engineering stress tolerance in crops.

Limitations of Genetic Engineering for Stress Tolerance

Because conventional breeding is a very time-consuming process, genetic engineering is now an important technology for many commercial applications aiming at plant improvement [1]. Today this mainly includes the production of plants with engineered herbicide or insect tolerance. However, it has been and still is extremely challenging to progress engineered plants with reported abiotic stress tolerance from research to field applications for farmers [134]. The main factors hampering the production of commercial solutions for the abiotic stress-related problems in agriculture are related to: (1) the availability of sequence information and transformation protocols, (2) the genetic diversity between model species and crop, (3) the multigenic character of stress tolerance, (4) the definition of stress tolerance and (5) the methods for the evaluation of stress tolerance [53, 135].

The first requirements for genetic engineering of plants include the availability of sequence information and transformation protocols. This important information is still lacking for many agricultural crops. For long time, this has limited (academic) research to model plant species. Although the use of model species such as *Arabidopsis* has proven its value, the genetic diversity within higher plants is the main factor that limits translatability of results obtained in model species into commercial crops. Even in those cases where the research is conducted in a more closely related model crop, it remains to be a challenge to translate the results into genetically diverse elite varieties used for commercialization.

Another factor limiting translatability from model species to crops is the complex, multigenic character of stress tolerance mechanisms. Despite the use of single genes for engineering stress tolerance, efficient and sustainable stress tolerance requires the cooperative action of many genes that are involved in various cellular processes that are not completely understood. Due to the initial success of transcriptomics, it has been accepted for long time that responses to abiotic stresses were almost exclusively regulated at the transcriptional level by a small set of core transcription factors. However, it is now clear that many other regulatory processes such as posttranscriptional control, translation, posttranslational, and epigenetic effects play an important role. In addition, some of these processes might not always be evolutionarily conserved but rather genetically diverse.

The early availability of the full genome sequence and many molecular analysis tools has turned *Arabidopsis* into the primary model species of choice for academic research in general, and for studying the response of plants to abiotic stresses in particular. However, the methods for evaluation of stress tolerance using *Arabidopsis* plants grown in a laboratory environment and agricultural crops in the field can be quite diverse. From an agronomic point of view, it is more relevant to study the effects of abiotic stresses on plant growth and yield over longer periods which cover the life span of a crop under field conditions. Appropriate field tests are therefore highly important because they allow studying plants in their natural environment in which they are exposed to combination of multiple stresses or cycles of the same stress [136]. In contrast to the field situation for crops, most studies on abiotic stress in *Arabidopsis* focus on short-term and strong stress treatments. Therefore, evaluation of stress tolerance is often focused on survival which is easier to screen. In contrast, yield-related traits are more difficult to measure but much more important for crops. Abiotic stress tolerance of crops should therefore be defined as the potential to produce high yields when exposed to mild(er), multiple stresses instead of the ability to survive a single, lethal stress.

Another limiting factor for successful field applications of plants with engineered protection against stress is the seemingly mutual exclusive characteristic of high

yield under normal conditions and high stress tolerance. Most reported experiments focused either on abiotic stress tolerance or on yield rather than including detailed analysis of both traits. More in-depth analysis of stress-tolerant plants under "normal" conditions often revealed a negative effect on growth, development, and yield traits. On the other hand, engineered plant with improved growth features, for example, by boosting a metabolic process, can be less adapted to maintain growth under abiotic stress conditions. Negative effects, often referred to as yield penalty, can be (at least partially) circumvented by temporally and spatially controlled expression of the target gene [137]. However, the availability of suitable promoters is still limited. Improved sequencing and bioinformatic tools for identification and construction of promoters allowing the expression of genes in a spatially and temporally controlled manner can overcome this bottleneck.

Phenotyping experiments with a set of genetically defined plants are often difficult to repeat because plants are largely influenced by the environment in which they grow. In fact, growth and yield (i.e., performance) of a plant under normal or stress conditions is the output of all integrated physiological processes in the plant. It is therefore not surprising that different laboratories often obtain different results for plant performance [138]. More effort is needed to describe and standardize the design of the experiments, of protocols for measuring traits, and of the growing conditions. Different methods to evaluate stress tolerance in *Arabidopsis* and a conceptual framework for phenotyping during breeding for drought tolerance have already been proposed, including defining the minimal information needed for carrying out a drought stress experiment [139, 140]. Improved and systematic phenotyping of plants will greatly contribute to overcome the limitations associated with the methods to evaluate stress tolerance.

Systematic Phenotyping of Plants

In the last decades, it has become clear that there is a substantial need for automation of research processes [141]. Automation not only allows increasing throughput, but also ameliorates standardization, reproducibility, and therefore the overall quality of an experiment. Automation increases the research value and also reduces "cost to practice" for companies aiming to apply the research findings toward the development of a superior and sustainable product. It can therefore be assumed that automation will help to improve the translation of research findings into valuable products for the customer.

Plant research has already embraced the development of high-throughput screening experiments that were used for drug or herbicide discovery. Such experiments focused on assessment of certain molecular function. Next to these screens, also high-content screening protocols were developed to asses such molecules in a biological assay. Although these technologies have proven value for single trait discovery, the efficient development of plants with complex, multifactorial traits such as increased tolerance to abiotic stresses is fully dependent on protocols and methods to automatically phenotype at the plant level. The performance of whole plants is assessed by studying their physiology or phenotype in tissue culture, environmentally controlled growth chambers, greenhouse, or the field. Various methods for automated phenotyping of plants or plant parts in well-defined environments have been published [141]. After the establishment of genomics, transcriptomics, proteomics, and metabolomics in plant sciences, which all greatly contributed to gene or lead discovery in the field of abiotic stress, plant phenomics needs to be developed and implemented [142–144].

Plant phenomics is expected to enable the efficient and reliable evaluation of a new trait solution and thereby enhance breeding for stress-tolerant varieties. Large, dedicated plant phenotyping centers such as the ACPFG in Adelaide (Plant Accelator™; http://www.plantaccelerator.org.au/), the CSIRO Plant Industry in Canberra (High Resolution Plant Phenomics Centre; http://www.plantphenomics.org.au/HRPPC), or the Research Centre in Jülich (Jülich Phenomics Centre; http://www.fz-juelich.de/icg/icg-3/jppc/) have been created. These research institutions can act as service providers for academia and industry by centralizing both high-tech infrastructure and highly skilled researchers with different background, including engineering, mathematics, computer science, and plant physiology/biology. An important biological question that probably can be answered by automated

phenomics studies is which phenotypes or combination of multiple phenotypes need or do not need to be collected in order to be able to correctly evaluate plant performance in the environment of interest. Moreover, parallel phenotyping of plants in one or multiple environments allows detection of associations between traits [145]. Identification of redundant information from (highly) associated phenotypes will help to reduce complexity and focus on essential phenotypes.

Well-described examples of automated platforms from academia or private companies to study the phenotype of model plants or crops include phenopsis, phenodyn, GROWSCREEN, TraitMill^{TM}, PlaRoM, and various LemnaTec products. Phenopsis is a phenotying platform for *Arabidopsis* that allows measuring multiple parameters, including plant growth, water use, and transpiration rates, which are associated to plant performance under normal and drought stress conditions [146]. Phenodyn was specifically constructed to perform similar experiments with monocots such as maize and rice [147]. GROWSCREEN is an automated method for growth analysis of a limited set of small seedlings [148]. TraitMill^{TM}, one of the first automated phenotyping systems, was developed to study traits in rice [149]. LemnaTec offers commercialized phenotyping systems for different purposes and has customers from both academia and industry all over the world (http://www.lemnatec.com). Although most phenotyping platforms focus on the shoot of the plant, several systems for root phenotyping were also developed [150, 151]. By using large field scanners, it is even possible to use similar approaches during field trials.

One common feature of all automated phenotyping systems is the use of digital cameras for imaging. The use of digital imaging has many advantages over traditional plant phenotyping efforts. While traditional experiments were often dependent on subjective visual scoring of plant traits or on labor-intensive, often destructive manual sampling, for example, for fresh and dry weight measurements, automated phenotyping by digital imaging is nondestructive, and therefore allows following the development of a single plant over time [152]. Another important advantage of digital imaging is that the raw data (digital images) can be easily stored and accessed later on for reanalysis, providing there is a good database in place. Obviously, adequate image analysis software is an essential feature in the concept of automated phenotyping. Several (semi-automated) image analysis tools are based on the free-ware ImageJ software, but other, more sophisticated software, for 2D or 3D analysis, were designed for measurement of leaf shape (LAMINA, [153]), root growth (GROWSCREEN_ROOT, [154]; ROOTEDGE, [155]; RHIZOSCAN, [156]; [157]; EZ-Rhizo, [158]); and hypocotyl growth and shape (HYPOTrace, [159]). The challenge is to find the most appropriate software that can extract the information relevant for the biological questions driving the experiment. Depending on the type of digital camera and the image analysis software, different types of information that describe the phenotype of the plant can be extracted: morphological or physiological and quantitative or qualitative. For example, conventional imaging can be used to measure color, shape, length, width, and size. Video imaging or consecutive imaging of the same plant or plant part over time even allows calculating plant growth. Thermal imaging (infrared thermography) is used to measure temperature and transpiration, while fluorescence and reflectance imaging allows measuring photosynthetic activity of a plant leaf or canopy. Leaf density in the canopy can be analyzed using light detection and ranging imaging that is based on laser scanning. It is now also possible to use typical clinical imaging applications, for example, magnetic resonance imaging (MRI, alternatively called nuclear magnetic resonance) for studying plant hydraulics (water or sap flow) and carbon biomass production [160]. X-ray computed tomography and a combined analysis using MRI and positron emission tomography allow the nondestructive analysis of root growth in soil environments [154]. All these types of image-derived information can be relevant to study abiotic stress tolerance mechanisms, but temperature and photosynthetic activity of leaves are among the most widely studied phenotypes in the response to abiotic stresses.

Imaging is particularly useful for the rapid, early-stage detection of abiotic stress in a plant because it allows detecting changes in the plant performance beyond the naked eye. It is therefore a unique way to diagnose plants. Understanding how plants sense stresses can be considered as a prerequisite for engineering stress tolerance. It can be assumed that plants

that have reduced stress-sensing capacities will continue to grow in mild stress conditions for longer time and thereby produce higher yields. In contrast, when subjected to severe or lethal stresses, the same plants could have a disadvantage because the investment into energy-demanding processes such as growth is high and tolerance mechanisms may be induced only late. Therefore, when exposed to severe stress conditions, plants ideally should be able to increase their stress-sensing ability.

Future Directions

Increased protection of plants against abiotic stresses involves a complex regulatory network controlling morphological, physiological, biochemical, and molecular changes. Understanding such changes has been of key importance in breeding. Breeding crop varieties with improved performance under suboptimal growing conditions is now one of the ambitious, but crucial objectives in modern plant biotechnology. Despite the great progress in the last decade, it is now apparent that abiotic stress tolerance is a complex trait involving genes with different biological functions. However, the number of genes that were actually successfully used to improve crop performance in the field is still low. Although many types of genes or processes have been the target of genetic engineering, certain areas are still unexplored. Interesting developments were recently made in the field of miRNA regulation and epigenetics [161]. Another unexplored field is that of small peptides and their role during abiotic stress signaling.

It is very unlikely that there will be one or a few solutions for current agronomic challenges caused by abiotic stresses, but rather specific solutions for specific cases. Most of the current findings originate from work in model plant species, but much more work is needed to translate such basic research findings to crops. Because of the complex nature of abiotic stress tolerance in plants, it appears that modifying one gene to induce stress tolerance will be in many cases not sufficient. Hence, the approach of using regulon biotechnology for crop improvement, modifying a functional process or even multiple interconnected functional processes instead of one single function, appears to be the way forward. However, this approach is often limited by the lack of knowledge on the network of molecular mechanisms underpinning stress tolerance. Conventional approaches were also hampered by experimental limitations, which can now (partially) be overcome by the advent of automated phenotyping platforms for both model and crop species. Based on the efforts made for phenotyping of *Arabidopsis* plants, it can be anticipated that this small weed will remain the primary model system of choice for academic/basic research in near future. However, "omics" platforms for crop species are becoming available and it will not take too long before research in model crop species will become intensified. Validation of results obtained in *Arabidopsis* in (model) crops will increase the value of the lead technologies and justify the large investments for field trials.

After the development of high-throughput, automated platforms to study plant phenotypes in various environments, it will be essential to combine the data obtained from the various "omics" platforms to intensify biological modeling. Modeling has proven to be useful to study specific processes, such as root growth and flowering. Efforts are now being made to model abiotic stress tolerance in plants [162]. Improved phenotyping technologies will not only allow better experimental validation of predictions drawn from such biological models, but also to improve existing models by including knowledge on the impact of external factors such as abiotic stresses. By including modeling in biological studies, our understanding of the plants' response to abiotic stress will reach a systems biology level, a new quality in plant sciences, which will accelerate the breeding process toward abiotic stress tolerance in all our major crops.

Bibliography

1. Fedoroff NV, Battisti DS, Beachy RN, Cooper PJ, Fischhoff DA, Hodges CN, Knauf VC, Lobell D, Mazur BJ, Molden D, Reynolds MP, Ronald PC, Rosegrant MW, Sanchez PA, Vonshak A, Zhu JK (2010) Radically rethinking agriculture for the 21st century. Science 327:833–834
2. Buchanan BB, Gruissem W, Jones RL (eds) (2000) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville
3. Ribaut JM, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations, and alternatives. J Exp Bot 58:351–360

4. Sreenivasulu N, Sopory SK, Kavi Kishor PB (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene 388:1–13
5. Witcombe JR, Hollington PA, Howarth CJ, Reader S, Steele KA (2008) Breeding for abiotic stresses for sustainable agriculture. Philos Trans R Soc Lond B Biol Sci 363:703–716
6. Vij S, Tyagi AK (2007) Emerging trends in the functional genomics of the abiotic stress response in crop plants. Plant Biotechnol J 5:361–380
7. Bhatnagar-Mathur P, Vadez V, Sharma KK (2008) Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Rep 27:411–424
8. Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227
9. Divya K, Jami SK, Kirti PB (2010) Constitutive expression of mustard annexin, AnnBj1 enhances abiotic stress tolerance and fiber quality in cotton under stress. Plant Mol Biol 73:293–308
10. Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399
11. Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. Curr Opin Plant Biol 7:323–328
12. Halliwell B (2006) Reactive species and antioxidants Redox biology is a fundamental theme of aerobic life. Plant Physiol 141:312–322
13. Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death. Plant Physiol 141:384–390
14. Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A, Tanaka K (2004) Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol Plant 121:231–238
15. Basu U, Good AG, Taylor GJ (2001) Transgenic Brassica napus plants overexpressing aluminium- induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminium. Plant Cell Environ 24:1269–1278
16. Gaber A, Yoshimura K, Yamamoto T, Yabuta Y, Takeda T, Miyasaka H, Nakano Y, Shigeoka S (2006) Glutathione peroxidase-like protein of Synechocystis PCC 6803 confers tolerance to oxidative and environmental stresses in transgenic Arabidopsis. Physiol Plant 128:251–262
17. Gong H, Jiao Y, Hu WW, Pua EC (2005) Expression of glutathione-S-transferase and its role in plant growth and development in vivo and shoot morphogenesis in vitro. Plant Mol Biol 57:53–66
18. Yoshimura K, Miyao K, Gaber A, Takeda T, Kanaboshi H, Miyasaka H, Shigeoka S (2004) Enhancement of stress tolerance in transgenic tobacco plants overexpressing Chlamydomonas glutathione peroxidase in chloroplasts or cytosol. Plant J 37:21–33
19. Murgia I, Tarantino D, Vannini C, Bracale M, Carravieri S, Soave C (2004) Arabidopsis thaliana plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. Plant J 38:940–953
20. Oberschall A, Deák M, Török K, Sass L, Vass I, Kovács I, Fehér A, Dudits D, Horváth GV (2000) A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. Plant J 24:437–446
21. Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 18:2051–2065
22. Yamamoto A, Bhuiyan MN, Waditee R, Tanaka Y, Esaka M, Oba K, Jagendorf AT, Takabe T (2005) Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and Arabidopsis plants. J Exp Bot 56:1785–1796
23. Zhao F, Zhang H (2006) Expression of Suaeda salsa glutathione S-transferase in transgenic rice resulted in a different level of abiotic stress resistance. J Agric Sci 144:547–554
24. Kornyeyev D, Logan BA, Payton P, Allen RD, Holaday AS (2001) Enhanced photochemical light utilization and decreased chilling-induced photoinhibition of photosystem II in cotton overexpressing genes encoding chloroplast-targeted antioxidant enzymes. Physiol Plant 113:323–331
25. Matsumura T, Tabayashi N, Kamagata Y, Souma C, Saruyama H (2002) Wheat catalase expressed in transgenic rice can improve tolerance against low temperature stress. Physiol Plant 116:317–327
26. Wang FZ, Wang QB, Kwon SY, Kwak SS, Su WA (2005) Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J Plant Physiol 162:465–472
27. Lee SH, Ahsan N, Lee KW, Kim DH, Lee DG, Kwak SS, Kwon SY, Kim TH, Lee BH (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J Plant Physiol 164:1626–1638
28. Prashanth SR, Sadhasivam V, Parida A (2008) Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant Avicennia marina in indica rice var Pusa Basmati-1 confers abiotic stress tolerance. Transgenic Res 17:281–291
29. Huang W, Ma X, Wang Q, Gao Y, Xue Y, Niu X, Yu G, Liu Y (2008) Significant improvement of stress tolerance in tobacco plants by overexpressing a stress-responsive aldehyde dehydrogenase gene from maize (Zea mays). Plant Mol Biol 68:451–463
30. Xue T, Li X, Zhu W, Wu C, Yang G, Zheng C (2009) Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. J Exp Bot 60:339–349
31. Van Aken O, Giraud E, Clifton R, Whelan J (2009) Alternative oxidase: a target and regulator of stress responses. Physiol Plant 137:354–361
32. Zhang Y, Xi D, Wang J, Zhu D, Guo X (2009) Functional analysis reveals effects of tobacco alternative oxidase gene (NtAOX1a) on regulation of defence responses against abiotic and biotic stresses. Biosci Rep 29:375–383

33. Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9:244–252
34. Katiyar-Agarwal S, Agarwal M, Grover A (2003) Heat-tolerant basmati rice engineered by over-expression of hsp101. Plant Mol Biol 51:677–686
35. Guo S-J, Zhou H-Y, Zhang XS, Li X-G, Meng Q-W (2007) Overexpression of CaHSP26 in transgenic tobacco alleviates photoinhibition of PSII and PSI during chilling stress under low irradiance. J Plant Physiol 164:126–136
36. Hong S-W, Vierling E (2000) Mutants of Arabidopsis thaliana defective in the acquisition of tolerance to high temperature stress. Proc Natl Acad Sci USA 97:4392–4397
37. Lee JH, Schöffl F (1996) An Hsp70 antisense gene affects the expression of HSP70/HSC70, the regulation of HSF, and the acquisition of thermotolerance in transgenic Arabidopsis thaliana. Mol Gen Genet 252:11–19
38. Malik MK, Slovin JP, Hwang CH, Zimmerman JL (1999) Modified expression of a carrot small heat shock protein gene, hsp17. 7, results in increased or decreased thermotolerance. Plant J 20:89–99
39. Park SM, Hong CB (2002) Class I small heat-shock protein gives thermotolerance in tobacco. J Plant Physiol 159:25–30
40. Queitsch C, Hong SW, Vierling E, Lindquist S (2000) Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. Plant Cell 12:479–492
41. Rhoads DM, White SJ, Zhou Y, Muralidharan M, Elthon TE (2005) Altered gene expression in plants with constitutive expression of a mitochondrial small heat shock protein suggests the involvement of retrograde regulation in the heat stress response. Physiol Plant 123:435–444
42. Yang JY, Sun Y, Sun AQ, Yi SY, Qin J, Li MH, Liu J (2006) The involvement of chloroplast HSP100/ClpB in the acquired thermotolerance in tomato. Plant Mol Biol 62:385–395
43. Maqbool A, Abbas W, Rao AQ, Irfan M, Zahur M, Bakhsh A, Riazuddin S, Husnain T (2010) Gossypium arboreum GHSP26 enhances drought tolerance in Gossypium hirsutum. Biotechnol Prog 26:21–25
44. Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218:1–14
45. Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular response to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol 3:217–223
46. Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273
47. Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol 118:1–8
48. Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599
49. Xiao B, Huang Y, Tang N, Xiong L (2007) Over-expression of a LEA gene in rice improves drought resistance under the field conditions. Theor Appl Genet 115:35–46
50. Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215–223
51. Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science 259:508–510
52. Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463–499
53. Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitation. Curr Opin Biotechnol 16:123–132
54. Kathuria H, Giri J, Nataraja KN, Murata N, Udayakumar M, Tyagi AK (2009) Glycinebetaine-induced water-stress tolerance in codA-expressing transgenic indica rice is associated with up-regulation of several stress responsive genes. Plant Biotechnol J 7:512–526
55. Jang IC, Oh SJ, Seo JS, Choi WB, Song SI, Kim CH, Kim YS, Seo HS, Choi YD, Nahm BH, Kim JK (2003) Expression of a bifunctional fusion of the Escherichia coli genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol 131:516–524
56. Garg AK, Kim J-K, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99:15898–15903
57. Quan R, Shang M, Zhang H, Zhao Y, Zhang J (2004) Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. Plant Biotechnol J 2:477–486
58. Park EJ, Jeknić Z, Pino MT, Murata N, Chen TH (2007) Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. Plant Cell Environ 30:994–1005
59. Abebe T, Guenzi AC, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol 131:1748–1755
60. Vendruscolo EC, Schuster I, Pileggi M, Scapim CA, Molinari HB, Marur CJ, Vieira LG (2007) Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J Plant Physiol 164:1367–1376
61. Shirasawa K, Takabe T, Takabe T, Kishitani S (2006) Accumulation of glycinebetaine in rice plants that overexpress choline monooxygenase from spinach and evaluation of their tolerance to abiotic stress. Ann Bot 98:565–571
62. Diamant S, Eliahu N, Rosenthal D, Goloubinoff P (2001) Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. J Biol Chem 276:39586–39591
63. Hare PD, Cress WA, Van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ 21:535–553
64. Tuteja N (2007) Mechanisms of high salinity tolerance in plants. Methods Enzymol 428:419–438

65. Obata T, Kitamoto HK, Nakamura A, Fukuda A, Tanaka Y (2007) Rice shaker potassium channel OsKAT1 confers tolerance to salinity stress on yeast and rice cells. Plant Physiol 144:1978–1985
66. Fukuda A, Chiba K, Maeda M, Nakamura A, Maeshima M, Tanaka Y (2004) Effect of salt and osmotic stresses on the expression of genes for the vacuolar H+-pyrophosphatase, H+-ATPase subunit A, and Na+/H+antiporter from barley. J Exp Bot 55:585–594
67. Laurie S, Feeney KA, Maathuis FJ, Heard PJ, Brown SJ, Leigh RA (2002) A role for HKT1 in sodium uptake by wheat roots. Plant J 32:139–149
68. Chung JS, Zhu JK, Bressan RA, Hasegawa PM, Shi H (2008) Reactive oxygen species mediate Na^+-induced SOS1 mRNA stability in Arabidopsis. Plant J 53:554–565
69. Lian HL, Yu X, Ye Q, Ding X, Kitagawa Y, Kwak SS, Su WA, Tang ZC (2004) The role of aquaporin RWC3 in drought avoidance in rice. Plant Cell Physiol 45:481–489
70. Pereira JF, Zhou G, Delhaize E, Richardson T, Zhou M, Ryan PR (2010) Engineering greater aluminium resistance in wheat by over-expressing TaALMT1. Ann Bot 106:205–214
71. Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J 37:645–653
72. Delhaize E, Ryan PR, Hebb DM, Yamamoto Y, Sasaki T, Matsumoto H (2004) Engineering high-level aluminum tolerance in barley with the ALMT1 gene. Proc Natl Acad Sci USA 101:15249–15254
73. Ariizumi T, Kishitani S, Inatsugi R, Nishida I, Murata N, Toriyama K (2002) An increase in unsaturation of fatty acids in phosphatidylglycerol from leaves improves the rates of photosynthesis and growth at low temperatures in transgenic rice seedlings. Plant Cell Physiol 43:751–758
74. Khodakovskaya M, McAvoy R, Peters J, Wu H, Li Y (2006) Enhanced cold tolerance in transgenic tobacco expressing a chloroplast omega-3 fatty acid desaturase gene under the control of a cold-inducible promoter. Planta 223:1090–1100
75. Orlova IV, Serebriiskaya TS, Popov V, Merkulova N, Nosov AM, Trunova TI, Tsydendambaev VD, Los DA (2003) Transformation of tobacco with a gene for the thermophilic acyl-lipid desaturase enhances the chilling tolerance of plants. Plant Cell Physiol 44:447–450
76. Sui N, Li M, Zhao SJ, Li F, Liang H, Meng QW (2007) Overexpression of glycerol-3-phosphate acyltransferase gene improves chilling tolerance in tomato. Planta 226:1097–1108
77. Zhang M, Barg R, Yin M, Gueta-Dahan Y, Leikin-Frenkel A, Salts Y, Shabtai S, Ben-Hayyim G (2005) Modulated fatty acid desaturation via overexpression of two distinct ω-3 desaturases differentially alters tolerance to various abiotic stresses in transgenic tobacco cells and plants. Plant J 44:361–371
78. Sohn SO, Back K (2007) Transgenic rice tolerant to high temperature with elevated contents of dienoic fatty acids. Biol Plant 51:340–342
79. Osakabe Y, Mizuno S, Tanaka H, Maruyama K, Osakabe K, Todaka D, Fujita Y, Kobayashi M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Overproduction of the membrane-bound receptor-like protein kinase 1, RPK1, enhances abiotic stress tolerance in Arabidopsis. J Biol Chem 285:9190–9201
80. Yang L, Ji W, Zhu Y, Gao P, Li Y, Cai H, Bai X, Guo D (2010). GsCBRLK, a calcium/calmodulin-binding receptor-like kinase, is a positive regulator of plant tolerance to salt and ABA stress. J Exp Bot 61:2519–2533
81. Ouyang SQ, Liu YF, Liu P, Lei G, He SJ, Ma B, Zhang WK, Zhang JS, Chen SY (2010) Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (Oryza sativa) plants. Plant J 62:316–329
82. Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2010) ABA perception and signaling. Trends Plant Sci 15:395–401
83. Melcher K, Ng LM, Zhou XE, Soon FF, Xu Y, Suino-Powell KM, Park SY, Weiner JJ, Fujii H, Chinnusamy V, Kovach A, Li J, Wang Y, Li J, Peterson FC, Jensen DR, Yong EL, Volkman BF, Cutler SR, Zhu JK, Xu HE (2009) A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. Nature 462:602–608
84. Santiago J, Dupeux F, Round A, Antoni R, Park SY, Jamin M, Cutler SR, Rodriguez PL, Márquez JA (2009) The abscisic acid receptor PYR1 in complex with abscisic acid. Nature 462:665–668
85. Koh S, Lee SC, Kim MK, Koh JH, Lee S, An G, Choe S, Kim SR (2007) T-DNA tagged knockout mutation of rice OsGSK1, an orthologue of Arabidopsis BIN2, with enhanced tolerance to various abiotic stresses. Plant Mol Biol 65:453–466
86. Diédhiou CJ, Popova OV, Dietz KJ, Golldack D (2008) The SNF1-type serine-threonine protein kinase SAPK4 regulates stress-responsive gene expression in rice. BMC Plant Biol 28(8):49
87. Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J 23:319–327
88. Xiang Y, Huang Y, Xiong L (2007) Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. Plant Physiol 144:1416–1428
89. Shou H, Bordallo P, Wang K (2004) Expression of the Nicotiana protein kinase (NPK1) enhanced drought tolerance in transgenic maize. J Exp Bot 55:1013–1019
90. Agarwal PK, Agarwal P, Jain P, Jha B, Reddy MK, Sopory SK (2008) Constitutive overexpression of a stress-inducible small GTP-binding protein PgRab7 from Pennisetum glaucum enhances abiotic stress tolerance in transgenic tobacco. Plant Cell Rep 27:105–115
91. Seki M, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr Opin Biotechnol 14:194–199
92. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417
93. Nakashima K, Yamaguchi-Shinozaki K (2005) Molecular studies on stress-responsive gene expression in Arabidopsis and improvement of stress tolerance in crop plants by regulon biotechnology. Jpn Agr Res Q 39:221–229

94. Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr Opin Biotechnol 17:113–122
95. Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25:1263–1274
96. Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803
97. Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol 47:141–153
98. Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Huner NP, Shinozaki K, Singh J (2005) The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in Brassica napus. Plant Cell Physiol 46:1525–1539
99. Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG, Hinchey BS, Kumimoto RW, Maszle DR, Canales RD, Krolikowski KA, Dotson SB, Gutterson N, Ratcliffe OJ, Heard JE (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci USA 104:16450–16455
100. Lee HE, Shin D, Park SR, Han SE, Jeong MJ, Kwon TR, Lee SK, Park SC, Yi BY, Kwon HB, Byun MO (2007) Ethylene responsive element binding protein 1 (StEREBP1) from Solanum tuberosum increases tolerance to abiotic stress in transgenic potato plants. Biochem Biophys Res Commun 353:863–868
101. Zhang G, Chen M, Li L, Xu Z, Chen X, Guo J, Ma Y (2009) Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. J Exp Bot 60:3781–3796
102. Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsch-Martinez N, Krishnan A, Nataraja KN, Udayakumar M, Pereira A (2007) Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene. Proc Natl Acad Sci USA 104:15270–15275
103. Gao S, Zhang H, Tian Y, Li F, Zhang Z, Lu X, Chen X, Huang R (2008) Expression of TERF1 in rice regulates expression of stress-responsive genes and enhances tolerance to drought and high-salinity. Plant Cell Rep 27:1787–1795
104. Xiang Y, Tang N, Du H, Ye H, Xiong L (2008) Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. Plant Physiol 148:1938–1952
105. Huang J, Sun SJ, Xu DQ, Yang X, Bao YM, Wang ZF, Tang HJ, Zhang H (2009) Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. Biochem Biophys Res Commun 389:556–561
106. Ye H, Du H, Tang N, Li X, Xiong L (2009) Identification and expression profiling analysis of TIFY family genes involved in stress and phytohormone responses in rice. Plant Mol Biol 71:291–305
107. Ma Q, Dai X, Xu Y, Guo J, Liu Y, Chen N, Xiao J, Zhang D, Xu Z, Zhang X, Chong K (2009) Enhanced tolerance to chilling stress in OsMYB3R-2 transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. Plant Physiol 150:244–256
108. Ogo Y, Itai RN, Nakanishi H, Kobayashi T, Takahashi M, Mori S, Nishizawa NK (2007) The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. Plant J 51:366–377
109. Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630
110. Yi SY, Kim JH, Joung YH, Lee S, Kim WT, Yu SH, Choi D (2004) The pepper transcription factor CaPF1 confers pathogen and freezing tolerance in Arabidopsis. Plant Physiol 136:2862–2874
111. Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 103:12987–12992
112. Huang XY, Chao DY, Gao JP, Zhu MZ, Shi M, Lin HX (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. Genes Dev 23:1805–1817
113. Vannini C, Campa M, Iriti M, Genga A, Faoro F, Carravieri S, Rotino GL, Rossoni M, Spinardi AM (2007) Evaluation of transgenic tomato plants ectopically expressing the rice Osmyb4 gene. Plant Sci 173:231–239
114. Seong ES, Baek K-H, Oh S-K, Jo SH, Yi SY, Park JM, Joung YH, Lee S, Cho HS, Choi D (2007) Induction of enhanced tolerance to cold stress and disease by overexpression of the pepper CaPIF1 gene in tomato. Physiol Plant 129:555–566
115. Hsieh TH, Li CW, Su RC, Cheng CP, Sanjaya TYC, Chan MT (2010) A tomato bZIP transcription factor, SlAREB, is involved in water deficit and salt stress response. Planta 231:1459–1473
116. Guo YH, Yu YP, Wang D, Wu CA, Yang GD, Huang JG, Zheng CC (2009) GhZFP1, a novel CCCH-type zinc finger protein from cotton, enhances salt stress tolerance and fungal disease resistance in transgenic tobacco by interacting with GZIRD21A and GZIPR5. New Phytol 183:62–75
117. Huang J, Wang MM, Jiang Y, Bao YM, Huang X, Sun H, Xu DQ, Lan HX, Zhang HS (2008) Expression analysis of rice A20/AN1-type zinc finger genes and characterization of ZFP177 that contributes to temperature stress tolerance. Gene 420:135–144

118. Shukla LI, Chinnusamy V, Sunkar R (2008) The role of microRNAs and other endogenous small RNAs in plant stress responses. Biochim Biophys Acta 1779:743–748
119. Mittler R, Feng X, Cohen M (1998) Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco. Plant Cell 10:461–473
120. Mittler R, Zilinskas BA (1994) Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. Plant J 5:397–405
121. Forment J, Naranjo MA, Roldán M, Serrano R, Vicente O (2002) Expression of Arabidopsis SR-like splicing proteins confers salt tolerance to yeast and transgenic plants. Plant J 30:511–519
122. Kant P, Kant S, Gordon M, Shaked R, Barak S (2007) STRESS RESPONSE SUPPRESSOR1 and STRESS RESPONSE SUPPRESSOR2, two DEAD-box RNA helicases that attenuate Arabidopsis responses to multiple abiotic stresses. Plant Physiol 145:814–830
123. Gong Z, Dong CH, Lee H, Zhu J, Xiong L, Gong D, Stevenson B, Zhu JK (2005) A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in Arabidopsis. Plant Cell 17:256–267
124. Kim YO, Kim JS, Kang H (2005) Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in Arabidopsis thaliana. Plant J 42:890–900
125. Kim YO, Pan S, Jung C-H, Kang H (2007) A zinc finger-containing glycine-rich RNA-binding protein, at RZ-1a, has a negative impact on seed germination and seedling growth of Arabidopsis thaliana under salt or drought stress conditions. Plant Cell Physiol 48:1170–1181
126. Romero C, Belles JM, Vaya JL, Serrano R, Culianez-Macia FA (1997) Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta 201: 293–297
127. Zhang Y, Yang C, Li Y, Zheng N, Chen H, Zhao Q, Gao T, Guo H, Xie Q (2007) SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive abscisic acid signaling in Arabidopsis. Plant Cell 19:1912–1929
128. Zhang YY, Li Y, Gao T, Zhu H, Wang DJ, Zhang HW, Ning YS, Liu LJ, Wu YR, Chu CC, Guo HS, Xie Q (2008) Arabidopsis SDIR1 enhances drought tolerance in crop plants. Biosci Biotechnol Biochem 72:2251–2254
129. Guo X, Wu Y, Wang Y, Chen Y, Chu C (2009) OsMSRA4.1 and OsMSRB1.1, two rice plastidial methionine sulfoxide reductases, are involved in abiotic stress responses. Planta 230:227–238
130. Wang Y, Ying J, Kuzma M, Chalifoux M, Sample A, McArthur C, Uchacz T, Sarvas C, Wan J, Dennis DT, McCourt P, Huang Y (2005) Molecular tailoring of farnesylation for plant drought tolerance and yield protection. Plant J 43:413–424
131. De Block M, Verduyn C, De Brouwer D, Cornelissen M (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. Plant J 41:95–106
132. Vanderauwera S, De Block M, Van de Steene N, van de Cotte B, Metzlaff M, Van Breusegem F (2007) Silencing of poly(ADP-ribose) polymerase in plants alters abiotic stress signal transduction. Proc Natl Acad Sci USA 104:15150–15155
133. Hauben M, Haesendonckx B, Standaert E, Van Der Kelen K, Azmi A, Akpo H, Van Breusegem F, Guisez Y, Bots M, Lambert B, Laga B, De Block M (2009) Energy use efficiency is characterized by an epigenetic component that can be directed through artificial selection to increase yield. Proc Natl Acad Sci USA 106:20109–20114
134. Mittler R, Blumwald E (2010) Genetic engineering for modern agriculture: challenges and perspectives. Annu Rev Plant Biol 61:443–462
135. Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci 10:615–620
136. Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci 11:15–19
137. Pino MT, Skinner JS, Park EJ, Jeknić Z, Hayes PM, Thomashow MF, Chen TH (2007) Use of a stress inducible promoter to drive ectopic AtCBF expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotechnol J 5:591–604
138. Massonet C, Vile D, Fabre J, Hannah MA, Caldana C, Lisec J, Beemster GT, Meyer RC, Messerli G, Gronlund JT, Perkovic J, Wigmore E, May S, Bevan MW, Meyer C, Rubio-Diaz S, Weigel D, Micol JL, Buchanan-Wollaston V, Fiorani F, Walsh S, Rinn B, Gruissem W, Hilson P, Hennig L, Willmitzer L, Granier C (2010) Plant Physiol 152:2142–2157
139. Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45:523–539
140. Salekdeh GH, Reynolds M, Bennett J, Boyer J (2009) Conceptual framework for drought phenotyping during molecular breeding. Trends Plant Sci 14:488–496
141. Kolukisaoglu Ü, Thurow K (2010) Future and frontiers of automated screening in plant sciences. Plant Sci 178:476–484
142. Urano K, Kurihara Y, Seki M, Shinozaki K (2010) 'Omics' analyses of regulatory networks in plant abiotic stress responses. Curr Opin Plant Biol 13:132–138
143. Mochida K, Shinozaki K (2010) Genomics and bioinformatics resources for crop improvement. Plant Cell Physiol 51:497–523
144. Finkel E (2009) Imaging. With 'phenomics', plant scientists hope to shift breeding into overdrive. Science 325:380–381
145. Saint Pierre C, Crossa J, Manes Y, Reynolds MP (2010) Gene action of canopy temperature in bread wheat under diverse environments. Theor Appl Genet 120:1107–1117
146. Granier C, Aguirrezabal L, Chenu K, Cookson SJ, Dauzat M, Hamard P, Thioux JJ, Rolland G, Bouchier-Combaud S, Lebaudy A, Muller B, Simonneau T, Tardieu F (2006) PHENOPSIS, an automated platform for reproducible

phenotyping of plant responses to soil water deficit in Arabidopsis thaliana permitted the identification of an accession with low sensitivity to soil water deficit. New Phytol 169:623–635
147. Sadok W, Naudin P, Boussuge B, Muller B, Welcker C, Tardieu F (2007) Leaf growth rate per unit thermal time follows QTL-dependent daily patterns in hundreds of maize lines under naturally fluctuating conditions. Plant Cell Environ 30:135–146
148. Walter A, Scharr H, Gilmer F, Zierer R, Nagel KA, Ernst M, Wiese A, Virnich O, Christ MM, Uhlig B, Jünger S, Schurr U (2007) Dynamics of seedling growth acclimation towards altered light conditions can be quantified via GROWSCREEN: a setup and procedure designed for rapid optical phenotyping of different plant species. New Phytol 174:447–455
149. Reuzeau C, Pen J, Frankard V, de Wolf J, Peerbolte R, Broekaert W, van Camp W (2005) TraitMill: a discovery engine for identifying yield-enhancement genes in cereals. Mol Plant Breed 3:753–759
150. Gregory PJ, Hutchison DJ, Read DB, Jenneson PM, Gilboy WB, Morton EJ (2003) Non-invasive imaging of roots with high resolution X-ray micro-tomography. Plant Soil 255:351–359
151. Yazdanbakhsh N, Fisahn J (2010) Analysis of Arabidopsis thaliana root growth kinetics with high temporal and spatial resolution. Ann Bot 105:783–791
152. Chaerle L, Lenk S, Leinonen I, Jones HG, Van Der Straeten D, Buschmann C (2009) Multi-sensor plant imaging: towards the development of a stress-catalogue. Biotechnol J 4:1152–1167
153. Bylesjö M, Segura V, Soolanayakanahally RY, Rae AM, Trygg J, Gustafsson P, Jansson S, Street NR (2008) LAMINA: a tool for rapid quantification of leaf size and shape parameters. BMC Plant Biol 22(8):82
154. Nagel KA, Kastenholz B, Jahnke S, van Dusschoten D, Aach T, Mühlich M, Truhn D, Scharr H, Terjung S, Walter A, Schurr U (2009) Temperature responses of roots: impact on growth, root system architecture and implications for phenotyping. Funct Plant Biol 36:947–959
155. Kasper TC, Ewing RP (1997) ROOTEDGE: software for measuring root length from desktop scanner images. Agron J 89:932–940
156. Berzin I, Cohen B, Mills D, Dinstein I, Merchuk JC (2000) RHIZOSCAN: a semiautomatic image processing system for characterization of the morphology and secondary metabolite concentration in hairy root cultures. Biotechnol Bioeng 70:17–24
157. French A, Ubeda-Tomás S, Holman TJ, Bennett MJ, Pridmore T (2009) High-throughput quantification of root growth using a novel image-analysis tool. Plant Physiol 150:1784–1795
158. Armengaud P (2009) EZ-Rhizo software: the gateway to root architecture analysis. Plant Signal Behav 4:139–141
159. Wang L, Uilecan IV, Assadi AH, Kozmik CA, Spalding EP (2009) HYPOTrace: image analysis software for measuring hypocotyl growth and shape demonstrated on Arabidopsis seedlings undergoing photomorphogenesis. Plant Physiol 149:1632–1637
160. Van As H (2007) Intact plant MRI for the study of cell water relations, membrane permeability, cell-to-cell and long distance water transport. J Exp Bot 58:743–756
161. Jia X, Mendu V, Tang G (2010) An array platform for identification of stress-responsive MicroRNAs in plants. Methods Mol Biol 639:253–269
162. Tardieu F, Tuberosa R (2010) Dissection and modelling of abiotic stress tolerance in plants. Curr Opin Plant Biol 13:206–212

Agroecological Basis for Managing Biotic Constraints

Claudio M. Ghersa
IFEVA-CONICET, Depto. de Recursos Naturales y Ambiente, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina

Article Outline

Glossary

Abiotic factor A nonliving component of the environment, such as soil, nutrients, light, fire, or moisture.

Adaptation (1) Any aspect of an organism or its parts that is of value in allowing the organism to withstand the conditions of the environment. (2) The evolutionary process by which a species' genome and phenotypic characteristics change over time in response to changes in the environment.

Agroecology The science of applying ecological concepts and principles to the design and management of sustainable agroecosystems.

Agroecosystem An agricultural system understood as an ecosystem.

Agroforestry The practice of including trees in crop- or animal-production agroecosystems.

Allelopathy An interference interaction in which a plant releases into the environment a compound that inhibits or stimulates the growth or development of other plants.

Beneficial insects – arthropods Beneficial insects are predators, parasites, or competitors of insect pests, helping to regulate pest populations without harm to crops.

Biomass The mass of all the organic matter in a given system at a given point in time.

Biotic factor An aspect of the environment related to organisms or their interactions.

Competition An interaction in which two organisms remove from the environment a limited resource that both require, and both organisms are harmed in the process. Competition can occur between members of the same species and between members of different species.

Consumer An organism that ingests other organisms (or their parts or products) to obtain its food energy.

Decomposer A fungal or bacterial organism that obtains its nutrients and food energy by breaking down dead organic and fecal matter and absorbing some of its nutrient content.

Disturbance An event or short-term process that alters a community or ecosystem by changing the relative population levels of at least some of the component species.

Diversity (1) The number or variety of species in a location, community, ecosystem, or agroecosystem. (2) The degree of heterogeneity of the biotic components of an ecosystem or agroecosystem (*see ecological diversity*).

Domestication The process of altering, through directed selection, the genetic makeup of a species so as to increase the species' usefulness to humans.

Dominant species The species with the greatest impact on both the biotic and abiotic components of its community.

Ecosystem A functional system of complementary relations between living organisms and their environment within a certain physical area.

Generalist A species that tolerates a broad range of environmental conditions; a generalist has a broad ecological niche.

Habitat The particular environment, characterized by a specific set of environmental conditions, in which a given species occurs.

Herbaceous Nonwoody.

Herbivore An animal that feeds exclusively or mainly on plants. Herbivores convert plant biomass into animal biomass.

Host An organism that provides food or shelter for another organism.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Intercropping Planting more than one crop in a field using a regular pattern that interleaves each crop in some pattern. A form of polyculture.

Integrated pest management Pest control using an array of complementary approaches including natural predators, parasites, pest-resistant varieties, pesticides, and other biological and environmental control practices.

Legume A plant in the Leguminosae (Fabaceae) family. Most species in this family can fix nitrogen.

Microclimate The environmental conditions in the immediate vicinity of an organism.

Multi-trophic relationships The organization of feeding and energy-transfer relationships that determine the path of energy flow through a community or ecosystem that involves organisms of different levels.

Mycorrhizae Symbiotic fungal connections with plant roots through which a fungal organism provides water and nutrients to a plant and the plant provides sugars to the fungi.

Organic matter Material containing molecules based on Carbon, usually referring to soil organic matter.

Parasite An organism that uses another organism for food and thus harms the other organism.

Parasitism An interaction in which one organism feeds on another organism, harming (but generally not killing) it.

Parasitoid A parasite that feeds on predators or other parasites.

Patchiness A measurement of the diversity of successional stages present in a specific area.

Patchy landscape A landscape with a diversity of successional stages or habitat types.

Phenotype The physical expression of the genotype; an organism's physical characteristics. Phenology is the study of periodic plant and animal life-cycle events and how these are influenced by seasonal and interannual variations in climate.

Polyculture Cropping systems in which different crop species are grown in mixtures in the same field at the same time.

Predation An interaction in which one organism kills and consumes another.

Predator An animal that consumes other animals to satisfy its nutritive requirements.

Primary production The amount of light energy converted into plant biomass in a system.

Productivity The ecological processes and structures in an agroecosystem that enable production.

Seed bank The total seed presence in the soil.

Shifting agriculture Farming systems that alternate periods of annual cropping with extended fallow periods. "Slash and burn" systems of shifting cultivation use fire to clear fallow areas for cropping.

Species richness The number of different species in a community or ecosystem.

Successional stages A condition characterized by a particular community of a succession, which is the process by which one community gives way to another.

Definition of the Subject

Agroecology provides guidelines to develop diversified agroecosystems that take advantage of the effects of the integration of plant and animal biodiversity. From a management perspective, the agroecological objective is to provide balanced environments, sustained yields, biologically mediated soil fertility, and natural pest regulation through the design of diversified agroecosystems and the use of low-input technologies.

Introduction

Constraints to agricultural production may be classified into four basic categories: abiotic, biotic, socioeconomic, and those related to crop management. The origin and importance of each constraint, their associated losses, and opportunities to alleviate them will vary for the crop, the input and management levels employed, and the environmental and socioeconomic characteristics of the broader farming system in which the crop is grown. Agronomists and plant protectionists usually address production constraints by focusing on management and input issues to alleviate yield losses caused by particular biotic factors (weed, pest, or disease) that are frequently overestimated, and when added up, exceed crops' yield potential. Moreover, plant breeders, especially with the recent addition of bioengineering and biotechnological tools, have assumed that better varieties alone are able to alleviate the impact of factors curtailing production. From an appreciation of constraints and losses, solutions or

opportunities have been proposed, prioritized, and placed into an agenda for action involving technology experimentation, training, socioeconomic and policy support, among other types of intervention. However, there is a need for broader more holistic integration that would jointly assess four broad categories of constraint: biotic, abiotic, management, and socioeconomic. In addition, it is important to focus on how, or through which process constraints to production are generated.

A concern with constraints studies that attempt to average out problems and losses over farms, villages, watersheds, or farming systems is the spatial variation encountered in the types and severity of constraints, in their associated losses, and achieved yields. Variation is often substantial and at a small scale (e.g., parts of the same small field and across field types on a farm). This is especially evident for smallholder farming systems that frequently exhibit historically variable and targeted inputs (e.g., fertilizers) and management superimposed on variability in biophysical factors such as soil types, water availability, weed and pest distribution [1].

Agroecology provides the basic ecological principles needed for studying, designing, and managing agroecosystems that are both productive and that are culturally sensitive, socially just, and economically viable. Instead of focusing on one particular component of the agroecosystem, agroecology emphasizes the interrelatedness of all of its components and the complex dynamics of ecological processes including all environmental and human elements. This approach is based on enhancing the habitat both aboveground and in the soil to produce strong and healthy plants by promoting beneficial organisms while adversely affecting crop pests (weeds, insects, diseases, and nematodes). From a management perspective, the agroecological objective is to provide balanced environments, sustained yields, biologically mediated soil fertility, and natural pest regulation through the design of diversified agroecosystems and the use of low-input technologies [2, 3].

Agroecologists recognize that intercropping, agroforestry, and other diversification methods mimic natural ecological processes, and that the sustainability of complex agroecosystems lies in the ecological models they follow. By designing farming systems that mimic nature, optimal use can be made of sunlight, soil nutrients, and rainfall [4]. The assumption is that by assembling a functional biodiversity, it is possible to subsidize key processes in the agroecosystem that impact on ecological services, such as the activation of soil biology, the recycling of nutrients, the enhancement of beneficial arthropods and antagonists (Table 1) [5]. Altieri [6] argues that there is evidence supporting that promotion of biodiversity within agricultural systems is the cornerstone strategy of system redesign, since: (1) higher diversity (genetic, taxonomic, structural, and resource) within the cropping system leads to higher diversity in associated biota, (2) increased biodiversity leads to more effective pest control and pollination, and (3) increased biodiversity leads to tighter nutrient cycling.

Agroecological Basis for Managing Biotic Constraints.

Table 1 Ecological processes to optimize in agroecosystems

• Strengthen natural pest-control system
• Decrease toxicity through elimination of agrochemicals
• Optimize metabolic function (organic matter decomposition and nutrient cycling)
• Balance regulatory systems (nutrient cycles, water balance, energy flow, population regulation, etc.)
• Enhance conservation and regeneration of soil-water resources and biodiversity
• Increase and sustain long-term productivity

There is evidence that agroecological diversified agrosystems improve their adaptive capacity and reduces vulnerability to natural disasters, climate change impacts, and new and emerging environmental and economic system stresses and shocks. This ability of withstanding the impact of factors that may reduce agroecosystem sustainability (systems resilience) can be accomplished through physical, biological, sociocultural, and political means. Aspects such as habitat and crop diversification, in situ conservation of local/indigenous seed and germplasm diversity, maintenance of natural enemies' species diversity, increased carbon sequestration, improved water capture and retention, etc., and diversification of farming systems and local economies; technical, legal, and social support networks for small-scale farmers, rural

communities, and indigenous people that reduce socioeconomic vulnerability and strengthen adaptive knowledge processes, etc., can be listed as means to increase sustainability. A study of 208 agroecologically based projects and/or initiatives throughout the developing world documented clear increases in food production over some 29 million hectares, with nearly 9 million households benefiting from increased food diversity and security. Promoted sustainable agriculture practices led to 50–100% increases in per hectare food production (about 1.71 t per year per household) in rain-fed areas typical of small farmers living in marginal environments; that is an area of about 3.58 million hectares, cultivated by about 4.42 million farmers. Such yield enhancements are a true breakthrough for achieving food security among farmers isolated from mainstream agricultural institutions [7].

Crop rotations, polyculture cover crops; intercropping, crop/livestock mixtures are some of the strategies that have been recognized as useful to restore agricultural diversity in both time and space. These strategies exhibit ecological features that have been recognized by different studies. For example, crop rotations incorporate temporal diversity into cropping systems, providing crop nutrients and breaking the life cycles of several insect pests, diseases, and weed life cycles [8]. Polycultures are complex cropping systems in which more than two crop species are planted within sufficient spatial proximity to result in competition or complementation, thus enhancing yields [9, 10]. Intercropping may include trees and animals creating agroforestry systems or mixed crop/livestock mixtures resulting in enhanced complementary relations between components increasing multiple use of the agroecosystem [11]. Moreover, animal integration in agroecosystems aids in achieving high biomass output and optimal recycling [12]. Cover crops based on the use of pure or mixed stands of legumes or other annual plant species under fruit trees improve soil fertility, enhance biological control of pests, and modify the orchard microclimate [13]. Altieri and Rosset [14] argue that including these, strategies in farming provide for diversified forms of agroecosystems that share the following features:

(a) Maintain vegetative cover as an effective soil and water conserving measure
(b) Provide a regular supply of organic matter adding manure, compost, and promotion of soil biotic activity
(c) Enhance nutrient recycling mechanisms with livestock systems based on legumes, etc.
(d) Promote pest regulation enhancing the activity of biological control agents achieved by introducing and/or conserving natural enemies and antagonists

Agroecosystems and Biological Constraints to Production

Plant and animal domestication and technological innovation processes, occurring at different rates depending on ecological and social factors during the expansion of agricultural land use, resulted in the development of sophisticated agricultural systems, which included various types of fallow-crop rotation farming, irrigation, land terracing, soil amendment and fertilizing [15, 16]. In these man-made systems tuned in novel ecosystems, farming became increasingly dependent on labor and capital (technological developments) as intensification maintained or even increased outputs. The population density of domestic animals and humans (slaves and peasants) available to satisfy production needs, such as planting, harvesting, and controlling weeds, became an important factor limiting yields and an important destination for agricultural products. Therefore food and fiber demands were not only driven by the nonagricultural fraction of the society, but were also increasingly demanded as labor populations grew to intensify land production. The social structural changes together with modern technological developments, such as steam and internal combustion engines that occurred since the beginning of industrialization made possible the replacement of animal and human labor by machinery and traditional by scientific agriculture. Mechanization of farming reduced the time needed to perform activities and was the means for augmenting the loads of energy inputs to the agricultural system in a way that increased productivity as never seen before. Postindustrial intensification that occurred during the twentieth century in most of the more developed countries, known as "The Green Revolution," became so efficient that the continuous increase in demands for food and fiber by the world's

population could be satisfied despite that during the century's last decades agricultural land shrank [16].

Food supply and human population growth are related, therefore farming intensification process can be seen as following predator prey pattern of change over time, in which population grows following the increase in food supply, and food supply follows labor/energy inputs to the agroecosystem. However, studies on agricultural development have shown that as society builds up its knowledge base through science and other methods to produce technological innovations, they may further increase yield through intensification of farming practices, even when food demands were well below supply, so intensive farming can be pushed just by technological developments [16]. Paradoxically, once expansion of agriculture stops and technological developments give way to intensification of production, technology innovation may at the same time reestablish the expansion model for agricultural production.

This traditional expansion–intensification model has typified agricultural production systems for the first 75 years of the last century. Gain of agricultural land to the sea by means of polders and drainage systems, the use of river damming to build irrigation oasis into desert lands, transformation of tropical rain forests into coffee, tea, sugar cane, and soybean areas, wetland drainage to produce annual crops are all examples on how technology expanded cropped lands. Perhaps the most interesting example on how technology may help in reestablishing the expansion model is the development of improved cultivars by means of plant breeding programs aimed at increasing crop tolerance to stress factors such as high or low temperatures, drought, pests, and disease and soil salinity. Once these characteristics are bread into crops, there is an expansion in the environmental ranges in which the crops are planted. Shifting agriculture and expansion of agricultural frontiers enabled the incorporation of highly fertile lands into production. These activities molded self-designed novel ecosystems [17, 18], the agroecosystems, which compensated and many times overcompensated the yield reductions due to soil erosion and increase in problems related to biotic constraints to production (weeds, pests, and diseases) that appeared in sites with longer agricultural histories [19, 20]. When settlements became fixed and expansion rate was reduced, intensification originally depended on the generation of spontaneous technological developments. Later, and especially in modern farming, agricultural and agronomy schools became nodal institutions integrating different and somehow related disciplines to generate the technological solutions to the continually emerging problems for agricultural production. Technological approaches tend to engineer and construct new systems, which use practices, like soil plowing, to destroy the natural vegetation and weeds creating the conditions for crop establishment and growth.

As discussed before, these new systems induced important modifications in the human social and economical structures. Individual and collective reactions to these changes repeatedly appeared in different sites and historical times, sometimes sustained on religious dogmas, others on philosophical views, as naturalism, or on scientific disciplines, such as ecology, that became important for modern societies. Integrated pest management (IPM) and agroecology originated as a reaction challenging the modern industrial farming practices. It emerged as a scientific-based philosophical view that questions the modern expansion–intensification agricultural model and its associated dogma of production at any cost. In the last decades, hundreds of research projects and technological development attempts, aimed at environmentally prone management, have taken place delivering significant information. However, the thrust is still highly technological, and focus is on alleviation or suppression of limiting factors or the symptoms disregarding malfunction of the agroecosystem.

Biotic constraints to agricultural production are broadly identified as weeds (plants), pests (animals), and diseases (fungi and bacteria), and their related biological interactions (namely, competition, herbivory, and predation and parasitism), which cause reductions in physical yields or yield quality. The prevalent philosophy is that pests, nutrient deficiencies, or other factors are the cause of low productivity, as opposed to the view that pests or nutrients only become limiting if conditions in the agroecosystem are not balanced [21]. This understanding of how production is sustained has diverted agriculturists from realizing that limiting factors only represent symptoms of a more systemic disease inherent to unbalances within the agroecosystem

and from an appreciation of the context and complexity of agroecological processes thus underestimating the root causes of agricultural limitations [6].

Pest, disease, and weed problems are strong site- and time-specific imposing a biotic constraint on a crop dimensions. The crop-loss impacts of one organism growing in a location may be entirely different from the losses incurred by the same organism on the same crop in other locales. Further, organism population dynamics, migration, invasion, and damage are driven by local conditions, such as temperature and rainfall, but also by the ecosystem and community complexities. Recognition of the systemic dimensions of biological constraints to production is needed to understand how climate–soil interactions, topography, natural, ecological, and land-use complexity, agricultural history, and cropping practices may determine their occurrence, importance, and frequency [22].

Agroecosystems that have evolved in tropical and subtropical climates have high levels of complexity, including many organisms and multi-trophic interactions. Those that have evolved under temperate or Mediterranean climates may also show complexity, but frequently are structurally simpler and may show strong seasonal variation caused by changes in resource availability during the cold/dry season that stops primary productivity. Topography may cause environmental gradients or patchiness adding complexity at regional and landscape scales. Agricultural land-use history, however, at both regional and landscape scales are important drivers of the agroecosystem complexity and organization of the communities that occur in the different crops. Agriculture in many regions of Europe, Middle-East, Asia, Andean regions in South America, and Central America has been practiced in the same sites through millennia, while in others, covering large extensions of Australia, South and North America, expansion of agriculture has occurred within the last centuries. During the last decades, land under agriculture in some of these regions with relatively new agroecosystems has shrunk, and intensification occurred in those areas that remained under cropping. In other regions, such as those in the Mato Grosso, Cerrado, and Chaco from Brasil, Paraguay, and Argentina, natural systems have been turned into cropland recently, and this transformation is still happening. These differences in agricultural histories are related to important differences in the organization of the biotic interactions in the agroecosystem. Different selection pressures occurring in agroecosystems select for different adaptations, which include coevolution of crops and organisms that challenge them as well as cropping practices [23]. Therefore, arable land complex communities have evolved in regions with long agricultural histories. These communities have strong interactions among organisms. Communities that were assembled in apparently homogeneous agroecosystems under long periods of monocultures can reach high levels of complexity. For example, Javanese rice fields, which are cropped as simple rice monocultures, can support large numbers of arthropods summed by 765 species that are important for biocontrol [24]. Vegetationally simple cropping systems in the UK have been shown to develop complex belowground biodiversity that may include 100 species of bacteria, 350 species of protozoa, 140 species of nematodes, and 24 distinct types of arbuscular mycorrhizae [25].

Size and variability in the above- and belowground community structures that functionally support the agroecosytem is important to discuss biotic constraints to production and the agroecological basis for managing them. The more complex and diverse ecosystems may sustain greater productivity and stability in ecosystem functions since they are supported by more than one species, and are then less vulnerable to changes in the populations of a particular species due to environmental stress or pest attack. However, complex biotic systems may also deliver more organisms, which may challenge the crop increasing the importance and stability of biotic constraints [26]. There are numerous studies showing that increased plant diversity enhances biological pest control [27, 28], but counter examples also exist where pests or disease levels increase due to the provision of highly palatable species or changes in canopy microclimate [27–30].

Many of the species originated in regions with long histories of agriculture, and even whole communities have migrated into regions in which crops and agroecosystems have recently expanded. In this way, the flora and fauna of the new agricultural areas of the world such as those in the Argentinean Pampas are dominated by species from the Mediterranean-European agricultural communities. In these new

areas, arable land communities may show instability due to the continuous introduction of new organisms and species shifts [18, 31, 32]. Changes in trading systems, land use, and cropping practices, experienced by many regions with long agricultural history, are enhancing instability of biotic communities caused by addition of organisms (*biotic invasions*), extinctions, and species shifts due to change in landscape [33]. The impacts of biotic complexity have significance, especially when cropping systems are simplified to monocultures, as many organisms would impose biotic limitations to production. Diverse cropping systems are typical of many traditional agricultural systems found around the world, particularly in risk-prone environments, but also with long histories of agricultural production [6]. It is also in these regions, primarily in the developing world, where the greatest emphasis on improving diverse production systems through intercropping and agroforestry is found. It is clear that there are trade-offs with more diverse agricultural systems, and that the kind of diversity matters greatly, but the question is how to design diverse systems that can meet multiple goals in an acceptable way [22].

System Management

As discussed previously, cultural and technological changes that took place during the second half of the twentieth century impacted on land-use patterns and agroecosystem design especially driven by the adoption of industrialized production technologies and reliance on increasing input loads of agrochemicals to alleviate agricultural production constraints. Despite the success in achieving significant increases in global food and fiber yields and yield stability, paradoxically, increasing environmental damage, loss of biodiversity, and associated traditional knowledge were often experienced in the new agroecosystems. In high-external input agriculture intensification of production due to technological development has increased by augmenting input levels that resulted in increased (biomass) production per unit of land and uniformity of the produce. In many cases, this intensification led to reductions in output stability and resource-use efficiency, and has enhanced overexploitation of natural resource base, and consequently reduced sustainability of agroecosystems [34]. There are many examples of reduction in sustainability by the elimination of growth limitations and of yield reduction factors that induced environmental homogenization and a decrease in genetic variation. Noteworthy are the studies showing that application of biocides to reduce biotic constraints to production impact negatively on organisms that are directly or indirectly beneficial for crop growth [34, 35] and, due to their effects on natural enemies of pests and diseases, further increase the need for biocides control [1].

Agroecological bases for reducing biological constraints to crop production depends on the possibility of managing diversity and disturbance at multiple spatial and temporal scales to use biotic interactions to provide desired agroecosystem service.

Arable Land Biotic Community Constraints

Small Scale Soil disturbance caused by plows and other cultivating devices have been used for centuries in different parts of the world to reduce weed competition as well as for reduction of some pests and diseases. Particular traits are selected by cultural practices in many organisms that survive and reproduce in arable lands and show functional patterns that can be identified [36]. Selection pressures imposed by agricultural practices may be sufficiently strong over extended periods of time so that weed populations can evolve into more competitive populations better adapted to agricultural field conditions than populations from nonagricultural areas [37] or populations from agricultural areas submitted to other type of management. In recent times, management approaches that include reduced tillage have strong impacts on the soil biota of plowed soils. It favors fungal food webs, increases abundance and diversity of predators that can reduce soils weed seed banks, and increases control of pests and diseases. It also changes the environmental conditions reducing germination of seed of many weeds or changing ontogenic processes and phenology of pests and diseases adapted to plowed-cultivated soil conditions. These effects may alleviate biotic constraints especially caused by weeds; its effects are not stable, and shifts in species happen after this practice is adopted for some time. The design and management of the agroecosystems complexity requires an understanding of general system behavior combined with species- and site-specific knowledge.

The performance of agroecological management could be improved since crops were selected in the ecological conditions likely to occur in the low-input farming. Adaptation to these conditions in which biotic interactions are expected to occur and characteristics such as increased competitiveness to weeds, disease, and pest resistance, enhanced ability to support beneficial rhizosphere microorganisms, and improved capacity to access soil nutrients could all potentially benefit crop growth. Noteworthy is that selecting for such traits and acquiring the necessary knowledge to understand the structure of particular agroecosystem structure dynamics can only be accomplished with long agricultural history to provide for coevolution and the existence of relatively strong interactions. In addition to this, if such a mix of characteristics can be identified, selecting it would be difficult and time consuming. Probably a more practical alternative to adaptation of a single crop to a complex biotic system would be the use of mixtures of cultivars within a field to improve crop production and disease management [38, 39].

Cropping systems may include crop sequences to favor beneficial biotic interactions, such as the use of crops that enhance the populations of beneficial rhizobacteria that reduce diseases and nematodes [40], accompanied by soil cultivation, fallow, and residue management practices aimed at maintaining soil cover and organic matter as well as arbuscular michorrhiza populations [41] and weed suppression [42]. Competitive crops species or mixtures may be used to reduce the impact of weeds on crop yields. However, holistic management approaches aimed at reducing weeds may integrate the use of other strategies such as manipulating crop seeding density and spatial arrangement, tillage, intercropping, use of allelopathic residues and suppressive mulches, and targeted use of biocontrol agents that reduce weed growth and fecundity during the growth cycle may be integrated in holistic weed management approaches. Also, various soil management techniques aimed at reducing weed seed survival such as reduced tillage, residue management, and organic matter inputs can be used and integrated with the use of weed-suppressive crop rotations that may also impact on seed survival due to enhanced seed predation or infection by pathogens [39].

In agricultural systems, species are selected to obtain yields but can also be used to manage diversity aiming at encouraging beneficial interactions and minimizing undesired ones [43]. For intercropping systems, species are usually chosen considering species or varieties that differ in rooting patterns, canopy types, phenology, etc., to avoid or reduce negative interference or that have positive interactions such as with the introduction of a legume. Well-designed intercrops can increase overall productivity [10, 44] and potentially reduce risk to farmers. A regional-scale field experiment with rice production in China shows how even a small increase in diversity can have a large impact on system function. In that experiment, simply interplanting two varieties of rice, rather than planting them in separate fields, led to a dramatic reduction in pest problems and pesticide use [45].

Large Scale As scale increases, so does the relative importance of species richness because greater numbers of species are needed for the maintenance of ecosystem functions [26]. Loss of diversity in agricultural landscapes has been linked to the disruption of ecological functions such as pest management, pollination services, resistance to plant invasion [46]. Spatial scale is particularly important for both occurrence of pest-related yield constraints to production and for management, because landscape features affect species interactions, microclimates, and weather patterns [24, 46–48]. Landscape structure and dynamics can have notable effects on pests by changing habitat patterns and immigration rates [46, 49–53]. In many regions of the world, agriculture shares space with other land uses forming structural mosaic of habitats with insects and other mobile organisms moving between them [33]. The development of multi-trophic arthropod communities depends on spatial processes (dispersal and foraging) that occur at larger scales than the farm, as well as temporal processes such as overwintering and reproduction. Habitat fragmentation caused by farming can disrupt both types of process and isolate small natural enemy populations from one another, increasing local extinctions [54].

Several biotic constraints to production could be alleviated if biological control maintained following several strategies that help to enhance indigenous populations of beneficial insects by providing food

resources (host prey, pollen and nectar, alternate prey) and shelter for overwintering. Habitat management involves vegetation diversification at multiple scales [55]. Use of insectary plantings or leaving strips of unharvested plants are examples of infield strategies, whereas wildflower borders, grassy buffer strips, windbreaks, and hedgerows are examples of field margin diversification techniques. Larger-scale distribution and connectivity of landscape features such as hedgerows, habitat fragments, and riparian vegetation can also impact levels of biological control as well as provide biodiversity conservation benefits [56]. Interplanting crops with flowering herbaceous plants is promoted as a farmscaping technique since pollen and nectar are essential to the fecundity and longevity of several natural enemy species [57, 58]. Planting of multispecies hedgerows along the edges of farm fields can provide stable habitat and resources for beneficials while fields are bare or crops are young. Biological control may not be enhanced by hedgerows if the availability of pollen and nectar is so high within the hedgerows so that natural enemies do not disperse into adjacent agricultural fields to feed on crop pests [59]; or if the hedgerow attracts new pests, non-pest prey that natural enemies prefer over the crop pest; or top predators that prey on the natural enemies of interest [60–63].

Natural enemy dispersal ranges, which can vary from a few meters to over a kilometer for some parasitoid species [64], will determine the effectiveness of various habitat patterns at enhancing biological control. Blackberry and prune trees provide habitat for alternative hosts of the parasitic wasp, *Anagros epos*, which later preys upon the vineyard leafhopper pest, *Erythroneura elegantula* [65, 66], but connecting border plantings to infield floral corridors may encourage greater natural enemy movement and biological control in vineyards [63]. Successful conservation biological control relies upon matching vegetational scale and pattern to the movement range of desired natural enemies in relation to their primary food sources. This requires an expansion beyond habitat management at the field level to incorporate larger landscape patterns and processes, a still relatively unexplored area. In addition to the size and distance between habitat patches, the "matrix" between patches is important for insect movement [67]. Many species that live in habitat patches also utilize resources outside the habitat patch, which is a desirable attribute for biological control allowing for natural enemies to migrate into agricultural fields. Structurally complex landscapes have been found to lead to higher levels of parasitism and lower crop damage [68, 69]; but this is not always the case even within the same region if parasitism rates also depend upon the presence of particular species or plant communities [70, 71].

Various strategies for increasing vegetation diversity within crop fields, including tolerating low levels of weed infestation and intercropping, have been exploited to reduce the density of herbivores attacking crops. Crops within diverse assemblages can be "harder to find and easier to lose" by herbivores, and better protected by natural enemies [72]. Diversified vegetation in field margins and mosaic patches across landscapes can support natural enemies that move into crop fields and provide biocontrol of pest herbivores [71]. Vegetation diversity may also affect management of phytopathogens.

Weed consumption by herbivores could be increased by reducing chemical pesticides to control agricultural pest and weeds, and then adopting resistant or repellent crops to generalist consumers found in agroecosystems. This management practice has been successful with some crop species toxic or deterrent to insects, negatively impacting weeds mainly consumed by herbivores [73]. This also is a good example on how food webs may be reorganized in response to human intervention. Native species of insects, rodents, birds, and other organisms can consume large numbers of weed seeds and promote reductions in requirements for chemical control tactics [74]. Interspersed strips of diverse, phenologically dissimilar crops may better conserve populations of weed seed consumers than crop monocultures [75]. Specialist pathogens have been used to suppress several weed species in pasture and rangelands, but in annual crops, the difficulty of maintaining appropriate environmental conditions for host infection and the need to achieve rapid weed suppression in a narrow time window has impeded the use of this approach [76].

Meadow strips enhanced pollinator diversity and plant reproductive success in adjacent fields in Switzerland [77]. Similarly, in Costa Rica, proximity to forested areas increased pollination and yield in coffee orchards [78]. Weeds in crop fields supply food for

many bird species [79], and hedgerows and other forms of field margin vegetation supply both habitat and food for wildlife species [80].

Timing and intensity of agricultural management such as the way in which soil cropping or irrigating activities are designed may alter habitat distribution and connectivity across the landscape that are very important for maintaining viable populations of different organisms [59, 61, 81]. For example, asynchronous tillage is important for maintaining beetle populations in arable cropland [82] as well as asynchronous flooding is important for sustaining natural enemy populations in rice fields [24]. Initial studies also found that creating a mosaic of crop fields and wetlands in different successional stages had great promise as a strategy for improving waterfowl habitat and sustaining crop production in a multiuse landscape [83]. Interestingly, maintaining rice fields flooded through the winter for waterfowl foraging habitat also provided beneficial agronomic impacts by increasing decomposition of rice straw and reducing grassy weed biomass [84]. Further, numerous studies have shown the interactive effects of landscape complexity and the impacts of agricultural management practices, with more benign practices (such as organic farming) having the greatest effects on increased biodiversity in simple landscapes [46, 85, 86].

Conclusions

Our understanding of biotic interactions taking place in agroecosystems and how they relate to production constraints is growing rapidly, aided by agroecological approaches and the integration of ecological methodologies and ecologists into agricultural research. Numerous studies provide data allowing for the characterization of agrosystems that reduce the importance of biological constraints to production. They include great spatiotemporal diversity of crops, discontinuity in monoculture (rotations, early varieties, etc.), a mosaic of small fields to ensure the juxtaposition of cultivated and noncultivated land, the presence of a dominant perennial crop (especially orchards), crops grown with high sowing density to limit weed populations, great genetic diversity in the crops (varieties grown in mixed or alternate rows of crops). Based on them, recommendations can be made concerning the management of cultivated plants and the choice of cropping techniques that consider the spatiotemporal dimension of cropping lands, the composition and abundance of the indigenous flora in and around the fields, soil type, the nature of the environment, and the type of farm.

Future Directions

It is clear that agroecosystems are self-designed, and their outputs are controlled and depend on the biotic interactions that evolve as they exist. If managing biotic constraints for crop production is to be sustained on agroecological bases, there is a need to understand how agricultural communities are structured taking into consideration the effects of diversity, species composition, and food web structure on ecosystem processes; the impacts of timing, frequency, and intensity of disturbance (at different complexity levels, i.e., whole ecosystem, community, population); and the importance of multi-trophic interactions. All of these aspects have to be observed and explicitly integrated at multiple spatial and temporal scales. The potential for a greater use of agroecological management approaches is high. However, given the variability of biological phenomena, the implementation of the agroecological strategy requires a planned spatiotemporal farm management. However, owing to the nature of these self-assembled ecosystems, there is some inherent unpredictability about responses to different management interventions. Effective synthesis of complex and often apparently contradictory information is still needed. Field-based research that includes monitoring of species performance, along with social learning mediated by farmer-researcher collaborations may help in this task.

Bibliography

1. Almekinders CJM, Fresco LO, Struik PC (1995) The need to study and manage variation in agro ecosystems. Neth J Agric Sci 43:127–142
2. Gliessman SR (1998) Agroecology: ecological processes in sustainable agriculture. Ann Arbor Press, Michigan
3. Altieri MA (1999) The ecological role of biodiversity in agroecosystems. Agric Ecosyst Environ 74:19–31
4. Pretty JN (1994) Regenerating agriculture. Earthscan, London
5. Altieri MA, Nicholls CI (1999) Biodiversity, ecosystem function and insect pest management in agroecosystems. In: Collins WW, Qualset CO (eds) Biodiversity in agroecosystems. CRC Press, Boca Raton, pp 69–84

6. Altieri MA (2002) Agroecology: the science of natural resource management for poor farmers in marginal environments. Agric Ecosyst Environ 93:1–24
7. Pretty J, Hine R (2000) Feeding the world with sustainable agriculture: a summary of new evidence: final report from "Safe-World" Research Project. University of Essex, Colchester
8. Sumner DR (1982) Crop rotation and plant productivity. In: Recheigl M (ed) CRC handbook of agricultural productivity, vol I. CRC Press, Boca Raton
9. Francis CA (1986) Multiple cropping systems. Macmillan, New York
10. Vandermeer J (1989) The ecology of intercropping. Cambridge University Press, Cambridge/London
11. Nair PKR (1982) Soil productivity aspects of agroforestry. ICRAF, Nairobi
12. Pearson CJ, Ison RL (1987) Agronomy of grassland systems. Cambridge University Press, Cambridge
13. Finch CV, Sharp CW (1976) Cover crops in California orchards and vineyards. USDA Soil Conservation Service, Washington, DC
14. Altieri MA, Rosset P (1996) Agroecology and the conversion of large-scale conventional systems to sustainable management. Int J Environ Stud 50:165–185
15. Chambers R (1983) Rural development: putting the last first. Longmans Scientific and Technical/Wiley, Essex, England/ New York, p 218
16. Vasey DE (1992) An ecological history of agriculture 10, 000 BC–AD 10, 000. Iowa State University Press, Ames
17. Jorgensen SE, Nielsen SN (1996) Application of ecological engineering principles in agriculture. Ecol Eng 7:373–381
18. Hobbs RJ, Salvatore A, Aronson J, Baron JS, Bridgewater P, Cramer VA, Epstein PR, Ewel JJ, Klink CA, Lugo D, Norton DO, Richardson DM, Sanderson ES, Valladares F, Villa M, Zamora R, Zobel M (2006) Novel ecosystems: theoretical and management aspects of the new ecological world order. Glob Ecol Biogeogr 15:1–7
19. Cox GW, Atkins MD (1979) Agricultural ecology: an analysis of world food production systems. W. H. Freeman, San Francisco, p 731
20. Cox CM, Garrett KA, Bockus WW (2005) Meeting the challenge of disease management in perennial grain systems. Renewable Agric Food Syst 20:15–24
21. Carrol CR, Vandermeer JH, Rosset PM (1990) Agroecology. McGraw-Hill, New York
22. Shennan C (2008) Biotic interactions, ecological knowledge and agriculture. Philos Trans R Soc Lond B Biol Sci 363:717–739
23. Ghersa CM, Roush ML, Radosevich SR, Cordray SM (1994) Coevolution of agroecosystems and weed management. Bioscience 44:85–94
24. Settle WH, Ariawan H, Astuti EH, Cahyana W, Hakim AL, Hindayana D, Lestari AS (1996) Managing tropical rice pests through conservation of generalist natural enemies and alternative prey. Ecology 77:1975–1988
25. Fitter AH, Gilligan CA, Hollingworth K, Kleczkowski A, Twyman RM, Pitchford JW (2005) Biodiversity and ecosystem function in soil. Funct Ecol 19:369–377
26. Hooper DU, Chapin FS III, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setälä H, Symstad AJ, Vandermeer J, Wardle DA (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecol Monogr 75:3–35
27. Andow DA (1991) Yield loss to arthropods in vegetationally diverse agroecosystems. Environ Entomol 20:1228–1235
28. Andow DA (1991) Vegetational diversity and arthropod population response. Annu Rev Entomol 36:561–586
29. Brown BJ, Ewel JJ (1987) Herbivory in complex and simple tropical successional ecosystems. Ecology 68:108–116
30. Prieur-Richard AH, Lavorel S, Linhart YB, Dos Santos A (2002) Plant diversity, herbivory and resistance of a plant community to invasion in Mediterranean annual communities. Oecologia 130:96–104
31. Ghersa CM, León RJC (1999) Succesional changes in the agroecosystems of the rolling pampas. In: Walker LR (ed) Ecosystems of disturbed ground. Elsevier, Amsterdam, pp 487–502
32. Radosevich SR, Holt JS, Ghersa CM (2007) Ecology of weeds and invasive plants. Wiley, New York
33. Baudry J, Poggio SL, Laurent C (2010) Agricultural landscape changes through globalisation and biodiversity effects. In: Primdahl J, Swaffied S (eds) Globalisation and agricultural landscapes: change patterns and policy trends in developed countries. Cambridge University Press, Cambridge, UK, pp 58–70
34. Edwards CA (1990) The importance of integration in sustainable agricultural systems. In: Edwards CA, Lal R, Madden P, Miller RH, House G (eds) Sustainable agricultural systems. Soil and Water Conservation Society, Ankeny, pp 249–264
35. Altieri MA (1987) Agroecology: the scientific basis of alternative agriculture. Westview, Boulder
36. Freckleton RP, Watkinson AR (2002) Are weed population dynamics chaotic? J Appl Ecol 39:699–707
37. Weinig C (2005) Rapid evolutionary responses to selection in heterogeneous environments among agricultural and nonagricultural weeds. Int J Plant Sci 166:641–647
38. Mundt CC (2002) Use of multiline cultivars and cultivar mixtures for disease management. Annu Rev Phytopathol 40:381–400
39. Shennan C (2008) Biotic interactions in agroecosystems. Philos Trans R Soc Lond B Biol Sci 363:717–739
40. Welbaum GE, Sturz AV, Dong ZM, Nowak J (2004) Managing soil microorganisms to improve productivity of agro-ecosystems. Crit Rev Plant Sci 23:175–193
41. Gosling P, Hodge A, Goodlass G, Bending GD (2006) *Arbuscular mycorrhizal* fungi and organic farming. Agric Ecosyst Environ 113:17–35
42. Anderson RL (2004) Sequencing crops to minimize selection pressure for weeds in the central great plains. Weed Technol 18:157–164
43. Moonen AC, Bàrberi P (2008) Functional biodiversity: an agroecosystem approach. Agric Ecosyst Environ 127:7–21

44. Mead R, Riley J, Dear K, Singh SP (1986) Stability comparison of intercropping and monocropping systems. Biometrics 42: 253–266
45. Zhu Y, Fen H, Wang Y, Li Y, Chen J, Hu L, Mundt CC (2000) Genetic diversity and disease control in rice. Nature 406:718–772
46. Tscharntke T, Klein AM, Kruess A, Steffan-Dewenter I, Thies C (2005) Landscape perspectives on agricultural intensification and biodiversity—ecosystem service management. Ecol Lett 8:857–874
47. Shennan C, Pisani Gareau T, Sirrine JR (2004) Agroecological approaches to pest management in the US. In: Pretty J (ed) The pesticide detox, solutions for safe agriculture. Earthscan, London, pp 193–211
48. Wilby A, Thomas MB (2002) Natural enemy diversity and pest control, patterns of pest emergence with agricultural intensification. Ecol Lett 5:353–360
49. Colunga GM, Gage SH, Dyer LE (1998) The insect community. In: Cavigelli MA, Deming SR, Probyn LK, Harwood RR (eds) Michigan field crop ecology, managing biological processes for productivity and environmental quality, Michigan State University Extension Bulletin E-2646. Michigan Agricultural Experiment Station, East Lansing, pp 59–70
50. Purtauf T, Roschewitz I, Dauber J, Thies C, Tscharntke T, Wolters V (2005) Landscape context of organic and conventional farms, influences on carabid beetle diversity. Agric Ecosyst Environ 108:165–174
51. Roschewitz I, Hucker M, Tscharntke T, Thies C (2005) The influence of landscape context and farming practices on parasitism of cereal aphids. Agric Ecosyst Environ 108:218–227
52. Roschewitz I, Gabriel D, Tscharntke T, Thies C (2005) The effects of landscape complexity on arable weed species diversity in organic and conventional farming. J Appl Ecol 42:873–882
53. Schmidt MH, Roschewitz I, Thies C, Tscharntke T (2005) Differential effects of landscape and management on diversity and density of ground-dwelling farmland spiders. J Appl Ecol 42:281–287
54. Kalkhoven JTR (1993) Survival of populations and the scale of the fragmented agricultural landscape. In: Bunce RGH, Ryszkowski L, Paoletti MG (eds) Landscape ecology and agroecosystems. Lewis, Boca Raton, pp 83–90
55. Landis DA, Menalled FD, Costamagna AC, Wilkinson TK (2005) Manipulating plant resources to enhance beneficial arthropods in agricultural landscapes. Weed Sci 53:902–908
56. Burel F, Baudry J, Butet A, Clergeau P, Delettre Y, Le Cœur D, Dubs F, Morvan N, Paillat G, Petit S, Thenail C, Brunel E, Lefeuvre JC (1998) Comparative biodiversity along a gradient of agricultural landscapes. Acta Oecol 19:47–60
57. Jervis MS, Kidd MAC, Fitton MD, Huddleson T, Dawah HA (1993) Flower visiting by hymenopteran parasitoids. J Nat Hist 27:287–294
58. Idris AB, Grafius E (1995) Wildflowers as nectar sources for *Diadegma insulare* (Hymenoptera, Ichneumonidae), a parasitoid of diamondback moth (Lepidoptera, Ypono- meutidae). Environ Entomol 24:1726–1735
59. Bugg RL, Ehler LE, Wilson LT (1987) Effect of common knotweed (*Polygonum aviculare*) on abundance and efficiency of insect predators of crop pests. NAL/USDA Report, Belsville
60. Pollard E (1971) Hedges. VI. Habitat diversity and crop pests, a study of Brevicoryne brassica and its syrphid predators. J Appl Ecol 8:751–780
61. Bugg RL, Pickett CH (1998) Introduction, enhancing biological control—habitat management to promote natural enemies of agricultural pests. In: Pickett CH, Bugg RL (eds) Enhancing biological control: habitat management to promote natural enemies of agricultural pests. The Regents of the University of California, Berkeley, pp 1–23
62. Rosenheim JA, Limburg DD, Colfer RG (1999) Impact of generalist predators on a biological control agent, *Chrysoperla carnea*, direct observations. Ecol Appl 9:409–417
63. Nicholls CI, Parrella M, Altieri MA (2001) The effects of a vegetational corridor on the abundance and dispersal of insect biodiversity within a northern California organic vineyard. Landscape Ecol 16:133–146
64. Corbett A (1998) The importance of movement in the response of natural enemies to habitat manipulation. In: Pickett CH, Bugg RL (eds) Enhancing biological control, habitat management to promote natural enemies of agricultural pests. University of California Press, Berkeley, pp 25–48
65. Doutt RL, Nakata J (1973) The Rubus leafhopper and its egg parasitoid, an endemic biotic system useful in grape pest mangement. Environ Entomol 2:381–386
66. Murphy BC, Rosenheim JA, Granett J, Pickett CH, Dowell RV (1998) Measuring the impact of a natural enemy refuge, the prune tree/vineyard example. In: Pickett CH, Bugg RL (eds) Enhancing biological control, habitat management to promote natural enemies of agricultural pests. University of California Press, Berkeley, pp 297–309
67. Ricketts TH, Daily GC, Ehrlich PR, Michener CD (2004) Economic value of tropical forest to coffee production. Proc Natl Acad Sci USA 101:12579–12582
68. Thies C, Tscharntke T (1999) Landscape structure and biological control in agroecosystems. Science 285:893–895
69. Pullaro TC, Marino PC, Jackson DM, Harrison HF, Keinath AP (2006) Effects of killed cover cropmulch on weeds, weed seeds, and herbivores. Agric Ecosyst Environ 115:97–104
70. Menalled FD, Marino PC, Gage SH, Landis D (1999) Does agricultural landscape structure affect parasitism and parasitoid diversity? Ecol Appl 9:634–641
71. Landis DA, Wratten SD, Gurr GA (2000) Habitat management to conserve natural enemies of arthrop pests in agriculture. Annu Rev Entomol 45:175–201
72. Gurr GM, Wratten SD, Altieri MA (eds) (2004) Ecological engineering for pest management: habitat manipulation for Arthropods. CSIRO, Collingwood, p 244
73. Liebman M, Davis AS (2000) Integration of soil, crop and weed management in low-external-input farming systems. Weed Res 40:27–47

74. Westerman P, Liebman M, Menalled FD, Heggenstaller AH, Hartzler RG, Dixon PM (2005) Are many little hammers effective?—velvetleaf (*Abutilon theophrasti*) population dynamics in two- and four-year crop rotation systems. Weed Sci 53:382–392
75. Heggenstaller AH, Menalled FD, Liebman M, Westerman PR (2006) Seasonal patterns in post-dispersal seed predation of *Abutilon theophrasti* and *Setaria faberi* in three-cropping systems. J Appl Ecol 43:999–1010
76. Ghorbani R, Leifart C, Seel W (2005) Biological control of weeds with antagonistic plant pathogens. Adv Agron 86: 191–225
77. Albrecht M, Duelli P, Muller C, Kleijn D, Schmid B (2007) The Swiss agri-environment scheme enhances pollinator diversity and plant reproductive success in nearby intensively managed farmland. J Appl Ecol 44:813–822
78. Ricketts TH, Daily GC, Ehrlich PR, Fay JP (2001) Countryside biogeography of moths in a fragmented landscape, biodiversity in native and agricultural habitats. Conserv Biol 15: 378–388
79. Marshall EJP, Brown VK, Boatman ND, Lutman PJW, Squire GR, Ward LK (2003) The role of weeds in supporting biological diversity within crop fields. Weed Res 43:77–89
80. Marshall EJP, Moonen AC (2002) Field margins in northern Europe: their functions and interactions with agriculture. Agric Ecosyst Environ 89:5–21
81. Baudry J, Papy F (2001) The role of landscape heterogeneity in the sustainability of cropping systems. In: Nösberger J, Geiger HH, Struik PC (eds) Crop science – progress and prospects. CABI, Oxon
82. Holland JM, Thomas CFG, Birkett T, Southway S, Oaten H (2005) Farm-scale spatiotemporal dynamics of predatory beetles in arable crops. J Appl Ecol 42:1140–1152
83. Shennan C, Bode CA (2002) Integrating wetland habitat with agriculture. In: Jackson LL, Jackson D (eds) The farm as a natural habitat. Island, Washington, DC, pp 189–204
84. van Groenigen JW, Burns EG, Eadie JM, Horwath WR, van Kessel C (2003) Effects of foraging waterfowl in winter flooded rice fields on weed stress and residue decomposition. Agric Ecosyst Environ 95:289–296
85. Benton TG, Vickery JA, Wilson JD (2003) Farmland biodiversity: is habitat heterogeneity the key? Trends Ecol Evol 18:182–188
86. Bengtsson J, Ahnstrom J, Weibull AC (2005) The effects of organic agriculture on biodiversity and abundance, a meta-analysis. J Appl Ecol 42:261–269

Agronomic Interactions with CO_2 Sequestration

Rattan Lal
OARDC/FAES Kottman Hall, Carbon Management and Sequestration Center, The Ohio State University, Columbus, OH, USA

Article Outline

Glossary

Carbon sequestration Transfer of atmospheric CO_2 into long-lived reservoirs

Mean residence time The duration during which CO_2-C resides in a specific pool (pool)

Soil quality Capacity of a soil to perform ecosystem services

Definition of the Subject

Atmospheric concentration of carbon dioxide (CO_2) can be stabilized, if not reduced, by reducing anthropogenic emissions, sequestering emissions, or both. Emission reduction implies identifying and using no-carbon (C) or low-C energy sources such as alternatives to fossil fuel including wind, solar, hydro, geo-thermal, biofuels, etc. Reductions in gaseous emissions can also be achieved by enhancing the energy use efficiency. In agronomic systems, involving practices to raise crops and livestock, enhancing energy efficiency implies a range of practices which increase agricultural productivity per unit input of energy-based resources (i.e., fertilizers, pesticides, irrigation). Another strategy is elimination or reduction in frequency and intensity of tillage operations and converting plow-based tillage to no-till farming or conservation agriculture. Use of solar and wind energy for grain drying, water pumping, and heating buildings can also reduce emissions.

Agricultural soils and ecosystems can also be used for sequestration of atmospheric CO_2 by enhancing photosynthesis, increasing net primary productivity (NPP), and converting some of the NPP into stable biomass (forest products) and the soil C pool. The biomass in forest, with a long mean residence time (MRT), has two distinct but related components: the above-ground biomass and the below-ground biomass. The above-ground biomass can be alive or the detritus material. The photosynthates transferred deep into the subsoil through a tap root system have a long MRT. Agroforestry systems, growing crops and raising livestock in combination with perennials (tree species), can enhance the ecosystem C pool by increasing both biomass-C and soil-C components.

Sequestering C in soil entails increasing soil organic C (SOC) pool and also the soil inorganic C (SIC) pool. The SOC pool has three related components: labile/active pool, intermediate pool, and the passive/recalcitrant pool with MRT of $<$ year, $<$decades to a century, and several millennia, respectively. The goal is to transfer the labile pool into intermediate and preferably passive pools through conversion to conversion tillage, use of manure/compost and other biosolids (biochar), and complex cropping/farming systems. Sequestration of SIC occurs through formation of secondary carbonates. In irrigated systems, however, leaching of bicarbonates is also an important mechanism of SIC transfer into the groundwater. Agronomic practices strongly interact with strategies of reducing emissions and sequestrating CO_2 in soils and biota. Therefore, the strategy of agronomic management is to identify the interactive agronomic practices which enhance ecosystem (biotic and pedologic) C pools.

Introduction

Limiting global warming to 2°C necessitates identification and adoption of diverse strategies which reduce

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

the net anthropogenic emissions of greenhouse gases (GHGs). Agriculture, hitherto a major source of GHGs, is an important industry which can be made more energy-efficient. Importance of improving agricultural production, by as much as 70% between 2010 and 2050, cannot be overemphasized in view of the need to reduce hunger, malnutrition, and poverty. Although a major challenge, making agriculture emission-neutral is a prudent long-term strategy. It is in this context that understanding of the agronomic interactions with CO_2 sequestration in soil is important.

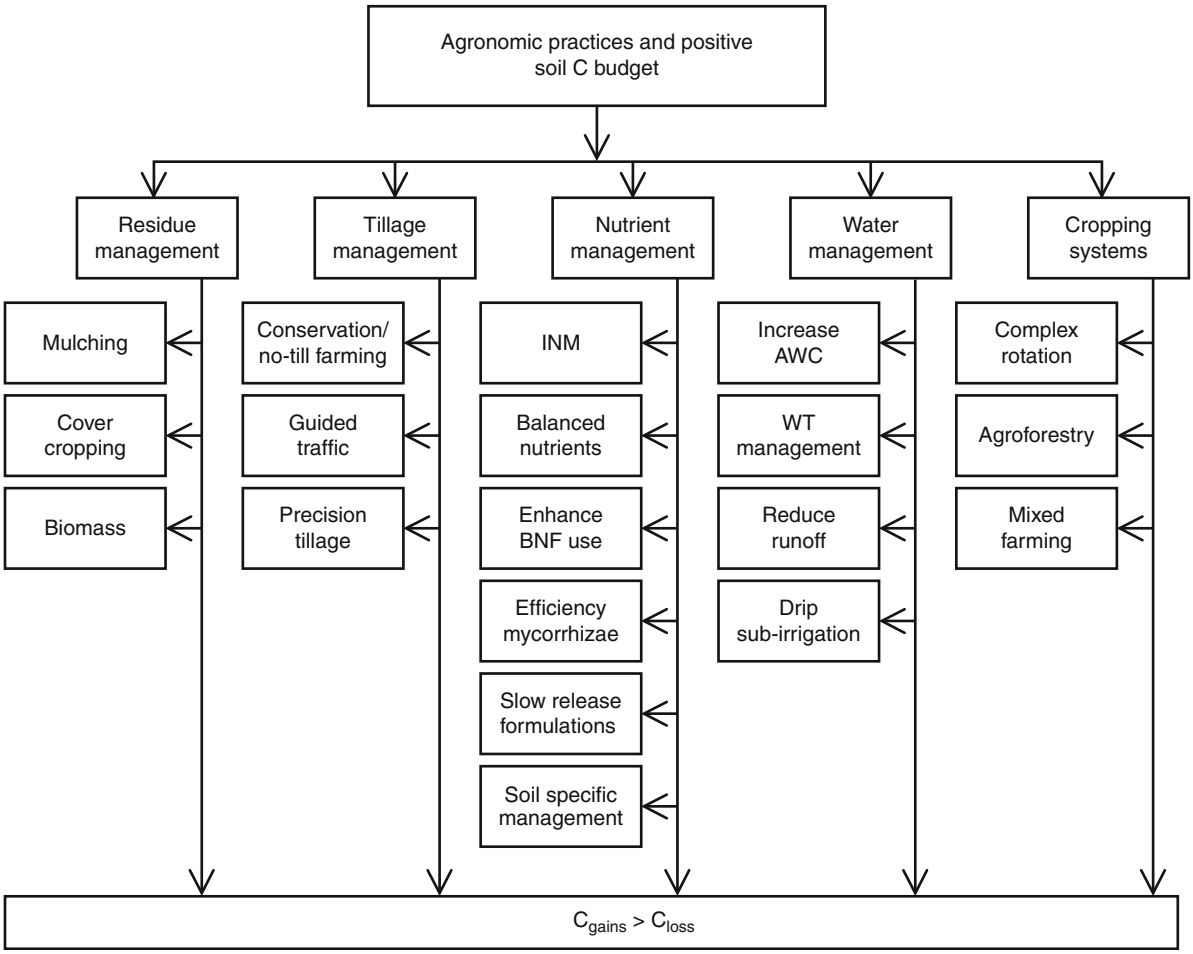
Agronomic systems are defined as site-specific management of soils and crops on the basis of eco-regional and physiographic characteristics, and in the context of socioeconomic and policy environments. These systems are strong determinants of agricultural production, sustainable use of resources, and their environmental impact. Pertinent examples of the environmental impacts of agronomic systems include accelerated soil erosion, nonpoint source pollution, emission of GHGs, and alterations in ecosystem and soil C pools. Most soils of agricultural ecosystems (i.e., croplands, grazing lands) are depleted of their SOC pool. In comparison with the natural/climax vegetation, the remaining SOC pool in agricultural soils may be 20–50% of the antecedent pool under undisturbed conditions [1]. The magnitude of loss is large in soils characterized by high than low antecedent pool, coarse than fine texture, in warm than cold climates, and in degraded/eroded than favorable quality. Furthermore, the magnitude of loss is more from soils managed by extractive farming than of intensive/science-based agriculture. The historic loss is also equivalent to the potential soil C sink capacity, a part of which can be realized through conversion to a restorative land use and adoption of recommended management practices (RMPs).

Sequestration of CO_2 in agroecosystems implies transfer of atmospheric CO_2 into biota and soils through photosynthesis and NPP in a manner such that the biomass-C is neither readily nor immediately returned back to the atmosphere. The strategy is to enhance the MRT of C in biota and soils. Principal determinants of MRT comprise of a wide range of factors including soil processes and plant/biomass characteristics. Important among soil processes are formation of: (1) stable micro-aggregates, (2) organo-mineral complexes including absorption on clay surfaces, and (3) recalcitrant organic polymers involving physical, chemical, and biological protection of soil organic matter (SOM). Another process of physical protection is transfer of SOM into the subsoil by illuviation as dissolved organic (DOC) such that it is away from the surface layer prone to accelerated erosion, intense mineralization, and other natural and anthropogenic perturbations. Important among plant characteristics which increase MRT are: (1) a deep root system and (2) a high concentration of recalcitrant compounds. This chapter is aimed at describing the processes and practices which moderate the agronomic interactions with CO_2 sequestration in soil as a recalcitrant humus of a long MRT.

Soil Carbon Budget

The strategy is to create a positive SOC budget so that input of biomass-C exceeds the losses. Important among agronomic practices which create a positive C budget (Fig. 1) are mulch farming, no-till/conservation agriculture, integrated nutrient management (INM) including slow release formulation of chemical fertilizers and biofertilizers, conservation and management of soil water to reduce losses by surface runoff and evaporation and increase soil-water storage, and use of complex cropping/farming systems including agroforestry and mixed farming systems. The strategy is to replace extractive farming practices, which deplete soil fertility and SOC pool, by science-based agriculture involving the widespread adoption of RMPs. Some RMPs outlined in Fig. 1 are generic, and no one practice is universally applicable because of the extreme diversity of soil types, ecoregions, and the human dimensions related to socioeconomic and political consideration. Site/soil-specific validation and adaption through fine-tuning of RMPs is essential.

While increasing the input of biomass-C, it is equally important to reduce its losses. Agronomic/soil processes which deplete the SOC pool are outlined in Fig. 2. The SOC pool is strongly depleted by accelerated soil erosion. The preferential/selective removal of SOC by runoff and erosion, as indicated by an enrichment

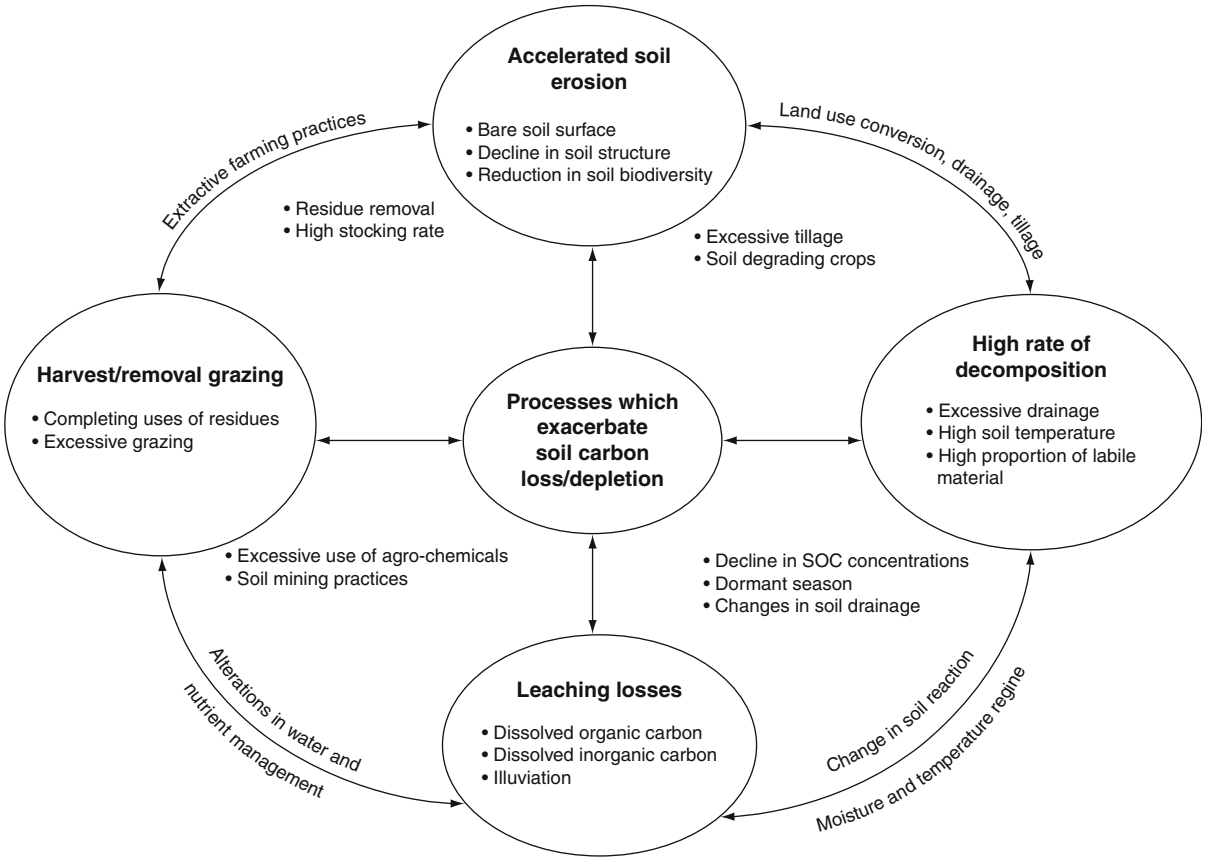


Agronomic Interactions with CO_2 Sequestration. Figure 1

Agronomic interactions which cause positive carbon budget (*INM* integrated nutrient management, *AWC* available water capacity, *WT* water table, *BNF* biological nitrogen fixation)

ratio of C in sediments ranging from 5 to 30 depending on soil/land and climate, is attributed to: (1) low density of SOC, (2) high concentration (stratification) in the surface layer, and (3) absorption on clay and thus removal along with the clay particles. Soil erosion is exacerbated by plowing, residue removal, uncontrolled and excessive grazing, and management practices which degrade soil structure and accentuate its vulnerability to climatic erosivity.

The SOC pool is also depleted by increase in the rate of mineralization. The latter increases with increase in soil temperature and changes in soil moisture regime. Conversion of natural to managed/agricultural ecosystems alters both the soil temperature and moisture regimes and accentuates the rate of mineralization. The latter, being a biochemical reaction, is approximately doubled with every 10°C increase in temperature (Vant Hoff rule). There is an optimal soil moisture regime for the mineralization/decomposition. Water table management and drainage of excessively wet soils increase the rate of mineralization. Excessive wetness increases methanogenesis and denitrification with an attendant increase in emissions of CH_4 and N_2O. In contrast, supplemental irrigation in arid and semi-arid climates can also accentuate mineralization and denitrification. In general, therefore, emissions of GHGs may be more from agricultural soils than those under natural ecosystems.



Agronomic Interactions with CO_2 Sequestration. Figure 2
Agronomic soil processes which deplete the soil carbon pool

Hidden Carbon Costs

Most agronomic inputs, especially in intensively managed systems, are based on use of fossil fuel combustion. Important among these are tillage operations, harvesting, drying, application of fertilizers, and other chemicals (pesticides) and irrigation. The hidden C cost (HCC) of these inputs are listed in Table 1. Agronomic practices with a significant input of HCC are tillage systems, and agricultural chemicals. Thus, a complete Life Cycle Analysis (LCA) is needed to determine the ecosystem C budget and assess the net C gains. The latter can be described by Eq. 1:

$$\text{Net C gain} = C_{input} - (C_{loss} + HCC) \quad (1)$$

C_{input} includes biomass addition such as shoot, leaves, detritus materials, roots, compost, manure, deposition through wind and water, etc. C_{loss} occurs mainly through erosion, decomposition, and leaching. Principal components of HCC are the energy-based inputs. Quantification of each of these components is essential to conducting LCA for specific soil, crop, ecoregion, and other site-specific factors.

It is often argued that HCC should not be deducted from the gross C gains because agronomic inputs (i.e., fertilizers, tillage, pesticides, manure, irrigation, and harvesting) are not used for C sequestration but for achieving food security to meet the food and other demands (i.e., feed, fiber, and raw materials) of the growing population. If HCCs are also considered, land managers/farmers are not adequately rewarded by payments through C trading.

Agronomic Interactions with CO_2 Sequestration.

Table 1 Hidden carbon costs of agronomic practices (Adapted from [2])

Agronomic practice		Carbon cost
I.	*Tillage (kg CE/ha)*	
	Moldboard plowing	15.2 ± 4.1
	Chisel plowing	7.9 ± 2.3
	Disking	8.3 ± 2.5
	Cultivation	4.0 ± 1.9
	Rotary hoeing	2.0 ± 0.9
II.	*Fertilizers (kg CE/kg)*	
	Nitrogen	1.3 ± 0.3
	Phosphorus	0.2 ± 0.06
	Potassium	0.15 ± 0.06
	Lime	0.16 ± 0.11
III.	*Pesticides (kg CE/kg)*	
	Herbicides	6.3 ± 2.7
	Insecticides	5.1 ± 3.0
	Fungicides	3.9 ± 2.2
IV.	*Irrigation (kg CE/ha/year)*	
	Surface	9.4–24.6
	Sprinkler	16.3–121.3
	Trickle	84.9

Farming Carbon

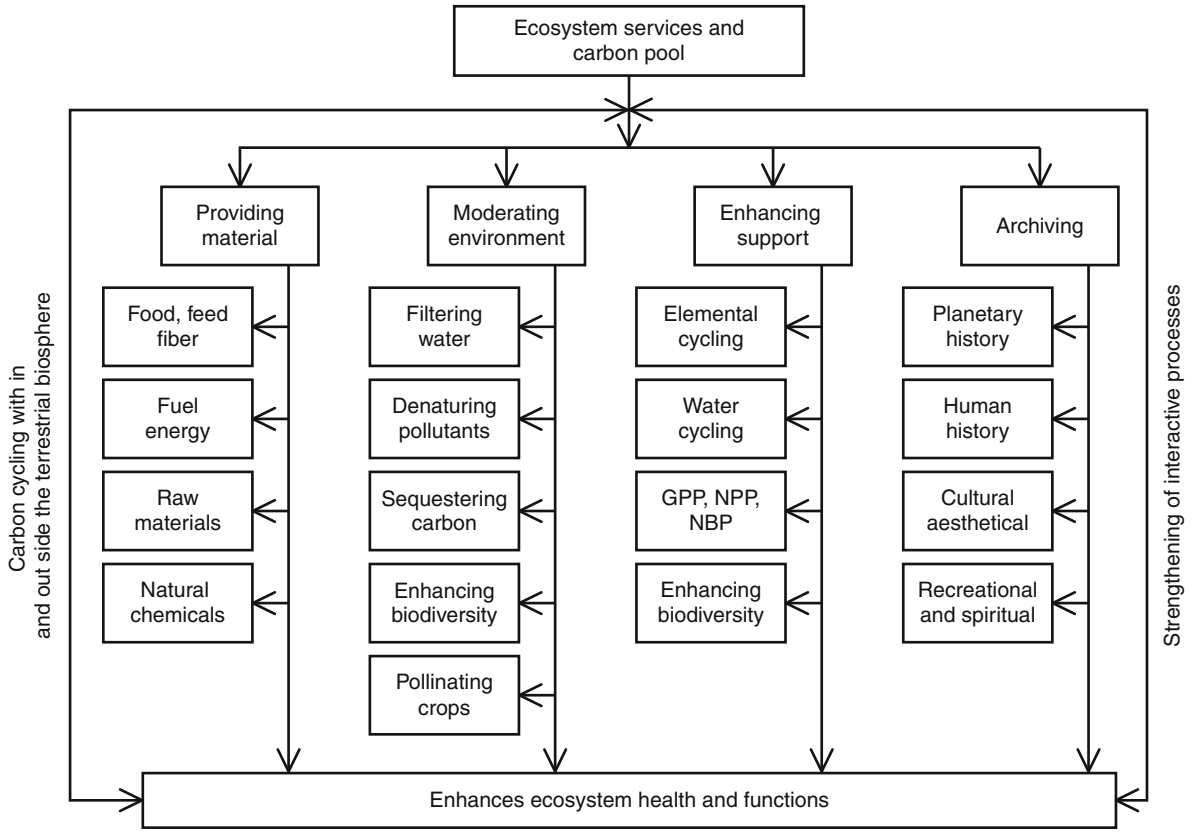
The term "farming carbon" implies growing/increasing C pool in soils and biota (trees) of managed ecosystems (agriculture, forestry, urban lands, wetlands) so that any increase in the ecosystem C pool can be traded in the market as a farm produce. Agronomic interactions, practices which enhance soil and the biotic C pools, are strong determinants of the C gains (or losses) from the ecosystem. Changes in the ecosystem C pool must be monitored by a standardized procedure so that the data are credible, reproducible, and verifiable by other procedures. A protocol for measurement, monitoring, and verification (MMV) is essential to implement C trading.

Ecosystem Services and Soil Carbon Sequestration

Sequestration of C in soils and biota generates and enhances numerous ecosystem services (Fig. 3). Important among these are: (1) providing materials of use to human (i.e., food), (2) moderating the environment (i.e., climate), (3) enhancing support, and (4) archiving human and planetary history [3, 4]. Soil and the ecosystem C pools are strong determinants of these services directly and indirectly. For example, quantity and quality of the SOC pool affect soil functions through (1) increasing soil aggregation and improving soil tilth, (2) increasing nutrient retention and availability, (3) moderating water retention and availability, (4) improving infiltration and reducing water runoff, (5) reducing soil erosion and nonpoint source pollution, (6) providing energy source and food to soil biota, (7) enhancing nutrient/elemental cycling, (8) accentuating use efficiency of input, (9) enhancing rhizospheric processes and micro-climatic environment, and (10) improving GPP and NPP.

Carbon Sequestration and Agronomic Production

It is because of numerous positive effects of organic carbon concentration in the root zone on soil quality that it is a strong determinant of the use efficiency of agronomic input and of crop growth and yield. Depending upon soil type and crop characteristics, there is a threshold value of 15–20 g/kg of SOC concentration in the root zone [5]. Crop growth and yield are strongly reduced when SOC concentration is below the threshold level (Fig. 4, [6, 7]). The yield response to SOC concentration in the root zone also depends on the management. The crop response (i.e., growth and yield) is generally stronger in agronomic practices based on low than high external inputs such as fertilizers, manure/compost, irrigation, etc. Indeed, the yield potential of elite varieties cannot be realized unless soil quality and the related agronomic interactions are optimized. Therefore, enhancing SOC concentration to above the threshold level is essential to improving agronomic yield in depleted/degraded soils of sub-Saharan Africa, South and Central Asia, and elsewhere in regions with low crop yields.



Agronomic Interactions with CO_2 Sequestration. Figure 3
Ecosystem services enhanced by soil carbon sequestration (*GPP* gross primary productivity, *NPP* net primary productivity, *NBP* net biome productivity)

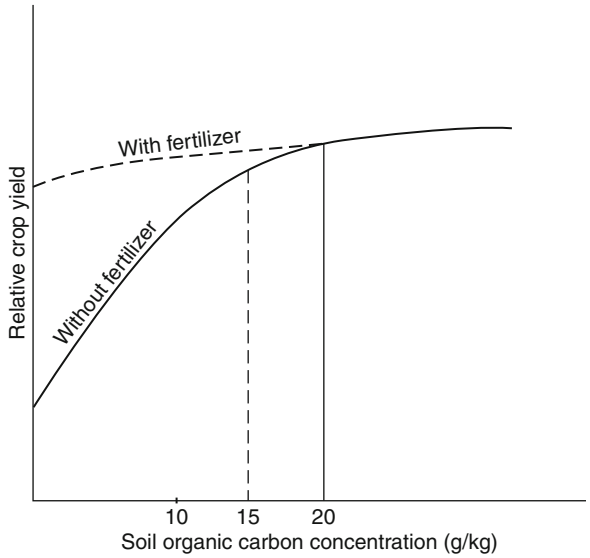
Future Directions

There is a strong need to understand the following:

- Soil processes and agronomic practices which create a positive C budget for diverse ecoregions
- Rate of C sequestration in relation to soil type, climate, and management
- Relationship between soil C pool/concentration and soil quality parameters, and agronomic yield
- Process, factors, and causes which enhance the mean residence time of C in soil
- Threshold value of C concentration for predominant soil type and crops
- Policy interventions to promote "carbon farming"

Conclusions

Creating a positive budget of C (and N) is important to C sequestration in the soil. Agronomic practices to create a positive C budget are those which enhance the inputs of biomass-C. Important among these are conservation/no-till agriculture, mulch farming, cover cropping, integrated nutrient management, harvesting/recycling of water, complex farming systems, and perennial culture. Restoration of eroded/degraded soils and ecosystems is important to C sequestration in the soil. Energy-based inputs (i.e., fertilizers, pesticides, and tillage) have high hidden



Agronomic Interactions with CO_2 Sequestration. Figure 4

A generalized response curve of agronomic yield response of crops to concentration of soil organic matter in the root zone

C costs. The strategy is to minimize losses of these inputs by erosion, leaching, volatilization, etc. Increase in soil organic C pool above the threshold level can enhance crop yield. Improvement in soil quality through increase in SOC pool is essential to increasing crop yields and agronomic production of soils in African, Asian, Caribbean, and the Andean regions.

Bibliography

1. Lal R (1999) Soil management and restoration for C sequestration to mitigate the greenhouse effect. Prog Env Sci 1:307–326
2. Lal R (2004) Carbon emissions from farm operations. Environ Int 30:981–990
3. Daily GC (1997) Nature's services: societal dependence on natural ecosystems. Island Press, Washington DC, 392 pp
4. MEA (2005) Ecosystems and human wellbeing: synthesis, Millennium ecosystem assessment. Island Press, Washington, DC, 155 pp
5. Aune JB, Lal R (1997) Agricultural limits in the tropics and critical limits of properties of Oxisols, Ultisols and Alfisols. Trop Agric (Trinadad) 74:96–103
6. Lal R (2010) Beyond Copenhagen: mitigating climate change and achieving food security through soil carbon sequestration. Food Security 2(2):169–177
7. Lal R (2010) Enhancing eco-efficiency in agroecosystems through soil C sequestration. Crop Sci 50:S120–S131

Animal Breeding and Genetics, Introduction

Ignacy Misztal
Department of Animal and Dairy Science Breeding and Genetics, University of Georgia, Athens, GA, USA

Informal animal breeding started thousands of years ago when hunter-gatherers started domesticating animals. Out of thousands of species available only few were domesticated as the requirements for domestication were numerous: plant diet, fast growth rate, ability to breed in captivity, good disposition, little tendency to panic, and ability to function well in groups [1]. An ancient farmer/herder took special care of well-behaving animals that provided good growth, or plenty of milk, or reliable draft, or lots of wool, etc., while eliminating the troublesome ones. The domestication greatly increased the nutritional output per unit of land although it also brought new problems, e.g., new diseases and wars. These problems were smaller than the benefits of the domestication as hunter-gatherers mostly disappeared. Some of the benefits could be due to a positive effect of animal products on IQ [2].

The natural selection maximizes survival under the natural conditions. Under domestication, the selection maximizes utility of a specie for a farmer while deemphasizing and thus reducing energy expenditure for characteristics less important or unimportant under domestication, e.g., fighting ability to select a mate or defend against predators, ability to cover long distances, etc. [3]. The degree of economically beneficial selection is environment dependent because some traits (e.g., resistance to harsh conditions) may be necessary in some environments while they are redundant in other environments. In the end, the improved animals have a smaller environmental imprint [4].

In ► Animal Breeding Methods and Sustainability, Agustin Blasco provides a historical perspective to animal breeding including the creation of breeds. Although the breeding has been practiced for many millennia, the science behind it is relatively recent. With new breeding tools, the genetic improvement accelerated resulting in much higher productivity per animal with lower cost per unit of animal product. High productivity of a few improved breeds raises a question of survival and consequently conservation of less improved breeds. Also, highly improved breeds may not be optimal in more demanding environments.

In "► Animal Breeding, Foundations of," Guilherme Rosa focuses on theories that made modern animal breeding possible. These are population genetics, quantitative genetics, mixed models, and related issues. He examines the infinitesimal genetic model where it is assumed that a trait is controlled by a large number of independent loci, and he mentions models involving quantitative trait loci (QTL) or major genes. Selection can be for a single trait with possibly undesirable response for some of the remaining traits, or it can be multitrait, where weights on traits are economically derived for a more balanced breeding.

The selection in animal breeding depends on models and often sophisticated computing to estimate parameters of those models. In "► Animal Breeding, Modeling in," Lawrence Schaeffer presents mixed models that are commonly used to analyze many traits in small and large populations. In particular, an animal model considers all phenotypes and pedigrees to provide best unbiased linear predictions of animals' breeding values (EBV). While the basic animal model may be sufficient for fairly accurate predictions, sometimes additional features are necessary to better reflect the complexity of data. Large differences in variability within the environments require a model that accounts for heterogeneous variances per environment, categorical traits are best analyzed by a threshold model; special models are needed to analyze data censored, e.g., by time.

While most of the progress in animal breeding was based on the infinitesimal model, the availability of genetic markers raised hopes of finding major genes. Subsequently, marker-assisted selection (MAS) would allow determining EBV for young animals without waiting for phenotypes. In his entry "► Animal Molecular Genetics from major genes to genomics," Asko Mäki-Tanila describes theories for finding markers or QTL and applications of MAS. While several large QTLs have been found, in general, the estimated values for large QTL seem to be inflated while many QTLs are below the detection level. Thus, the total contribution of large QTLs seems to be small for most traits.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Currently, individuals can be genotyped inexpensively for a large number of SNP markers. The use of these markers for breeding is called the genomic selection. The genomic selection is successful in populations with small effective population sizes where the genomic information provides more accurate relationships.

A selection is usually performed in a specific environment, which is defined as a combination of climate, nutrition, management, and stability. Animals excelling in one environment may perform poorly in another environment and vice versa, or show a genotype by environment interaction (GxE). In "► Animal Genetic in Environment Interaction," Erling Strandberg describes terms useful for quantification of GxE such as plasticity and homeostasis. GxE can be analyzed by reaction norms. We are interested in GxE only when genotypes rank differently in different environments.

The traditional selection is by selecting the "best" individuals. However, these individuals may indirectly be selected for poor group performance, e.g., aggression, and such animals may inhibit the growth of the other animals. In "► Socially affected traits, Inheritance and genetic improvement," Peter Bijma presents cases where the group performance is more important than the individual performance, proposes models that can be used to breed "socially adapted" animals, and describes results of experiments with such breeding. The social performance is most important for animals bred in cages such as poultry and pigs where the aggression can result in physical harm or even death. An intuitively obvious solution to aggression of allowing more space per animal in fact increases fighting and subsequently causes economic losses, as extra space allows for more fighting.

The next few entries are devoted to breeding for specific species.

In the entry ► Poultry Breeding, Yoav Eitan and Morris Soller document how, over the past 100 years, the chicken meat changed from being one of the most expensive to the least expensive. Such a progress required a good choice of initial breeds, intensive selection, and diligent research to discover sources of new problems and their mitigation, and constantly adapting management to address these problems. The success with chicken is possible only when the environment can be tightly controlled as highly adapted animals retain minimal flexibility to handle less than the optimal conditions. An important issue in chicken is animal welfare. While some stresses are unavoidable, like in nature, reducing the avoidable distress can be good economics.

In the entry ► Pig breeding for increased sustainability, Pieter Knapp examines broader issues in breeding using pigs as an example. The first issue is diversity. Should different breeds be preserved or is it unimportant? What is the optimal size of the breeding population to sustain genetic variability and maximize the genetic progress over the long run? Pigs have been singled out as causing environment pollutions. Can breeding minimize the pollution? One way to minimize pollution and environmental damage is to breed efficient animals with good feed conversion ratio (FCR). Can one radically improve FCR? Is animal welfare a liability or an asset? The entry by Pieter Knapp contains extensive references.

Filippo Miglior, Sarah Locker, and Roger Shanks take a look into dairy breeding in their entry ► Dairy Cattle Breeding. An intensive selection for milk made Holsteins the highest producing and the most popular dairy breed in temperate countries. Most of the progress in Holsteins, and to a lesser intent, in other breeds, is through the extensive use of highly select sires through artificial insemination. The selection of sires is in fact global because national sire evaluation from some 30 countries are now pooled together by an international agency "Interbull," and semen of bulls ranked by this agency are available worldwide. Strong selection for production had an undesirable effect on fitness, including reduced fertility and survival. This side effect is remedied now by increased emphasis on fertility and survival in the selection index, and by crossbreeding. Lately, genomic selection greatly changed the breeding scheme in dairy.

Matthew Spangler describes beef breeding in the USA in his entry ► Breeding in Beef Cattle. As opposed to dairy, the beef population consists of many breeds, and most animals sold for beef are crossbreds. Beef is initially raised in ranches and later brought to feedlots for a short time for accelerated growth. Animals are genetically evaluated based on growth at a few age points, for carcass characteristics and for fertility. Important issues in beef evaluation are heterosis and recombination loss in crosses. FCR in beef is much lower than in poultry or pigs; however, beef can utilize land unsuitable for crops.

Thorvadur Árnason, in his entry ► Breeding in Horses, presents issues in breeding horses, predominantly for racing. Such breeding is successful as the trend for maximum speed is positive. As only selected individuals are run in races, breeding for speed requires accounting for censoring, i.e., lack of records for an important part of the population. This is done by treating as phenotype a categorical variable of "has" or "does not have" records. An issue specific for horses is increased inbreeding due to very intensive selection and small populations. Large increase in inbreeding has negative effects on many traits and also increases the chances of propagating a recessive gene.

SWP Cloete looks at aspects of ► breeding in developing countries and tropics. Increased production due to the animal breeding and limited population growth was successful in developing countries in creating surpluses of animal products. In developing countries, the animal breeding was less successful while the population exploded, resulting in shortages of animal products. The breeding was less successful because of specific challenges in developing countries and especially the tropics, and much smaller R&D. Traits especially important in the tropics are disease resistance (including tick and trypanosome), draft resistance, and ability to produce under periodic feed shortages. Subsequently, animals bred in the developed world may not survive in the tropics; however, their crosses sometimes do well. This entry contains a large number of references.

Even though the animal breeding is successful, there is a question whether the current pace of progress can be maintained. For example, FCR cannot be decreased below some 1.5 kg feed/1 kg of meat, unless the extra selection results in increased water content. Also, increased milk production at a cost of reduced fitness (less fertility, lower survival, more susceptibility to diseases) can at one point decrease overall profitability. Peer Berg ponders long-term challenges in animal breeding in his entry titled ► Animal Breeding, Long-term Challenges. Low effective population sizes make whole populations less biosecure. Too optimized genotypes may require huge facilities that could destroy the environment and rural life. Also, new requirements for welfare may require changing breeding goals. However, most long-term challenges are not well known.

Bibliography

1. Diamond J (2005) Guns, germs and steel. W.W. Norton, New York, 518 pp
2. Neumann CG, Murphy SP, Gewa C, Grillenberger M, Bwibo NO (2007) Meat supplementation improves growth, cognitive, and behavioral outcomes in Kenyan children. J Nutr 137(4):1119–1123
3. De Jong G, Van Noordwijk AJ (1992) Acquisition and allocation of resources: genetic (co)variances, selection, and life histories. Am Nat 139:749–770
4. Von Braun J (2010) The role of livestock production for a growing world population. http://www.lohmann-information.com/content/l_i_45_2010_10.pdf

Animal Breeding Methods and Sustainability

Agustin Blasco
Institute of Animal Science and Technology, Universidad Politécnica de Valencia, Valencia, Spain

Article Outline

Glossary

Breed A population of animals with common morphological characteristics that is recognized as a breed by the administration, by a breeders association, or by other groups of people.

Best linear unbiased prediction (BLUP) It is the most common statistical method used in breeding evaluation.

Quantitative trait locus (QTL) A gene having influence in a quantitative trait.

Markers Fragments of the DNA molecule for which their position is known.

Response to selection Genetic progress.

Definition of the Subject

After domestication, animals were selected in different environments and for different traits leading to the modern breeds. Long before the appearance of the science called now as "Genetics," animal breeding had been practiced by humans following intuitive criteria, less efficient than the scientific ones, but criteria that had provided success along many generations of selection [1]. The lack of a theory explaining inheritance slowed down animal breeding for many years, but with the rediscovery of Mendel's rules at the beginning of the twentieth century and the development of quantitative genetics in the 1920s and 1930s animal breeding had the tools needed for its development. Animal breeding methods were developed in the 1930s and 1940s, and the first animal breeding companies and cooperatives started in using scientific methods for animal selection [2]. The development of artificial insemination in cattle in the 1940s and frozen semen in the 1950s led to the modern schemes of progeny test, in which bulls are proved with a high number of daughters, and semen of the best bulls is available worldwide. Large companies of animal breeding were created in the 1960s for poultry and pigs, and nowadays they dominate the market of reproducers, particularly in the avian case. In 1953 the structure of the DNA was published, leading to a quick development of all molecular genetics techniques. Today, DNA information is widely used as a complementary tool to the statistical methods based on data from records, to estimate the genetic values of the candidates to selection [3]. Although the commerce of genes is now extended worldwide, there is a recent interest in conserving breeds in danger of extinction due to this globalization. These breeds are a genes reserve for ensuring possible changes in the future market. Besides, some breeds can be helpful for developing sustainable systems in areas in which modern developed animals cannot be bred because of the lack of resources, climate, or other reasons [4].

Introduction

Long before the appearance of the science that is now called "Genetics," animal breeding had been practiced by humans following intuitive criteria, less efficient than the scientific ones, but criteria that had provided success along many generations of selection. Darwin himself was impressed by the achievements of farmers, and artificial selection was a source of inspiration for his theory of evolution [5].

> We cannot suppose that all the breeds were suddenly produced as perfect and as useful as we see now them; indeed, in several cases, we know that this has not been their history. The key is man's power of accumulative selection: nature gives successive variations; man adds them up in certain directions useful to him. In this sense he may be said to make for himself useful breeds.
>
> C. DARWIN
> On the origin of species (1859, p. 30)

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Animal breeding starts with domestication. Although there are several theories about the domestication process, it is generally admitted that selective breeding led to modern domestic animals, a hypothesis corroborated by the experiment of domestication of wild silver foxes started by Dimitri Belyaev in 1959 and still continued. After 40 years of selection for quiet temperament, silver foxes, which are aggressive to humans in the wild, became as friendly as dogs [6]. As a correlated response, some physical appearance also changed, and some bones of the skull were modified in the same direction as dogs when compared with wolves [7]. Modern molecular techniques permit to reconstruct the history of domestication [8]. After domestication, animals were selected in different environments and for different traits, leading to the modern breeds. References to animal breeding can be found in ancient Greek and Roman authors' works [9]; however, modern breeding practices start with the self-taught work of Robert Bakewell (1725–1795), who produced new breeds and had a high reputation as breeder [1]. He focused his work in the performances of his cattle and sheep, hiring rams, recording the offspring and keeping the sons of the best males. He fixed few and clear breeding objectives mating the best females with the best males. However, he disregarded the damaging effects of inbreeding and due to this, he had fertility troubles with his new breeds, but he is still considered as the first farmer practicing modern animal breeding.

The lack of a theory explaining inheritance slowed down animal breeding for many years. The theory of blending inheritance, sustaining that offspring was intermediate between parents, could not explain the persistence of genetic variability. Some hybrid breeders had noticed that crossing hybrids they can recover discrete traits that were present in the parental population [10], but Gregor Mendel (1822–1884) was the first in calculating the frequencies in which the observed traits were transmitted, allowing him to propose the first rules of genetic inheritance [11]. Although Mendel was conscious of the importance of his research, his work, published in a context of hybrid plant production, was largely ignored until it was rediscovered at the beginning of the twentieth century, and it was widely used to explain the inheritance of discrete observable traits. Mendel's rules worked well for discrete traits like yellow or green color, but many traits like milk production or body weight showed a continuous variation and seemed to follow different inheritance rules. The biometrician school, founded by Karl Pearson (1857–1936), was using and developing statistical methods, and rejected Mendel's rules, considering them as a special case of inheritance for some discrete characters [12]. Mendel was aware about the fact that the simple rules he discovered could not be applied to continuous variation, but he suggested that in these cases many inheritance factors might act simultaneously producing all intermediate indistinguishable classes. After some exam of this possibility, it was disregarded by the biometricians, and a bitter dispute about the mechanisms of inheritance started until Fisher (1890–1962), in a seminal paper [13], used statistical methods to reconcile Mendel's laws on inheritance with the continuous variation observed by biometricians. (For a history of early development of genetics and this dispute, see [12].)

The work of Fisher in this and subsequent papers started both modern statistics and modern quantitative genetics, but the methods of this new science had still to be applied to animal breeding. This task was accomplished by Lush (1896–1982), who harmonized breeding practices with the knowledge provided by the new discipline. Lush defined concepts like heritability, and proposed methods of selection including the information of relatives, weighed according to the genetic contribution predicted by Mendel's rules and quantitative genetics. The several editions of his book "Animal breeding plans" contributed to spread the new knowledge among scientists, technicians, and breeders [14]. Modern indexes of selection for several traits were developed for plants by Fairfield Smith [15] closely following some indications given by Fisher, and Hazel [16] applied them to animal breeding allowing on one side to use family information and on the other side to weigh all traits of economic interest according to the predicted benefits that the offspring would give.

The development of artificial insemination permitted having offspring of the same sires in many farms (see [17] for a history of its development). As environmental effects were different depending on the farm circumstances, data had to be corrected in order to evaluate the animals properly. Corrections for environmental effects like parity, season, length of lactation, etc., had been made before, but then the problem was

more complex. Several methods were developed to pre-correct the data before genetic analysis was made, but it was Henderson (1911–1989), who proposed a method for integrating the genetic values and the environmental ones in the same statistical model. This allowed the prediction of genetic values at the same time that corrections for environmental values were made [18]. The development of computers allowed using all relatives in the evaluation, and some computing difficulties derived from the use of all relatives were solved by Henderson himself [19]. Nowadays his method called best linear unbiased prediction (BLUP) is the standard method in animal breeding evaluation. BLUP needs the variance components (genetic and environmental) for predicting the genetic values. To estimate them is a difficult task, because data come from different farms and different environments and they should be corrected as before. Paterson and Thompson [20] showed how to correct for the environmental effects and how to estimate the genetic variance components at the same time. Their method is called REML (Residual or Restricted Maximum Likelihood) and it is a standard for variance component estimation.

Animal breeding was dominated by REML and BLUP – and they are still the most common methods – until the development of modern computers allowed the use of Bayesian methods. These methods use probabilities for inferences, which give them several advantages and permit to express the uncertainty about the unknowns in a natural way. For example, it is easier to understand that the probability of a breed having a higher growth rate than another is 93% than to understand that when estimating the difference in growth rate between breeds, in an infinite number of repetitions of the experiment, new samples will be higher than the actual sample in a 7% of the cases (which is the definition of a *P*-value of 7%). A review of Bayesian methods compared with classical statistical methods in animal breeding can be found in [21]. Bayesian methods were introduced by Daniel Gianola in the 1980s [22], but they lead to complicated integrals that could not be solved even by approximate methods. The rediscovery of a numerical method called Monte Carlo Markov Chains permitted to overcome this problem and to use Bayesian methods, leading to a high development and extension of them in animal breeding (see [23] for a detailed exposition of the methods).

With the arrival of DNA analysis techniques, a new field was open for research. Transgenesis looked as a promising area, but its real usefulness in animal breeding has been discussed [24]. Molecular markers, however, have been widely used in animal breeding as a complementary tool in genetic programs. They have been also used for capturing major genes; unfortunately, most traits are not controlled by major genes and molecular markers have had a limited success in this area [25]. Recently, simple molecular markers consisting in a single nucleotide substitution in the DNA chain (SNP) have been made easy and cheap to detect. This permits to use several thousand markers in each individual, thus all genes controlling a trait can potentially be associated to SNPs [26]. A main problem of this procedure is that these associations between SNPs and genes are lost after few generations of selection [27], but new associations can be reestimated. Nowadays genomics is being examined as a promising tool for many genetic programs, particularly in species like dairy cattle in which there is a continuous recording, the trait is expressed only in females, and generation intervals are large. In this case, genomics can be used for a better evaluation of young bulls that still have no offspring. Other uses of genomics will appear in the forthcoming years and it will be probably established as a useful complementary tool to current genetic programs.

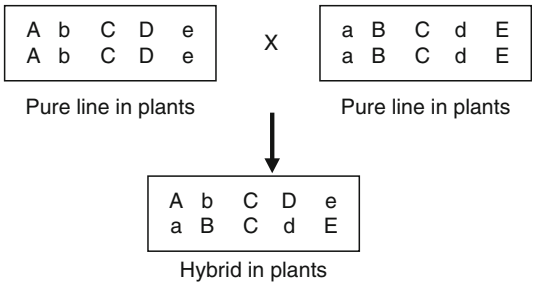
Animal Breeding and Sustainability

Animal breeding consists essentially in selecting animals kept in close reproduction systems, often accompanied with crosses between these groups of animals [2]. Historically, the groups of animals kept in close reproduction were breeds, although modern intensive meat production of prolific species is now based in selection of synthetic lines. These lines are called "synthetic" because they do not correspond to traditional breeds, but have been generated by crossing animals from different breeds or crossing commercial "hybrids" (which are not hybrids in a genetic sense, as it will be seen later). This procedure allows obtaining a large genetic variability available for selection on productive traits. The relevance of breeds for sustainability lies in that some breeds can be particularly well adapted to local conditions, although this does not mean that local breeds are always better for local conditions than

foreign breeds. A foreign breed can be better adapted or can be economically more interesting than a local breed. This is common mainly in species like poultry, pigs, or rabbits, which are usually kept in better environmental conditions than beef, sheep, or goats, but it also happens in ruminants. For example, Nelore cattle, a foreign breed in Brazil, has had a high success and now it is extensively implanted there [28].

Definition of Breed

There is no consensus about the definition of what a breed is. Many definitions of breed have been compared [29–31], and the only common requirement to all of them is the genetic homogeneity, which applies essentially to external traits. It can be said that *a breed is a group of animals with some common external characteristics defined by some people who consider this group of animals to be a breed*. A breed requires some people who decide the external characteristics used to define the breed; often they also attribute some "average performances" to the breed. The problem with this definition is that it depends too much on external characteristics that may be very useful for dog or ornamental animals, but not necessarily for animals producing meat or milk in an efficient way. Some breeds were historically selected for improving some traits and they have been established as the most productive ones in intensive production systems; Leghorn hens for white eggs, Friesian cows for dairy cattle and Landrace and Large white in pig production are now widely established. However, the word "Leghorn" or "Landrace" only define the external appearance of the breeds; there are many types of Landrace in the world, depending on the traits for which they have been selected, and the few multinational companies that control the eggs market use specific highly productive Leghorn lines, therefore the concept of "breed" is often of little utility. Other words used in animal breeding that can lead to confusion are "pure breed" and "hybrid." In plants, a hybrid is the cross of two pure lines. A pure line is homozygous for all its genes, and all individuals have the same genotype, all hybrids have also the same genetic composition, and the cross of two plant hybrids produces very different plants due to the segregation of all the alleles (Fig. 1).



Animal Breeding Methods and Sustainability. Figure 1 Pure lines and hybrids in plants. Couples of letters indicate genes; capital letters indicate one allele of a gene and small letters another allele of the same gene

There are no "pure lines" in animals in the same sense as in plants. Pure lines in plants have been produced by self-fertilization or by fertilization of close relatives, something that is not possible in animals. Some attempts of creating highly inbred lines in pigs and poultry were done in the 1940s and 1950s, without positive results, because inbreeding produces infertility and abnormalities to a degree that prevents its use in animal breeding [2]. "Pure lines" in animals are only groups of animals in closed reproduction that will not be homozygous for all their genes, therefore animal "hybrids" will be crosses between lines or breeds with no genetic homogeneity. Moreover, it is a frequent practice in animal breeding to open the lines to some animals from other commercial lines in order to reduce inbreeding. This practice is also useful to capture genes that would be in lower frequency in the recipient line and that may be in higher frequency in the imported animals [32]. As "animal hybrids" are only crossed animals, they can be used to produce new "animal pure lines" with high genetic variability available for selection; for example, several rabbit breeds used for commercial purposes were originated by crossing commercial "hybrids" [33].

Breeds were created by humans after domestication by selecting traits they particularly liked. New breeds can be created nowadays. Apart from pets, many companies of pigs, rabbits, and poultry now use synthetic breeds without giving special importance to external characteristics, with the exception of the functional ones.

Breed Conservation

Some breeds, local or not, can perform better than some intensively selected lines in systems in which food is less rich in protein or energy, or less balanced than in intensive systems. Some breeds can also perform better in some areas in which climate or breeding conditions are very different from the ones of current intensive production systems. There are more reasons for conserving breeds [34]: keeping genes that may be useful in the future, supporting sustainable animal production systems for food security, maintaining genetic variability for further use, conserving cultural heritage, etc. However, when a breed is useful, it does not normally need special aids for conservation, since it produces some profit and then it is kept for obtaining benefits. Help is needed especially for breeds that are not profitable, but there are reasons for inferring that they have genes that may be useful in the future. A question then would be whether the object of conservation should be breeds or genes, that is, whether it can be created as synthetic breeds having the genes of interest instead of spending funds in several programs for conserving several breeds. Although focusing the problem in keeping genes seems to be simpler, this can produce some problems. A first problem is that creating synthetic breeds may lead to undesirable gene interactions, difficult to manage for both the survival of the breed and the transmission of the interesting genes. Another problem would be the difficulties in integrating new synthetic breeds in areas in which farmers would not be prepared or accustomed to manage [35].

One of the main objectives of breed conservation, keeping genes for the future, has been discussed [35]. This objective is too vague unless the concrete purpose for using these genes in the future is envisaged. When a breed is a tool for making meat, milk, or eggs, conservation should be focused on whether this tool works now or whether there are expectations for using this tool in the future. This is an important point, because the extinction of a breed is completely different from the extinction of species. Breeds extinction, which can be created, transformed, or recovered, should not be compared with losing unrecoverable species created by natural evolution and forming part of a peculiar ecosystem.

The more concrete objective of maintaining genetic variability can be attractive for two reasons. First, genetic variability is needed for selection. Second, genetic variability implies a gene reserve that may also be useful when a rapid change in selection objectives is needed, for example, the current fertility problem of Holstein, partially caused by the increasing levels of inbreeding, can be managed by crossing Holstein with more fertile breeds [36, 37]. We should, however, notice that the genes of interest in animal breeding control economically relevant traits, thus keeping genetic variability is not an objective if the trait is near its optimum (100% of survival, for example). Genetic variability can be divided in between breeds variability and variability within breeds. It is important to know how much of the total existing genetic variability can be found between and within breeds, because if most of the genetic variability is contained within breeds, there is no genetic reason for conserving many breeds. For example, measuring the number of SNPs per kb in chicken, the International Chicken Genome Sequencing Consortium [38] detected "surprisingly little difference in diversity in comparisons between red jungle fowl and domestic lines, between different domestic lines, and within domestic lines." For productive traits, it is generally admitted that about 50% of genetic variability is between breeds and 50% within breeds [39, 40]. Some methods of measuring genetic variability, like estimating genetic distances between breeds by molecular markers, have among other problems that they do not consider within breed genetic variability. The core of the argument for maintaining between breeds genetic variability is that some breeds have genes that other breeds do not have or have in low frequency, and these genes may be useful in the future. It is a type of "insurance argument": insurance against changes in market or environmental conditions, and safeguard against potential emerging disasters as emergent diseases [4]. There is nothing wrong in keeping every breed in danger when having an unlimited amount of financial resources, but when resources are scarce, for example, in developing countries, a precise analysis of the foreseen benefits is needed.

Breeds and Sustainable Systems

By animal breeding sustainable systems, it is generally understood that farming systems are capable of maintaining their productivity indefinitely without damaging the environment [41]. This definition does not prevent having intensive systems with highly productive animals integrated in an industrial food chain, but sustainability is often associated to some kind of traditional farming at small scale in which waste is recycled, local breeds and local sources of food used and a rather high amount of hand labor is needed. Local breeds have a key role in this second type of sustainable systems, particularly when the environmental conditions are harsh or the food resources are not particularly good. This second type of sustainable systems is in general much less efficient for producing meat or animal products than intensive systems. There are, however, some reasons for establishing them:

1. *There are harsh environments in which no other systems will work properly.* A common example is cattle in swamp tropical areas. This applies essentially to cattle, sheep, and goats, and not necessarily to pigs, rabbits, or poultry, which have been kept in much better conditions traditionally.
2. *Using these systems in poor areas avoids land abandoning and migration of people to urban areas, avoiding desertification.* If life in these areas is hard for humans, this type of sustainable system should be considered as a temporary solution, because people living there deserve a better life.
3. *Sustainable systems are more environmentally friendly and produce a better animal welfare.* Although this reason is frequently invoked, this may or may not happen, and each case should be critically examined. Intensive industrial egg production can use enriched cages and manure process ensuring both welfare and sustainability. Moreover, animals in intensive systems arrive to commercial slaughter weight much earlier, thus they can produce less CO_2 and pollutants *per unit of product* than animals bred in extensive production systems, including pollution producing for transport, machinery, etc. A report ordered by the British government to the University of Cranfield [42] shows how this happens in poultry meat production, being organic chickens more contaminant *per kg of meat produced*, although results are more variable in pig production (for most pollutants, organic pigs contaminate less per kg of product). The same can be said about welfare: free-range hens are not necessarily happier than hens in enriched cages [43]. Looking for better animal welfare is not a particular task of industrial systems; it affects non-intensive systems as well.
4. *Some of these systems provide farmers an independence from big multinational companies.* This may be true, but is not necessarily good. Feeding people is a priority of poor countries, and the cheapest way may be to buy the genes to multinational companies. Genetics is very cheap; the genetic cost of 1 kg of pork, chicken, or rabbit meat is less than a 1% of the total cost of the meat as it will be seen in next section, and the same can be said about the genetics of 1 l of milk. Few companies provide the cheapest animal protein in the world (eggs and poultry meat and, up to a certain extent, pork meat), and genetics of dairy cattle is now managed in what is a world nucleus in practice. Poor countries need efficient genetic material for meat production even if this does not ensure genetic independence from multinational companies; this happens in industrial products and in other sectors (cars, industrial products, energy, etc.), and there is no reason for not accepting this in animal breeding.
5. *Some breeds are better adapted to local environment.* As said before, some breeds can be particularly well adapted to local conditions, although this does not mean that local breeds are better for local conditions than foreign breeds. There are spectacular examples of foreign breeds particularly well adapted, as Nelore cattle in Brazil. Besides, adaptation is a bigger problem in some species than in others. Poultry, pigs, and rabbits have been raised in better environments than sheep or goats, thus intensive commercial breeds have less adaptation problems than in other species. Local food sources are often of lower quality than the usual food provided for highly productive breeds, and it has been said that local breeds can take a better profit of it. This is highly speculative, since the available

Animal Breeding Methods and Sustainability. Table 1 Sow reproductive performance of PIC pigs in Philippines [44]

	Ave total born per litter	Ave born alive per litter	Ave weaned pigs per litter	Ave birth weight (kg)	Ave 30 day weight (kg)
Large farm sector	10.7	10.2	9.2	1.4	7.7
Small farm sector	11.9	11.4	11.1	1.5	8.8

Source: From Gibson et al. [44]

information for these local breeds is normally scarce or null. Moreover, highly productive breeds of pigs, poultry, or rabbits can be bred with success in developing countries, even by small farmers [44]. Table 1 shows that small farms in rural conditions can obtain a similar profit as better farms using the same genetic material of a big multinational company (PIC). Local breeds of cattle, sheep, and goats may be better adapted in some harsh environments, although it is important to check whether this is true and when it is true.

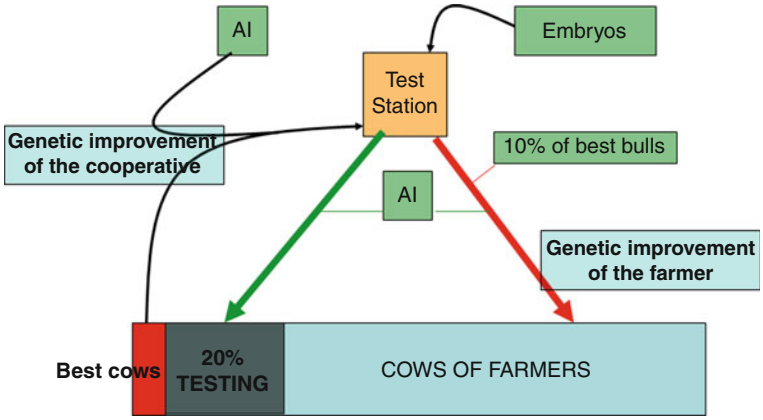
6. *Local breeds produce better quality products.* The question is too general to give a simple answer. It is rather obvious that an Iberian pig (local breed) produces a much better cured ham than a Large White pig. Production of high quality products is one among several reasons for keeping breeds that are less efficient in producing meat or meat products. It is nevertheless convenient to check whether this better quality is detectable by the consumer. Some products like fresh cheese are not easy to differentiate, and local breeds sometime only show an external appearance of the animals different from the main breeds used for cheese production. It is also important, as St. Clair Taylor has stressed many times [45], that comparisons between breeds are performed at the same stage of maturity. As breeds have often different adult size and growth rate, if they are slaughtered at the same commercial weight, they can be compared at different stage of maturity, thus differences between them can be due to the fact that one breed is younger, in physiological terms, than the other. For example, a breed can have a better meat quality than another only because at the same commercial weight it is slaughtered at a more mature stage.

Animal Breeding Methods and Schemes

Breeding Companies: Organization and Diffusion of Genetic Progress

Animal breeding can be practiced at small scale by farmers or small farmers associations, but this affects only the local breeds and its efficiency is low [21]. Nowadays animal breeding is generally in the hands of multinational companies or large cooperatives, although there are still medium sized ones performing animal breeding at a smaller scale. There are two types of schemes, based on recording data on farm or concentrating on all animal improvement in a small nucleus and diffusing later the genetic progress. The first scheme applies mainly to dairy cattle, and the second one to pigs, poultry, and rabbits.

The standard example of the first scheme is dairy cattle. A 20% of the cows of a cooperative are inseminated with semen of young bulls that are going to be tested. The daughters are then inseminated with semen from other bulls in order to have lactation. Milk, protein, fat and cell count of the milk, and sometimes longevity, are recorded for each of the daughters, and these data are used to decide which 10% of the bulls being tested will pass to the catalogue of the cooperative (Fig. 2), to be used by the farmers to inseminate their cows in order to replace their stock. Each bull being tested provides semen for 1,000 cows in order to be sure that most of them will have at least 100 daughters, in order to achieve a high precision in the estimation of their breeding value [46]. This implies that an association created for bulls testing should have at least 100,000 cows in order to include a couple of bulls per year in their catalogue. Nowadays there are many practices: big cooperatives test their bulls; some associations test few bulls that are available



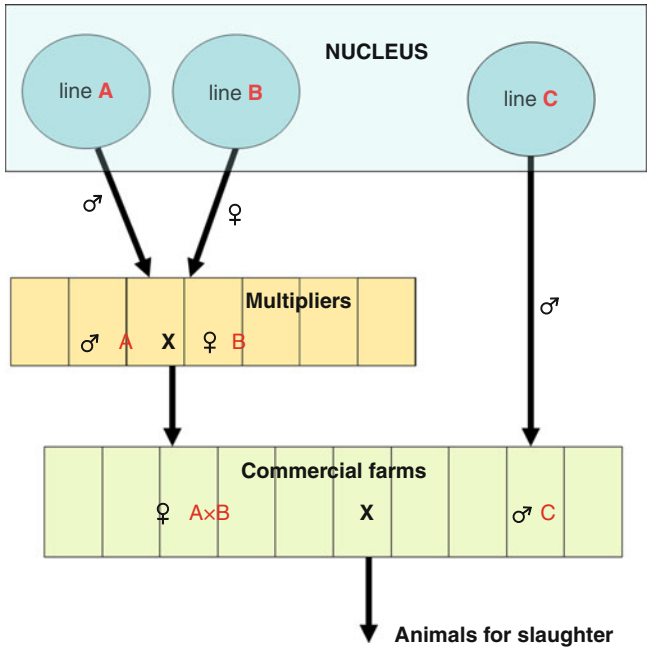
Animal Breeding Methods and Sustainability. Figure 2
Schemes of genetic evaluation and gene diffusion for dairy cattle

in their catalogue after having 60 or 70 daughters, and import semen and embryos making them available to the members; some companies test bulls and then commercialize the semen; etc. In a global society in which frozen semen can be bought worldwide and records are collected in different countries, a global genetic evaluation has been established by an association called Interbull that publish their world evaluation for all sires of different countries.

Selection is made from the records, but since a bull being tested will inseminate 1,000 cows, a previous strong selection is made when deciding which bulls will go to the test station to be proved. To do this, the best cows of the association are inseminated with the best semen available to produce the bulls to be proven. Nowadays it is also possible to buy embryos from the best cows evaluated in the world and the best semen available. Genomics is used here to help in the evaluation of these bulls that will arrive to the station. A particularity of the system is that individual farmers can make their own genetic improvement. Catalogues contain an accurate prediction of the genetic value of bulls for many traits, thus a farmer having particular problems with protein content of the milk, functional conformation, or other trait, can buy semen from bulls particularly well evaluated for these traits, improving the genetic level of his farm in the aspects he particularly needs.

The other scheme commonly used in animal breeding is the nucleus-multiplier scheme [47, 48]. Here all improvement is concentrated in a farm, from which it is spread to commercial farms through multiplication steps. This is the typical scheme for pigs, poultry, and rabbits (Fig. 3). Usually two lines are selected in closed reproduction, and males of one of the lines and females from the other are sent to farms called "multiplication units," in which both are crossed to produce the crossed female sent to the farmers. Typically, these lines are selected for prolificacy and they may be selected for other traits. A third line is selected to produce the males that the farmers will use (called "terminal sires"); in this case, the line is not selected for prolificacy because this is a trait attributed to the dam, in which males seem to have little influence. Commonly, there is only one nucleus of selection in each company, and multipliers are spread in several countries. Multipliers act usually under a contract with the company; they buy parental stock for multiplication and they are in charge of providing facilities for breeding and commercializing the product: This system has allowed a rapid development of the business. There are some variations of the scheme; terminal sires are sometimes the product of a cross between two lines C and D, and sometimes there is a multiplication step more, in which other multipliers receive females A $\times$ B to be crossed by a male from other line E to produce females (A $\times$ B) $\times$ E for the commercial farmers.

Multiplication permits to reduce the cost of selection, for example, in pigs, a female coming from one



Animal Breeding Methods and Sustainability. Figure 3
Scheme of selection and gene diffusion in pigs, poultry, and rabbits

of the lines of the nucleus and entering a multiplier can cost 600 € from which 500 € is the cost of the genetics and the rest is the cost of producing a pig. This female will produce about 15 crossbred females for production farms during her life, the rest of them being culled for various reasons (leg problems, diseases, etc.). This means that the 500 € of the genetic cost should be divided by the 15 females, giving 33 € of genetic cost for the farmer. If each female produces an average of 50 pigs for slaughter during his life, the cost of genetics for slaughtered pig is about 67 cents per pig, less than 1 cent for kg. These figures are extreme in poultry production, in which each female of the nucleus can produce nearly 100 females for the multiplication step, and each female of a multiplier can provide about the same quantity for commercial farms.

Statistical Methods of Selection

Statistical methods used in animal breeding are essentially based in the infinitesimal model [49]. In this model, traits are determined by many genes independently distributed, having each one a small effect on the trait. A first consequence of the model is that genetically good animals can produce by chance some genetically poor sons, since by chance a son can inherit most of the alleles producing poor performances, whereas other sons can be genetically better than the parents if they get good versions of the alleles. As an average, all possible offspring of a parent will define how good this parent for breeding is. This is known as "breeding value" or additive value of the parent. The genetic value of an animal is not exactly this because genes can interact between them or among them producing better or worse individuals than the sum of their individual effects. These interactions are known as "dominance" when they appear between the two alleles of one gene or "epitasis" when they appear between alleles of different genes. Interactions can also occur between genotypes and environment, when the best genotypes in an environment (e.g., in the farm where the animals are selected) are not the best in other environments (e.g., in commercial farms). The development of artificial insemination in cattle and the prominent situation in the market of large

companies selling parent stock along the world has made the interactions between genotype and environment an important area of research in modern animal breeding [50].

Another consequence of the infinitesimal model is that it permits to invoke a theorem of statistics known as the *central limit theorem*, which permits considering the traits genetically distributed according to multivariate normal distributions. The multivariate normality has many advantages, for example, zero correlation implies independence between variables (which does not occur in other distributions), variables are determined by few parameters, and all relationships between variables are linear. Statistical methods in animal breeding are based thus in linear regression techniques. The most common models applied in animal breeding are called "mixed models" because they estimate simultaneously the breeding values, considered as random effects, and the environmental values, considered as fixed effects.

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e}$$

where $\mathbf{y}$ is a vector with the data, $\mathbf{b}$ is a vector containing the environmental effects (season, herd, parity, etc.), $\mathbf{u}$ is a vector with the breeding values, and $\mathbf{e}$ is a vector with the residuals. $\mathbf{X}$ and $\mathbf{Z}$ are known design matrixes containing 1s and 0s indicating the presence or absence of the effects. Fixed effects remain when repeating the experiment, and random effects change each repetition. Due to this, random effects are not usually estimated in classical statistical theory, but geneticists are interested in the value of these random effects, because they are the breeding values that, *as an average*, will be transmitted to the offspring; thus the best animals can be selected by taking offspring only from the ones with better predicted breeding values. The covariance structure of the breeding values is known due to our knowledge of Mendel's rules for gene transmission. For example, half brothers share *as an average* half of the genetic information of their father. This allows calculating the genetic covariance matrix between random effects $\mathbf{G}$ after knowing which part of the observed variance is due to the genes and which part to the environment. The most common method to estimate these variance components, correcting at the same time for the environmental effects, and using the same model as for estimating breeding values, is called REML (Restricted or residual Maximum Likelihood) [20].

The data need not be normally distributed; in these cases, the model gives the best linear solution. Directly solving this model for many individuals, for example, several thousands or millions of data in dairy cattle, would not be possible, but an equivalent system of equations allows finding the solutions easily [18]. This system is known as Mixed Model equations, and the solution is known as the best linear unbiased prediction (BLUP) of the random genetic values.

$$\begin{bmatrix} \mathbf{X'X} & \mathbf{X'Z} \\ \mathbf{Z'X} & \mathbf{Z'Z} + \mathbf{G}^{-1} \end{bmatrix} \begin{bmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{u}} \end{bmatrix} = \begin{bmatrix} \mathbf{X'y} \\ \mathbf{Z'y} \end{bmatrix}$$

There is a technical difficulty in solving the mixed model equations, because inverting a large matrix as $\mathbf{G}$ is difficult. However, there is an easy way for directly calculating $\mathbf{G}^{-1}$, allowing a general use of these equations in animal breeding programs [19]. The model can be complicated adding repeated data, effects corresponding to single genes, and many other possibilities. It can also be used for many traits simultaneously. When several traits are used, the random effects are correlated not only due to the relationships between individuals, but also due to the genetic correlations between traits, originated when some genes have influence not in one trait but in several ones. Multitrait genetic variances and covariances can be estimated by REML as before, but Bayesian techniques, using a numerical procedure known as Markov Chain Monte Carlo (MCMC), have been particularly useful in complex situations, for example, when some traits have repeated data and other traits not and consequently the design matrixes X, Z are not the same for both traits. Bayesian methods also permit to transform multivariate problems in series of unvaried estimations. Bayesian techniques with MCMC have been rapidly developed in the field of animal breeding, mainly for complex models, for example, when traits have different distributions, for censored data, for robust models, etc. (see [21] for a scope of their use and a comparison with classical methods and [23] for detailed description of Bayesian procedures).

In the case of using many traits, the objective is maximizing the economical benefit, which is obtained weighing each trait by economic weights. These weights can be calculated with more or less sophisticated

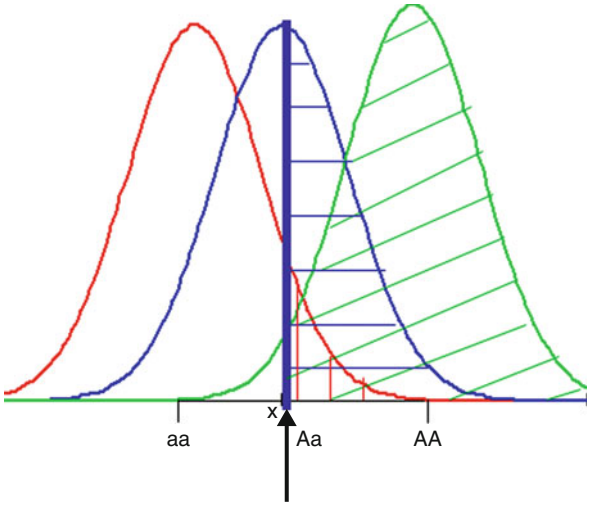
models [51, 52], but in essence they represent the amount of benefits, measured in economical units, obtained by improving one unity of the trait, for example, the number of euros of benefit for producing 1 kg of milk.

The Use of Molecular Genetics in Animal Breeding

Molecular genetics has influenced modern genetic programs. Two different aspects, will be commented here, transgenic animals and molecular markers, including in the late genomic selection. A critical review and discussion about the uses of transgenesis and cloning in animal breeding, with references to markers, can be found in [24]. Genomic selection is very recent and its possibilities and development are still under discussion.

Transgenesis The first transgenic mice growing twice than normal created an enormous expectation about what could be done with transgenic animals [53], particularly in the field of animal production. However, few transgenic animals are now available, and the economical advantage of transgenic animals is small [24]. Although apparently it is economically viable to produce transgenic products useful for human health, the application of transgenic animals in medicine will not be considered here.

To apply transgenesis in animal production, genes with major effects are needed, but unfortunately, most economically interesting traits are determined by many genes of small effects. Sometimes there are genes with major effects for some traits, for example, for fat deposition in pigs, but classical selection has fixed yet the favorable alleles in commercial populations, thus they are not particularly useful now. When a trait of an economic interest has a major gene segregating in the population of study, this gene can be easily captured by selection. This can be shown by computer simulation [54, 55] but a simple example can help in understanding this. In Fig. 4, it can be seen the phenotypical distribution of a trait is controlled by a single gene. When selecting the best 50% of the animals, copies of the allele "A" are selected with preference. Therefore, in few generations of selection the gene will be in high frequencies or will get fixed. If the frequency of the favorable allele is low, the process takes more



Animal Breeding Methods and Sustainability. Figure 4 Phenotypic distribution of a trait determined by a major gene with a high additive effect. Selection of the best 50% individuals

generations, but in general, it hardly will compensate to use transgenesis to capture it. Marker-assisted selection can be used for augmenting more rapidly the frequency of such genes of major effects, as it will be commented in next section.

Some major genes that are present in a breed or a line but not in other can be easily introduced by introgression without requiring transgenesis. The breed with the gene of interest G is crossed with the breed objective O, and then backcross is made by crossing O with the animals of the G × O cross that carry the gene of interest. After several backcrosses, the gene is introgressed. An example of gene introgression is often performed with the Boorola gene in sheep that augments litter size, due to the high prolificacy of the carriers that permit an easy identification. When the carriers do not clearly show the gene of interest, genetic markers can be used to help the introgression [56].

The process of transgenesis is extremely inefficient. Genes are placed at random, thus the gene can be inserted in an inappropriate tissue or it can happen that genes around the inserted gene modify the expression of it. Transgenes are not always expressed and they are not always transmitted to descents. Moreover, many animals are needed for obtaining a viable embryo expressing the genes

transferred. For example, 36,500 embryos were needed to obtain 18 transgenic calves expressing the trait, and the cost of each transgenic cow was higher than 500,000 dollars [57]. Lentivirus vectors can produce transgenic animals more efficiently in some species and at a lower cost, but they still suffer the former problems [58].

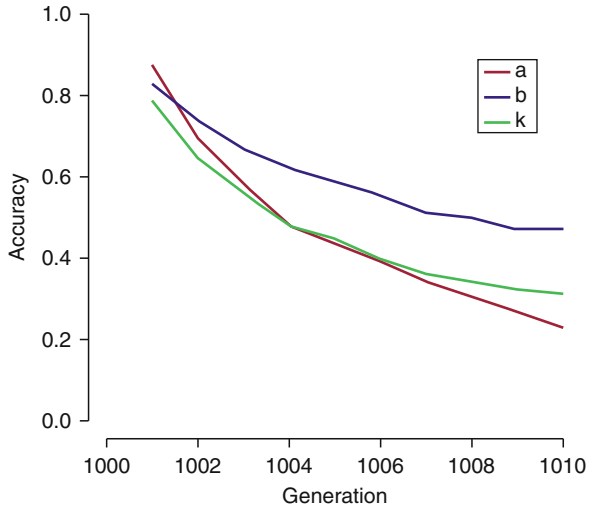
Transgenic animals should be tested to prove that they are commercially viable [24]. They should be tested for the trait that is the object of transgenesis, because it should be proved that the transgene is expressed in the animal and in the offspring for several generations. They should be also tested for commercial traits, since a transgenic line might be good for a trait but might have a poor productivity for other economic traits. The overall productivity should be evaluated. Transgenic animals may have poor fitness, sensitivity to diseases for which non-transgenic animals are resistant and poor performances in other traits that might affect longevity; it is also frequent that transgenic animals have reproductive problems.

Once the major gene has been transferred in an animal, a whole population or line having this gene has to be constituted. In the nucleus-multiplier scheme, inbreeding depression will increase when creating the transgenic nucleus, since mating with relatives during several generations are needed to spread the gene [59]. The process of evaluation of transgenic animals, and the diffusion of the transgene in a line, increases the genetic lag between the transgenic line and the commercial lines, due to the genetic improvement made during this time by its competitors. Diffusion of a transgenic animal in dairy cattle, in which a world nucleus of selection is much higher than in prolific species, and generation interval is large due to progeny testing (6 years), has also been studied. It has been calculated that in a population of 10,000,000 cows, three generations later after the introduction of the transgenic founder (18 years later), the presence of the gene in the population would be between 1% and 4% [60]. The genetic lag produced, the fact that a transgenic animal may be genetically inferior for other traits not controlled by the gene transferred, the complications of the processes and the scarce number of gene candidates for being transferred, makes transgenesis little attractive, even if it would be a less expensive and more successful technique [24].

Genetic Markers and Genomic Selection Genetic markers are parts of the DNA molecule that can be identified in individuals. They may be close to a gene of interest, so they can be used to select the favorable version of a gene affecting a quantitative trait. Genes controlling a quantitative trait are called QTL (quantitative trait loci), and occasionally they can have a large effect and can be selected with the help of a marker. However, generally quantitative traits are controlled by many genes with small effects, thus the effectiveness of markers has been rather limited [25]. The situation has dramatically changed since it has been possible to obtain a large number of markers at low cost and since they can be associated to many of the genes controlling traits even having small effects. There are several types of markers; the simplest one is the Single Nucleotide Polymorphism (SNP), which marks a place in the genome in which there is variability in a single nucleotide. Nowadays there are microchips allowing detection of about 50,000 SNPs in a genome; the number of SNPs that can be easily detected is increasing to 500,000 and soon it will be possible to genotype the whole genome of livestock species at reasonable prices. Prediction equations can be fitted, in which a set of SNPs will be used for predicting breeding values. Taking data from 1,000 to 4,000 animals (calling this *training population*), the model to be fitted can be

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + \cdots + b_{50,000}\,x_{50,000}$$

where $x_1, x_2, \ldots, x_{50,000}$ are the variables indicating the presence of one polymorphism (AA, Aa, aa) of each SNP (usually indicated by 1, 0, −1, or by 0, 1, 2), and $b_1, b_2, \ldots, b_{50,000}$ are the regression coefficients to be estimated. These equations cannot be solved by least squares given the high number of SNPS in relation to the data available for the prediction, and Bayesian techniques should be used. The use of prior information allows solving these big equation systems, and depending on how prior information is included, the Bayesian methods differ [61]. This method can also be used for several traits [27]. Many of these SNPs are noninformative, and there are some techniques to select only informative SNPs [61, 62]. There is now a promising research area for selecting informative SNPs for prediction, often using nonparametric statistics [62].



Animal Breeding Methods and Sustainability. Figure 5 Loss of accuracy of genomic selection on parameters a, b, and k of Gompertz growth curve. Selection acts on trait a, and the loss of accuracy of traits b and k are due to genetic correlations with trait a (From Blasco and Ibáñez-Escriche [27])

Genomic selection has been proposed for traits that are difficult or expensive to measure (e.g., adult weight [24], index of conversion [63], mortality [64]). It has also been proposed in dairy cattle, in which the traits of interest are expressed in dams but selection acts mainly in sires, and the generation interval is very long [65]. It may be useful for other traits like litter size, difficult to select due to their low heritability, but studies are needed to determine its usefulness in these cases, because very low heritabilities will give poor prediction equations since the records will then be determined mainly by the environment.

A main problem of genomics is that the association of SNPs with the genes responsible of the trait quickly disappears in few generations of selection, thus the prediction equations have to be reestimated and new training populations are needed. Figure 5 shows an example of the loss in accuracy of the prediction of genetic values. It can be observed that accuracy is practically halved in four generations of selection.

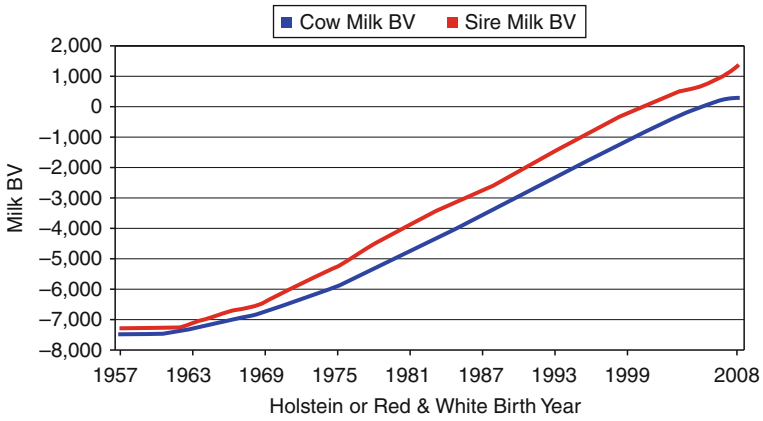
This limits the use of genomic selection in current programs, because in some species the generation interval is short (6–9 months in rabbits or hens, 1 year in pigs), and a continuous reestimation can be difficult or expensive. Finding when and how genomics can be included in current genetic programs is one of the most important research areas nowadays.

Future Directions

The Future Evolution of Methods and Schemes

Prediction of breeding values from records seems to be well established with the methods briefly exposed in section "Statistical Methods of Selection" and it does not seem that dramatic changes will occur in the future at short or medium term. The revolution in methods for estimating breeding values is in the area of genomics. The possibility of having information from several thousands of markers at a reasonable price, now from several hundred thousands and in the near future from the whole genome, has brought the problem of how to manage all these data, and prediction methods are examined from other areas of knowledge as artificial intelligence, using nonparametric or semiparametric methods, Bayesian methods, etc.

Schemes of selection are also changing due to the globalization of the market of genes. Today the best cows of the world are not dedicated to produce milk but embryos that are sexed, frozen, and commercialized. Some of the deficiencies of current dairy cattle programs such as long generation intervals can be partially solved by using genomic selection and having a quicker and better evaluation of the bulls being tested. Larry Schaeffer suggested that genomic evaluation can substitute progeny test, dramatically shortening generation intervals [66], but it is doubtful that farmers will accept genomics evaluation as they accept now tests mainly based in offspring records [65]. It can also happen that private companies will compete with others or that breeders can organize brands in which semen is not identified, like in pigs, as Maurice Bichard suggests [67], but it looks unlikely, since farmers like to perform their own genetic improvement at farm level by buying semen from accurately tested sires. Poultry genetics is now in the hands of two large holdings, and the only change envisaged in their structure is related to possible troubles with laws about competence. Pig companies tend also to be bigger, but they will probably coexist with nucleuses of smaller companies well established in local markets, and with large pig production companies producing parental stock for



Animal Breeding Methods and Sustainability. Figure 6
Genetic values of sires and cows in USA in the last 50 years (From USDA http://aipl.arsusda.gov/eval/summary/trend.cfm)

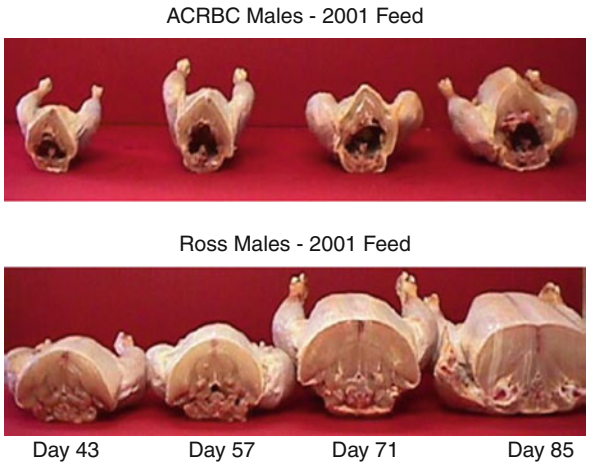
themselves. Both pigs and poultry companies will introduce genomics in their programs not only as a complementary tool for selection but also as a commercial strategy, using modern methodologies as an added value to their products. The interest in meat quality traits and quality of animal products will probably increase. Companies will also stress the sustainability of their productions and the good welfare of their animals, thus there will probably be an increasing interest in traits like robustness and disease resistance. The new emphasis in sustainability will give importance to breed conservation programs, which will receive more attention and will get substantial public funds. Nevertheless, no dramatic changes in objectives are envisaged in the near future. Changes in genetic objectives are slow and the product of the selection arrives with delay to the market, thus this prevents short-term selection policies.

The Limits to Genetic Progress

The theory of selection limits was developed by Alan Robertson (1920–1989) [68]. Classic quantitative genetics theory predicts the extinction of genetic variability by selection, and consequently the end of genetic progress. Frequencies of favorable genes increase with selection until they are close to 100%, and the genetic response is necessarily low, or genes are fixed by genetic drift, which occurs more likely when they are at high or low frequencies and when the selected population is small. Mutation can introduce new genetic variability, but useful mutations are rare and they were disregarded in the classical theory of limits of selection. A decline in genetic response is thus expected until genetic variability is exhausted, and some experiments arrived to a plateau after showing response to selection along 20 or 30 generations in drosophila [69] and mice [70]. However, there is little evidence of any loss of genetic variability in commercial populations [71, 72]. Heritability of milk production in dairy cattle is not decreasing with time but augmenting! [73] and this is not only due to a better control of environmental variance or methods of correction, but also to the continuously maintained response to selection in the last 50 years (Fig. 6).

Long-term genetic responses have been observed in both plants and animals, and there are several examples of continuous genetic progress in all livestock species. A part of the success of the phenotypic trends observed in animals is due to improvements in nutrition, but when comparing chicken broilers fed with food as prepared in 1957 and as prepared in 2001, most of the observed differences are due to genetic improvement [74]. Figure 7 shows carcasses of poultry from an unselected line and a selected line of the same company, fed with modern food.

Broilers show a continuous growth, egg mass production continuously increases, pigs' lean growth selection has dramatically decreased the amount of fat of the carcasses, and in general all selection programs



Animal Breeding Methods and Sustainability. Figure 7 Carcasses of 1957 and 2001 of an unselected and a selected line of poultry fed with the same food [75]

continue having success [72]. The reasons for this apparent non-limits to selection are selection pressure on genes produced by mutation (which has a heritability of about 0.1% [72, 76]) or epistatic interactions, but even when epistatic interactions are important, additive variance typically accounts for over half, and often close to 100%, of the total genetic variance [77]. Bill Hill moved further the classical theory of limits of selection showing how new mutations with selective advantage can increase genetic variability [78]. An experiment corroborating the theory showed how totally homozygous lines produced artificially in drosophila melanogaster could recover genetic variability by selection [79].

Are there limits to the genetic progress? Some traits have biological limits but still genetic progress can be obtained acting on related traits, for example, it is not possible to produce more than one egg per day, but it is possible to increase the laying period, and most of the new response to artificial selection in egg mass comes from this [72]. Highly productive animals can increase the incidence of pathological problems like ascites in broiler chicken or fertility in dairy cattle, but selection on these unfavorable traits [80] or crossbreeding [36] can be performed to continue the progress. Selection including traits different from strictly productive ones should be considered to avoid undesirable consequences of the continuous genetic progress [81]. Apart from some obvious limits (e.g., traits measured in percentage, like survival, cannot surpass 100%), it seems that genetic response can be directed to overcome the biological limits presented when selection acts only in one or few productive traits.

Bibliography

Primary Literature

1. Wood J, Orel V (2001) Genetic prehistory in selective breeding. Oxford University Press, Oxford
2. Johansson I, Rendel J (1972) Genetics and animal breedng. Oliver & Boyd, Edinburgh
3. Hayes BJ, Bowman PJ, Chamberlain AJ, Goddard ME (2009) Genomic selection in dairy cattle: progress and challenges. J Dairy Sci 92:433–443
4. Gandini G, Oldenbroek K (2007) Strategies for moving from conservation to utilisation. In: Oldenbroeck K (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 55–74
5. Darwin C (1859, 1964) On the origin of species (a facsimile of the first edition). Harvard University Press, Cambridge, MA
6. Trut LN (1999) Early canid domestication: the farm-fox experiment. American Scientist 87:160–169
7. Trut LN, Plyusnina IZ, Oskina IN (2004) An experiment on fox domestication and debatable issues of evolution of the dog. Russ J Genet 40:644–655
8. Achilli A, Olivieri A, Pellecchia M, Uboldi C, Colli L, Al-Zahery N, Accetturo M, Pala M, Kashani BH, Perego UA, Battaglia V, Fornarino S, Kalamati J, Houshmand M, Negrini R, Semino O, Richards M, Macaulay V, Ferretti L, Bandelt HJ, Ajmone-Marsan P, Torroni A (2008) Mitochondrial genomes of extinct aurochs survive in domestic cattle. Curr Biol 18:R157–R158
9. Russell N (1986) Like enged'ring like. Cambridge University Press, Cambridge
10. Sageret A (1826) Considérations sur la production des hybrides des variantes et des variétés en général et sur celles de la famille des Cucurbitacées en particulier. Ann Sci Nat 8:294–314
11. Mendel JG (1866). Versuche über Plflanzenhybriden Verhandlungen des naturforschenden Vereines in Brünn, Bd. IV für das Jahr, 1865 Abhandlungen: 3–47. English translation at http://www.mendelweb.org/Mendel.html
12. Provine (2001) The origins of theoretical population genetics. The University of Chicago Press, Chicago
13. Fisher RA (1918) The correlations between relatives on the supposition of Mendelian inheritance. Trans R Soc Edinb 52:399–433
14. Lush JL (1937) Animal breeding plans. Collegiate Press, Ames
15. Smith F (1937) A discriminant function for plant selection. Ann Eugen 7:240–250
16. Hazel LN (1943) The genetic bases for constructing selection indexes. Genetics 28:476–490

17. Foote RH (2002) The history of artificial insemination: selected notes and notables. J Anim Sci 80:1–10
18. Henderson CR (1964) Selection index and expected genetic advance. In: Hanson WD, Robison HF (eds) Statistical genetics and plant breeding. NAS, NRC, Washington, pp 141–163
19. Henderson CR (1976) A rapid method for computing the inverse of a relationship matrix. J Dairy Sci 58:1727–1730
20. Patterson HD, Thompson R (1971) Recovery of interblock information when block sizes are unequal. Biometrika 58:545–554
21. Blasco A (2001) The Bayesian controversy in animal breeding. J Anim Sci 79:2023–2046
22. Gianola D, Fernando RL (1986) Bayesian methods in animal breeding theory. J Anim Sci 63:217–244
23. Sorensen D, Gianola D (2002) Likelihood Bayesian and MCMC methods in quantitative genetics. Springer, New York
24. Blasco A (2008) The role of genetic engineering in livestock production. Livest Sci 113:191–201
25. Dekkers JCM (2004) Commercial application of marker- and gene assisted selection in livestock: strategies and lessons. J Anim Sci 82:E313–E318, Suppl
26. Meuwissen THE, Hayes BH, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
27. Blasco A, Ibáñez-Escriche N (2010) Modifying growth curve parameters by multitrait genomic selection. 9th world congress on genetics applied to livestock production. Leipzig, 1–6 August 2010
28. Porter E (ed) (2002) Mason's world dictionary of livestock breeds, types and varieties. CABI Publ, Wallingford
29. Rodero E, Herrera M (2000) El concepto de raza. Un enfoque epistemológico. Arch Zootecnia 49:5–16
30. Hall SJG (2004) Livestock biodiversity. Blackwell, Oxford
31. Woolliams J, Toro MA (2007) What is genetic diversity? In: Oldenbroeck K (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 55–74
32. Falconer D, Mackay TFC (1996) Introduction to quantitative genetics. Longman, Edinburgh
33. Baselga (2004) Genetic improvement of meat rabbits. Programmes and diffusion. In: Proceedings of the 8th world rabbit congress, Puebla, Mexico, pp 1–13, 7–10 September 2004. Available at http://world-rabbit-science.com/WRSA-Proceedings/Congress-2004-Puebla/Puebla-2004-a.htm#Genetics
34. Oldenbroeck K (ed) (2007) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen
35. Blasco A (2008) Breeds in danger of extinction and biodiversity. Rev Bras Zootecnia 37:101–109, Special Supp
36. Madalena FE (2008) How sustainable are the breeding programs of the global main stream dairy breeds? The Latin-American situation. Livest Res for Rural Develop 20, article19. Avalilabe at http://www.lrrd.org/lrrd20/2/mada20019.htm
37. Hansen LB (2006) Monitoring the worldwide genetic supply for dairy cattle with emphasis on managing crossbreeding and inbreeding. 8th world congress on genetics applied to livestock production, Belo Horizonte, Brasil, 13–18 August 2006. Avalilable at http://www.wcgalp8.org.br/wcgalp8/articles/paper/1_484-1695.pdf
38. International Chicken Polymorphism Map Consortium (2004) A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. Nature 432:717–722
39. Hall SJG (2004) Livestock biodiversity. Blackwell, Oxford
40. Bennewitz J, Eding H, Ruane J, Simianer H (2007) Selection of breeds for conservation. In: Oldenbroeck K (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 131–146
41. World Commission on Environment and Development (1987) Our common future. Oxford University Press, Oxford
42. Williams AG, Audsley E, Sandars DL (2006) Determining the environmental burdens and resource use in the production of agricultural and horticultural commodities. Main Report. Defra Research Project IS0205. Bedford: Cranfield University and Defra. Available at http://randd.defra.gov.uk/Document.aspx?Document=IS0205_3959_FRP.doc
43. Webster J (2005) Animal welfare: limping towards Eden. Blackwell, Oxford
44. Gibson JP, Steane DE, Plastow GS, Warkup C (2006) Meeting the needs of rich and poor: the needs, opportunities and trends in pig genetic improvement. 8th world congress on genetics applied to livestock production, Belo Horizonte, Brasil, 13–18 August 2006. Available at http://www.wcgalp8.org.br/wcgalp8/articles/paper/6_1040-2147.pdf
45. Taylor CSt (1985) Use of genetic size-scaling in evaluation of animal growth. J Anim Sci 61:118–143
46. Goddard ME, Wiggans GR (1999) The genetics of dairy cattle. In: Fries R, Ruvinsky A (eds) The genetics of cattle. CABI Publ, Wallingford
47. Bichard M (1977) Economic efficiency of pig breeding schemes: a breeding company view. Liv Prod Sci 4:245–254
48. Hunton P (1990) Industrial breeding and selection. In: Crawford RD (ed) Poultry breeding and genetics. Elsevier, Amsterdam
49. Bulmer G (1980) The mathematical theory of quantitative genetics. Oxford University Press, Oxford
50. Knap PW, Wang L (2006) Robustness in pigs and what we can learn from other species. 8th world congress on genetics applied to livestock production, Belo Horizonte, Brasil, 13–18 August 2006. Available at http://www.wcgalp8.org.br/wcgalp8/articles/paper/6_203-1825.pdf
51. Blasco (1995) Los pesos económicos en mejora genética animal. ITEA 91A:59–79
52. Weller I (1994) Economic aspect of animal breeding. Chapman & Hall, London
53. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611–615
54. Fournet F, Elsen JM, Barbieri ME, Manfredi E (1997) Effect of including major gene information in mass selection: a

stochastic simulation in a small population. Genet Sel Evol 29:35–56
55. Villanueva B, Pong-Wong R, Grundy B, Wooliams JA (1999) Potential benefit from using an identified major gene in BLUP evaluation with truncation and optimal selection. Genet Sel Evol 31:115–133
56. Visscher PM, Haley CS (1995) Utilizing genetic markers in pig breeding programmes. Anim Breedg Abstr 63:1–8
57. Niemann H, Kues WA (2003) Application of transgenesis in livestock for agriculture and biomedicine. Anim Reprod Sci 79:291–317
58. Pfeifer A, Hofmann A, Kessler B, Wolf E (2004) Response to whitelaw: lentiviral transgenesis in livestock. Trends Biotechnol 22:159
59. Gama LT, Smith CS, Gibson JP (1992) Transgene effects, introgression strategies and testing schemes in pigs. Anim Prod 54:427–440
60. Hoeschele I (1990) Potential gain from insertion of major genes into dairy cattle. J Dairy Sci 73:2601–2618
61. Gianola D, De los Campos G, Hill WG, Manfredi E, Fernando R (2009) Additive genetic variability and the Bayesian alphabet. Genetics 183:347–363
62. Gianola D, De los Campos G (2008) Inferring genetic values for quantitative traits non-parametrically. Genet Res 90:525–540
63. González-Recio O, Gianola D, Rosa GJM, Weigel KA, Kranis A (2009) Genome-assisted prediction of a quantitative trait measured in parents and progeny: application to food conversion rate in chickens. Genet Sel Evol 41:3. Available at http://www.gsejournal.org/content/41/1/3
64. González-Recio O, Gianola D, Long N, Weigel KA, Rosa GJM, Avendaño S (2008) Nonparametric methods for incorporating genomic information into genetic evaluations: an application to mortality in broilers. Genetics 178:2305–2313
65. König S, Simianer H, Willam A (2009) Economic evaluation of genomic breeding programs. J Dairy Sci 92:382–391
66. Schaeffer LR (2006) Strategy for applying genome-wide selection in dairy cattle. J Anim Breed Genet 123:218–223
67. Bichard M (2002) Genetic improvement in dairy cattle – an outsider's perspective. Livest Prod Sci 75:1–10
68. Robertson A (1960) A theory of limits in artificial selection. Proc R Soc B 153:243–249
69. Robertson FW (1955) Selection response and the properties of genetic variation. Cold Spring Harb Sympos Quant Biol 20:166–177
70. Roberts RC (1966) The limits to artificial selection for body weight in the mouse. II The genetic nature of the limits. Genet Res 8:361–375
71. Hill WG (2008) Estimation, effectiveness and opportunities of long term genetic improvement in animals and maize. Lohmann Information 43:3–20. Available at http://www.lohmann-information.com/content/l_i_43_artikel1.pdf
72. Hill WG, Bünger L (2004) Inferences on the genetics of quantitative traits from long-term selection in laboratory and farm animals. Plant Breed Rev 24, part 2:169–210
73. Van Tassell CP, Wiggans GR, Norman HD (1999) Method R estimates of heritability for milk, fat, and protein yields of United States dairy cattle. J Dairy Sci 82:2231–2237
74. Havenstein GB, Ferket PR, Qureshi MA (2003) Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1509–1518
75. Havenstein GB (2006) Performance changes in poultry and livestock following 50 years of genetic selection. Lohmann Information 41:30–37. Available at http://www.lohmann-information.com/content/l_i_41_2006-12_artikel5.pdf
76. Hill WG (1982) Predictions of response to artificial selection from new mutations. Genet Res 40:255–278
77. Hill WG, Goddard ME, Visscher PM (2008) Data and theory point to mainly additive genetic variance for complex traits. PLoS Genet 4(2):e1000008. Available at http://www.plosgenetics.org/article/info:doi/10.1371/journal.pgen.1000008
78. Hill WG (1982) Rates of change in quantitative traits from fixation of new mutations. Proc Natl Acad Sci USA 79:142–145
79. López M, López-Fanjul C (1993) Spontaneous mutation for a quantitative trait in *Drosophila melanogaster*. 1. Response to artificial selection. Genet Res 61:107–116
80. Pavlidis HO, Balog JM, Stamps LK, Hughes JD, Huff WE, Anthony NB (2007) Divergent selection for ascites incidence in chickens. Poult Sci 86:2517–2529
81. Rauw WM, Kanis E, Noordhuizen-Stassen EN, Grommers FJ (1998) Undesirable side effects of selection for high production efficiency in farm animals: a review. Liv Prod Sci 56:15–33

Books and Reviews

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland
Nicholas FR (2010) Introduction to veterinary genetics. Wiley-Blackwell, Oxford
Willis MB (1998) Dalton's introduction to practical animal breeding. Blackwell, Oxford

Animal Breeding, Foundations of

Guilherme J. M. Rosa
Departments of Animal Sciences, Biostatistics & Medical Informatics, Dairy Science, University of Wisconsin, Madison, WI, USA

Article Outline

Glossary

Bayesian inference Statistical inference approach based on the combination of prior information and evidence (i.e., observations) for estimation or hypothesis testing. In Bayesian analysis the prior information is updated with the experimental data to generate the posterior distribution of unknowns, such as model parameters. The name "Bayesian" comes from the use of the Bayes' theorem in the updating process.

Breeding value A measure of the genetic merit of an individual for breeding purposes.

Genetic correlation The correlation between traits that is caused by genetic as opposed to environmental factors. Genetic correlations can be caused by pleiotropy (genes that affect multiple traits simultaneously) or by linkage disequilibrium between genes affecting the different traits.

Genomic selection Genomic selection is a form of marker-assisted selection in which genetic markers covering the whole genome are used such that all quantitative trait loci (QTL) are in linkage disequilibrium with at least one marker.

Heritability (narrow sense) The fraction of the phenotypic variance that is due to additive genetic effects.

Infinitesimal genetic model A genetic model that assumes that a trait is influenced by a very large (effectively infinite) number of loci, each with infinitesimal effect.

Linkage disequilibrium Nonrandom association of alleles at two or more loci, leading to combinations of alleles (haplotypes) that are more or less frequent in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies.

Mixed models A mixed-effects model (or simply mixed model) is a statistical model containing both fixed and random effects. Such models are useful in a wide variety of disciplines in the physical, biological, and social sciences, especially for the analysis of data with repeated measurements on each statistical unit or with measurements taken on clusters of related statistical units.

Population genetics The study of allele frequency distribution and change under the influence of the four main evolutionary processes: selection, genetic drift, mutation, and migration.

Quantitative genetics The study of complex traits (e.g., production and reproductive traits, disease resistance) and their underlying genetic mechanisms. It is effectively an extension of simple Mendelian inheritance in that the combined effect of the many underlying genes results in a continuous distribution of phenotypic values or of some underlying scale or liability thereof.

Definition of the Subject

The term *Animal Breeding* refers to the human-guided genetic improvement of phenotypic traits in domestic animals such as livestock and companion species [1]. Animal breeding is based on principles of *Quantitative Genetics* [2–4] and aims to increase the frequency of favorable alleles and allelic combinations in the population, which is achieved through selection of superior individuals and specific mating systems strategies. Selection methods and mating strategies are developed by combining principles of quantitative and population genetics with sophisticated statistical methods and computational algorithms for integrating phenotypic, pedigree, and genomic information, along with the utilization of reproductive technologies that allow for

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

larger progeny cohorts from superior animals as well as shorter generation intervals.

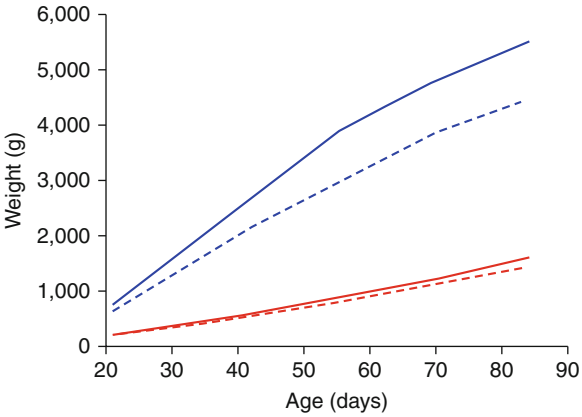
Through selection and mating of superior animals the frequency of favorable alleles is increased, so the overall additive genetic merit of a population is increased through successive generations [5]. Selection can be regarded as the most important tool for the improvement of lines or breeds within a specific species in terms of additive genetic effects. Such lines or breeds can be then intermated such that nonadditive genetic effects such as dominance and epistasis can be exploited through specific inter- and intralocus allelic combinations [1–4].

The theoretical foundations of population and quantitative genetics can be traced back to the work of R. A. Fisher, J. B. S. Haldane, and S. Wright. The rational animal breeding has its origins in the work of J. L. Lush, who made substantial contributions to animal genetics and biometrics research and is generally referred to as the father of modern scientific animal breeding [1].

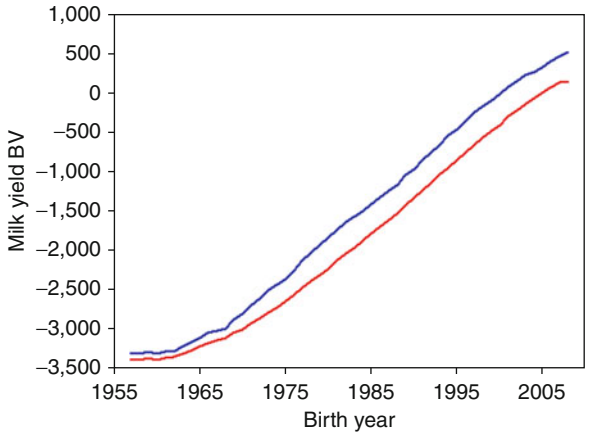
More recent theoretical developments in population and quantitative genetics have been fostered by researchers such as C. C. Cockerham, C. W. Cotterman, J. F. Crow, W. J. Ewens, W. G. Hill, M. Kimura, G. Malécot, T. Nagylaki, and B. S. Weir, among others. A landmark in the area of animal breeding and genetics is the development of mixed model methodology, first proposed by C. R. Henderson, which has been used extensively in many applications in the field, ranging from breeding value prediction under the infinitesimal assumption to gene mapping and segregation analysis. Most recently, Bayesian methods, Monte Carlo, and resampling techniques have been employed to fit and evaluate complex models in different contexts, including nonlinear systems, generalized models, survival analysis, and situations in which the number of parameters or covariates surpasses the number of observations, such as in association analysis and whole-genome marker-assisted selection using high density panels of single nucleotide polymorphism (SNP) markers.

Introduction

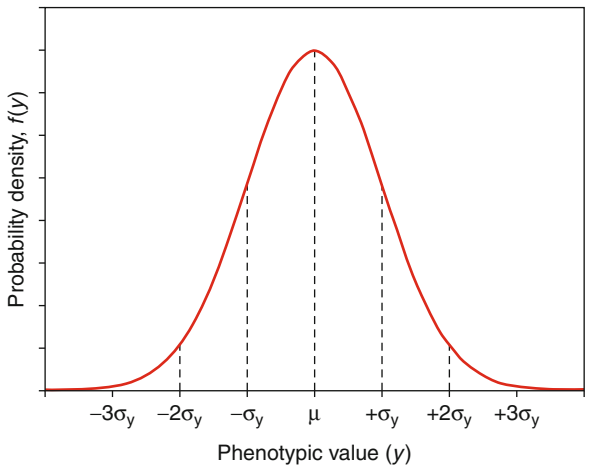
Since domestication, artificial selection has greatly changed the shape, size, and production and reproduction performance of livestock and companion animal species. For example, there is an incredible diversity of canine breeds – and between dogs and their wolf ancestors – from differences in overall appearance to behavior and their ability to perform specific tasks. Although to a lesser degree, the same can be observed in many other companion animal species, such as cats and horses. With livestock species, tremendous genetic changes have been accomplished as well, markedly in the last 50 years or so. For example, Fig. 1 depicts the average growth curves of broilers from selected and control populations. These results refer to a population of birds selected for over 40 years for increased growth rate and another population kept without artificial selection, with both groups derived from the same base population, starting in 1957 [6]. In the experiment presented in Fig. 1, the two groups of birds were fed diets typical of 1957 and 2001, such that the interaction between genetics and feed, as well as the genetic contribution to the phenotypic differences observed, could be assessed. It is seen that the 2001 genetics group presented an average body weight of about 4 kg at 56 days of age, while its 1957 counterpart weighed only 800 g or so. Moreover, it is shown that 85–90% of this fivefold improvement is accounted for by genetics with the remaining 10–15% to nutrition.



Animal Breeding, Foundations of. Figure 1
Average growth curves of commercial broilers. *Blue* and *red lines* represent birds with "2001" and "1957" genetics, respectively. *Solid* and *dashed lines* represent birds fed diets typical of 2001 or 1957, respectively (Adapted from [6])



Animal Breeding, Foundations of. Figure 2
Genetic trend for milk yield in the US Holstein and Red and White populations. Males and females average breeding values are in *blue* and *red*, respectively; genetic base refers to cows born in year 2005 (*Source*: AIPL – USDA; http://www.aipl.arsusda.gov/)



Animal Breeding, Foundations of. Figure 3
Probability density function of a normally distributed trait with mean $\mu = E[y_i]$ and variance $\sigma_y^2 = \mathrm{Var}[y_i]$, i.e., $y_i \sim N(\mu, \sigma_y^2)$

Similar levels of genetic improvement can also be observed in many other species, such as swine, beef and dairy cattle, and some species of fish. For example, as illustrated in Fig. 2, the average breeding value for milk yield in the US Holstein, and Red and White populations has increased over 3,500 kg in the last 50 years.

Such genetic improvements have been accomplished mostly through the selection and breeding of superior animals, which can be chosen using specific statistical methods such as those discussed on the following sections. In this chapter, the discussion will focus on methods developed for normally distributed (Gaussian) traits, under the infinitesimal assumption, i.e., that traits are affected by a large (virtually infinite) number of genes of small effects [2–4], although this assumption is somewhat alleviated in marker- assisted selection, which is discussed later.

Principles of Selection

Basic Genetic Model for Quantitative Traits

The basic genetic model can be expressed as [2, 3, 7]:

$$y_i = \mu + g_i + e_i \qquad (1)$$

where y_i is the phenotypic value of animal i (i.e., the animal's performance for a specific trait); μ is the population mean (average performance of the animals); g_i is the genotypic value of the animal, expressed as a deviation from the mean; and e_i is a term representing environmental factors affecting the animal's performance, also expressed as a deviation from the mean. Hence, it is assumed that $E[g_i] = 0$ and $E[e_i] = 0$, such that $E[y_i] = \mu$, where $E[.]$ represents the expectation function. Moreover, the variance of y_i is given by $\mathrm{Var}[y_i] = \sigma_y^2 = \sigma_g^2 + \sigma_e^2$, where $\sigma_g^2 = \mathrm{Var}[g_i]$ and $\sigma_e^2 = \mathrm{Var}[e_i]$ are the genetic and environmental variances, respectively. Normally distributed traits, i.e., phenotypic traits with a bell-shaped distribution, are generally represented as $y_i \sim N\left(\mu, \sigma_y^2\right)$. Such distribution has a probability density function that can be described as [2, 4]:

$$f(y_i) = \frac{1}{\sqrt{2\pi\sigma_y^2}} \exp\left\{-\frac{1}{2\sigma_y^2}(y_i - \mu)^2\right\},$$

for $-\infty < y_i < \infty$, $-\infty < \mu < \infty$, and $\sigma_y^2 > 0$, which can be represented as in Fig. 3. To simplify the notation used throughout the text, it is noted that either random variables or their realizations will be represented with lower case letters. However, the context should make it clear to the reader when a letter represents one or the other.

The genetic component g_i of Model (1) can be partitioned into additive (a_i) and nonadditive (c_i) genetic effects, i.e., $g_i = a_i + c_i$, where a_i is also called "breeding value," and c_i refers to the "gene combination value," which encompasses interaction effects between alleles within each locus (i.e., dominance effects) or between alleles in different loci (i.e., epistatic effects).

Hence, Model (1) can be expressed as:

$$y_i = \mu + a_i + c_i + e_i \tag{2}$$

where $a_i \sim N(0, \sigma_a^2)$, $c_i \sim N(0, \sigma_c^2)$, and $e_i \sim N(0, \sigma_e^2)$, with all these terms assumed independent from each other. The phenotypic variance can be then expressed as $\text{Var}[y_i] = \sigma_y^2 = \sigma_a^2 + \sigma_c^2 + \sigma_e^2$, from which two important definitions are derived. The first one is called broad sense heritability, given by $\text{H}^2 = \sigma_g^2/\sigma_y^2$, where $\sigma_g^2 = \sigma_a^2 + \sigma_c^2$, which gives the proportion of the phenotypic variance that is due to genetic effects. The second, called narrow sense heritability, refers to the specific contribution of additive genetic effects to the phenotypic variance, i.e., $h^2 = \sigma_a^2/\sigma_y^2$. These two quantities, particularly narrow sense heritability, will be further discussed and used in the next sections.

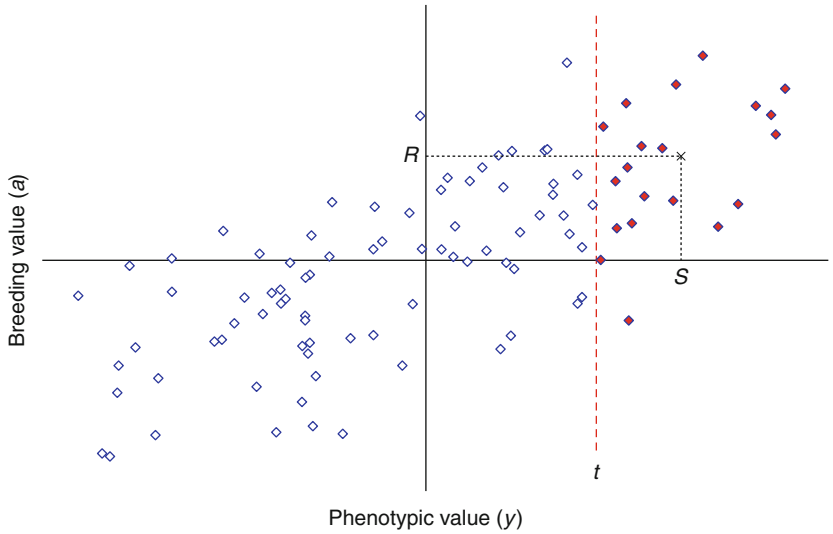
The breeding value of an individual (a_i) is equal to the sum of additive effects of individual alleles within and across loci, and it is sometimes called "additive genetic deviation" or "additive genetic effect." Because individual alleles, and therefore independent allele effects, are passed from parent to offspring, the breeding value of an individual is important for predicting its progeny's performance and so it is central to selection of superior animals [1, 3]. The gene combination value (c_i) is the difference between the genetic merit (g_i) of an animal and its breeding value, i.e., $c_i = g_i - a_i$, so it is often called "nonadditive genetic deviation." Because the component c_i involves interactions between alleles (both within and between loci), and only a single allele (as opposed to a pair of alleles) in each locus is transmitted from parents to offspring, nonadditive effects are not transmitted in a predictable manner. Hence, while average breeding value in a population can be changed through selection of superior animals, the gene combination value should be explored through specific mating systems. Here, the discussion will focus on selection approaches and the genetic improvement of a population in terms of additive genetic effects only. For a discussion on mating systems, such as inbreeding and outbreeding strategies, see for example, [1, 7, 8]. Additional discussion on inbreeding depression and heterosis (or hybrid vigor) can be found in [3, 4].

As discussed previously, the breeding value of an individual is equal to the sum of its independent allele effects. Because a parent passes a random sample of half of its alleles to its progeny, an animal's breeding value is twice what is often called "transmitting ability" or "expected progeny difference" [1, 5]. The expected breeding value of an offspring (a_o) is then equal to the average of its parents' breeding values (the same as the sum of its parents' transmitting abilities), i.e., $E[a_o|a_s, a_d] = \frac{a_s + a_d}{2}$, where a_s and a_d represent the (realized) breeding values of the offspring's sire and dam, respectively. However, there will be variability in terms of breeding values within a full-sib family because of the random sampling of parents' alleles that each offspring receives, the so-called Mendelian sampling [4].

The breeding value of an individual can be expressed as a function of its parents' breeding values as $a_o = 0.5a_s + 0.5a_d + \delta$, where δ refers to the Mendelian sampling component. It is interesting to notice that the variance of breeding values in a specific generation is equal to $\text{Var}[a_o] = 0.25\text{Var}[a_s] + 0.25\text{Var}[a_d] + \text{Var}[\delta]$. Assuming the same additive genetic variance across generations and for both sexes (i.e., $\text{Var}[a_o] = \text{Var}[a_s] = \text{Var}[a_d] = \sigma_a^2$), it is shown that the Mendelian sampling variance is equal to half the additive genetic variance, i.e., $\text{Var}[\delta] = \sigma_a^2/2$.

Phenotypic Selection

The most traditional approach of genetic improvement of livestock (and more generally any domestic animal or plant species) is based on selection of animals with the best performance, or "phenotypic selection" [1–4]. Accordingly, given a group of animals supposedly reared in similar environmental conditions, only those with the highest performance are allowed to breed to produce the next generation. As discussed previously (Model 2), the performance of each animal is a combination of its breeding value and all other nonadditive genetic effects and



Animal Breeding, Foundations of. Figure 4
Scatter plot of breeding values versus phenotypic values. Each dot represents a specific animal and those colored in *red* are selected animals with performance (i.e., phenotypic value) above a specified threshold (*t*). *S* and *R* represent the average phenotypic and breeding values of the selected (*top*) animals, respectively

environmental factors, such that a superior performance does not always represent superior breeding value. Nonetheless, whenever $\sigma_a^2 > 0$, there will be a positive correlation between performance and breeding value, and the effectiveness of phenotypic selection (i.e., selection response) will increase with such correlation.

To illustrate this concept consider Fig. 4, in which a scatter plot of breeding values and phenotypes (centered on zero, i.e., $y_i - \mu$) for a few fictitious animals is presented. As indicated before, in this chapter the discussion will be focused on selection approaches and the genetic improvement of a population in terms of additive genetic effects only, such that Model (2) can be conveniently reexpressed as:

$$y_i = \mu + a_i + \varepsilon_i \qquad (3)$$

where $\varepsilon_i = c_i + e_i$ represents all nonadditive genetic and environmental effects affecting the phenotypic value y_i, assumed $\varepsilon_i \sim N(0, \sigma_\varepsilon^2)$.

Assuming that each effect in Model (3) is independent from each other, the covariance between phenotype and breeding value is given by:

$$\mathrm{Cov}[y_i, a_i] = \mathrm{Cov}[\mu + a_i + \varepsilon_i, a_i] = \mathrm{Var}[a_i] = \sigma_a^2,$$

such that the correlation between phenotype and breeding value is:

$$r_{y_i,a_i} = \frac{\mathrm{Cov}[y_i, a_i]}{\sqrt{\mathrm{Var}[y_i]\mathrm{Var}[a_i]}} = \frac{\sigma_a^2}{\sigma_y \sigma_a} = \frac{\sigma_a}{\sigma_y} = \sqrt{h^2},$$

i.e., the square root of the (narrow sense) heritability.

As the breeding values of animals are unknown in practice, what phenotypic selection does is to predict (or estimate) the animals' breeding values based on their own performance. The prediction is based on the regression of breeding values on phenotypes, and the regression coefficient (slope) is given by:

$$b_{a_i \bullet y_i} = \frac{\mathrm{Cov}[y_i, a_i]}{\mathrm{Var}[y_i]} = \frac{\sigma_a^2}{\sigma_y^2} = h^2.$$

This means that an animal's estimated breeding value (EBV) based solely on its performance (and with a single measurement only) can be expressed as:

$$\hat{a}_i = h^2(y_i - \mu).$$

The correlation between such EBV (which is a linear transformation of y_i) and the true breeding value (a_i) is $r_{\hat{a}_i,a_i} = \frac{\mathrm{Cov}[\hat{a}_i,a_i]}{\sqrt{\mathrm{Var}[\hat{a}_i]\mathrm{Var}[a_i]}} = \frac{h^2\sigma_a^2}{\sqrt{h^4\sigma_y^2\sigma_a^2}} = h$, which

is generally referred to as "prediction accuracy" in the animal breeding literature [5]. The square of the accuracy in this case is equal to the heritability of the trait and is often called "prediction reliability." The prediction accuracy (and consequently the reliability) can be increased by using additional sources of information on an animal (such as repeated measurements of the trait or performance of progeny and other relatives) when estimating its breeding value. An example is with selection indexes and mixed model methodology, which will be discussed later in this chapter.

As indicated in Fig. 4, the selected animals (i.e., the best performing animals) will have an average phenotypic value equal to S and an average breeding value equal to *R*. The expected average breeding value (and also the expected phenotypic performance) of the progeny of the selected animals is also *R*, as illustrated in Fig. 5, and the ratio R/S is equal to the heritability (h^2) of the trait under selection. The genetic progress after one generation of selection is then given by:

$$R = h^2 S,$$

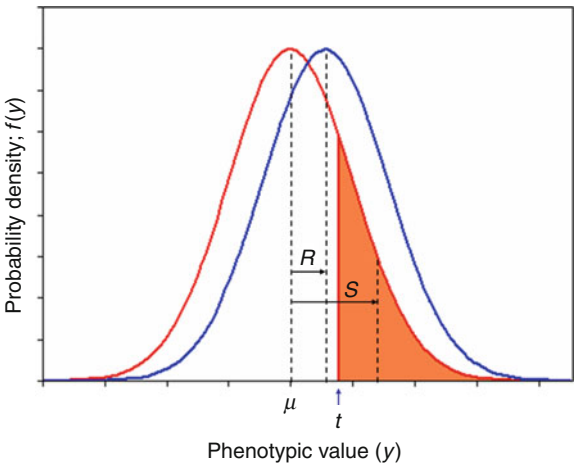
where $R = \mu_P - \mu$ and $S = \mu_S - \mu$, with μ_P, μ_S, and μ representing the average phenotypic performance of the progeny (generation 1), of the selected animals, and of the selection candidate (generation 0) populations, respectively.

The selection differential (S) can also be expressed as $S = i\sigma_y$, where $i = \frac{\mu_S - \mu}{\sigma_y}$ is called "selection intensity," and represents the selection differential in terms of phenotypic standard deviations. In addition, as *R* represents the genetic progress expected in a single generation of selection, the genetic improvement per unit of time is then given by $R^* = R/L$, where *L* is the generation interval. Hence, the expected genetic progress when phenotypic selection on a single trait is employed is [1, 3]:

$$R^* = \frac{h^2 i \sigma_y}{L},$$

which, given that $\sigma_y = \sigma_a / h$, can be expressed also as:

$$R^* = \frac{h i \sigma_a}{L}.$$



Animal Breeding, Foundations of. Figure 5
Probability density of the distribution of phenotypic values in the candidates-for-selection (*red*) and the progeny (*blue*) populations. The candidates-for-selection group represents the parental population (or generation 0), from which the top performing animals (above the threshold *t*) are selected and mated to produce the next generation, or progeny (generation 1). The difference between the phenotypic average of the selected animals and that of the generation 0 is called selection differential (represented by *S*), and the difference between the phenotypic mean of the progeny and that of the generation 0 is called genetic progress, or genetic response (represented by *R*)

This equation is a special form of the so-called "breeder's equation" (or "key equation"), for the case of phenotypic selection. In its general form, the breeder's equation is expressed as [5]:

$$R^* = \frac{\text{Accuracy} \times \text{Intensity} \times \text{Variation}}{\text{Generation interval}},$$

meaning that the genetic progress per unit of time is proportional to the accuracy of breeding values prediction, to the selection intensity, and to the genetic variation, and inversely proportional to the generation interval.

Hence, to increase the genetic progress in a population (e.g., breed or line) through selection, animal breeders (and similarly plant breeders) work to improve the four components of the equation above. As the genetic variability is a natural characteristic of a population and cannot be easily changed, genetic

progress is generally incremented by improving prediction accuracy (e.g., by using specific statistical techniques to combine different sources of information regarding the animals' genetic merit), by increasing the selection intensity, and by shortening the generation interval, which can be accomplished using molecular genetics techniques (e.g., the use of marker-assisted selection) and biotechnology approaches (e.g., artificial insemination).

It is important to mention that the breeder's equation discussed here can be extended for more complex scenarios, such as when males and females contribute differently for some components of the equation [5]. For example, prediction accuracies and selection intensity are generally higher for males if artificial insemination is used. Another important issue to mention here is that selection not only shifts the mean of the breeding values in a population but also changes the genetic variance (and heritability). A primary cause of the change in genetic variance is due to the fact that selected parents represent one tail of the phenotypic distribution, therefore their phenotypic variance is smaller than that of the whole candidates-for-selection population. This leads to a reduction in both the phenotypic and additive genetic variances in the progeny population, which is known as the "Bulmer effect" [2]. In addition, as selection modifies allele frequencies toward the fixation of favorable alleles, selection in one direction over many generations is also expected to reduce the genetic variation. Additional discussion on effects of selection on variance and other short-term and long-term consequences of artificial selection can be found, for example, in [2, 3].

In the remainder of this chapter specific statistical techniques (such as the selection index, BLUP, and genomic selection) for the improvement of accuracy, intensity, and generation interval, and consequently the increase of genetic progress from artificial selection, will be discussed.

Correlated Response and Indirect Selection

If two traits x and y are genetically correlated, direct selection on one of the traits (say y) will also cause a genetic change in the other trait (trait x), which is called "correlated response" [3]. Correlated response to selection ($R_{x\bullet y}$), that is, genetic change in trait x as a consequence of direct selection on trait y, can be predicted by:

$$R_{x\bullet y} = b_{x\bullet y} R_y,$$

where R_y is the genetic progress of trait y through direct selection on itself, and $b_{x\bullet y}$ is the genetic regression coefficient, given by:

$$b_{x\bullet y} = \frac{\mathrm{Cov}(a_x, a_y)}{\sigma^2_{a_y}},$$

where $\mathrm{Cov}(a_x, a_y)$ is the genetic covariance between traits x and y.

The genetic correlation between two traits x and y is given by:

$$\rho_{a_x,a_y} = \frac{\mathrm{Cov}(a_x, a_y)}{\sigma_{a_x}\sigma_{a_y}},$$

such that $\mathrm{Cov}(a_x, a_y) = \rho_{a_x,a_y}\sigma_{a_x}\sigma_{a_y}$ and the genetic regression can be expressed as:

$$b_{x\bullet y} = \frac{\rho_{a_x,a_y}\sigma_{a_x}\sigma_{a_y}}{\sigma^2_{a_y}} = \rho_{a_x,a_y}\frac{\sigma_{a_x}}{\sigma_{a_y}}.$$

Using this term, and recalling the selection response formula discussed before, given by $R_y = h_y i_y \sigma_{a_y}$, the correlated response can then be expressed as:

$$R_{x\bullet y} = \rho_{a_x,a_y}\frac{\sigma_{a_x}}{\sigma_{a_y}} h_y i_y \sigma_{a_y} = \rho_{a_x,a_y}\sigma_{a_x} h_y i_y,$$

or, given that $\sigma_{a_x} = h_x \sigma_{y_x}$, it can be finally written as:

$$R_{x\bullet y} = \rho_{a_x,a_y} h_x h_y i_y \sigma_{y_x}.$$

Such an equation can be used either to monitor potential genetic changes in correlated traits when performing direct selection on a specific trait of economic importance or, alternatively, to explore indirect selection strategies using indicator traits [5]. The latter use may be of interest when a trait of economic importance (e.g., trait x) is difficult or expensive to measure, or it is expressed later in an animal's life, so it may be advantageous to select on a correlated trait (e.g., trait y), which would be the indicator trait. To assess the effectiveness of indirect selection relative to direct selection, one may look at

the ratio of expected genetic progress per unit of time in each scenario, i.e.,

$$\frac{R_{x\bullet y}}{R_x} = \frac{\rho_{a_x,a_y}\sigma_{a_x}h_y i_y/L_y}{h_x i_x \sigma_{a_x}/L_x} = \frac{\rho_{a_x,a_y}h_y i_y L_x}{h_x i_x L_y} = \rho_{a_x,a_y}\frac{h_y}{h_x}\frac{i_y}{i_x}\frac{L_x}{L_y}.$$

So, it can be seen that this ratio can be higher than 1 (meaning that the indirect selection is more effective than the direct selection) depending on the genetic correlation between the economic and the indicator traits, the ratios of their heritabilities, and their potential selection intensities and generation intervals.

Selection Index

In section "Phenotypic Selection," selection based on a single measurement on each animal was discussed. However, it is not always possible to observe the phenotype for all animals, such as traits that are expressed in only one sex or that require the sacrifice of animals to be measured, etc. In addition, even when it is possible to measure the phenotypic trait in each animal, information from relatives can be used to obtain earlier or more reliable predictions of breeding values. In this section, the prediction of breeding values using different sources of information (e.g., multiple measurements of the trait in each animal and progeny performance) will be discussed and a methodology (the selection index) that combines multiple sources of information into a single prediction for each animal will be presented.

When multiple measurements of the same trait are recorded (e.g., milk yield in multiple lactations), breeding values can be predicted using the average of observations ($\bar{y}_i$) from each animal as $\hat{a}_i = b_{a_i\bullet\bar{y}_i}(\bar{y}_i - \mu)$. However, to derive the genetic regression of breeding value on average phenotypic value, Model (3) must be expanded to include an additional term, which is discussed next.

It can be shown empirically that the covariance (or resemblance) between repeated measurements on the same animal is larger than σ_a^2, which is what would be expected under the assumptions of Model (3). This additional source of covariance between records for the same animal refers to environmental factors that affect all records similarly, the so-called "permanent environmental effects" [1, 4]. Under these circumstances, the Model (3) can be extended to:

$$y_{ij} = \mu + a_i + p_i + \varepsilon_{ij} \tag{4}$$

where y_{ij} represents the observation j ($j = 1, \ldots, n_i$) on animal i, with n_i being the total number of records on animal i; μ and $a_i \sim N(0, \sigma_a^2)$ are as defined previously; p_i refers to the permanent environmental effects affecting records on animal i, assumed $p_i \sim N(0, \sigma_p^2)$; and $\varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2)$ represent residual effects (nonadditive genetic and temporary environmental effects) associated with observation y_{ij}. In addition, it is assumed that all random terms in Model (4) are independent from each other, i.e., $\mathrm{Cov}(a_i, p_i) = \mathrm{Cov}(a_i, \varepsilon_{ij}) = \mathrm{Cov}(p_i, \varepsilon_{ij}) = \mathrm{Cov}(\varepsilon_{ij}, \varepsilon_{ij'}) = 0$ for any i, j, and j' ($j \neq j'$).

Under these settings, the average phenotypic value of an animal is given by $\bar{y}_{ij} = \mu + a_i + p_i + \bar{\varepsilon}_{ij}$, where $\bar{\varepsilon}_i = \frac{1}{n_i}\sum_{j=1}^{n_i}\varepsilon_{ij}$, such that its variance is given by $\mathrm{Var}[\bar{y}_i] = \sigma_a^2 + \sigma_p^2 + \sigma_\varepsilon^2/n_i$, and the covariance between a_i and $\bar{y}_i$ is $\mathrm{Cov}(a_i, \bar{y}_i) = \sigma_a^2$. In this case, the regression of breeding values on phenotypic means is given by:

$$b_{a_i\bullet\bar{y}_i} = \frac{\mathrm{Cov}[a_i, \bar{y}_i]}{\mathrm{Var}[\bar{y}_i]} = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_p^2 + \sigma_\varepsilon^2/n_i}.$$

An important definition related to repeated measurements refers to repeatability (r), which is given by the intraclass correlation, i.e., the ratio of the within-individual (or between repeated measurements) to the phenotypic variances [1, 4]:

$$r = \frac{\sigma_a^2 + \sigma_p^2}{\sigma_y^2} = \frac{\sigma_a^2 + \sigma_p^2}{\sigma_a^2 + \sigma_p^2 + \sigma_\varepsilon^2},$$

and measures the correlation between records on the same animal.

Noting that $r = 1 - \frac{\sigma_\varepsilon^2}{\sigma_a^2+\sigma_p^2+\sigma_\varepsilon^2}$, the variance of the average phenotypic value of an animal can be expressed as a function of the repeatability as $\mathrm{Var}[\bar{y}_i] = [r + (1-r)/n_i]\sigma_y^2$, such that the genetic regression becomes:

$$b_{a_i\bullet\bar{y}_i} = \frac{\sigma_a^2}{[r + (1-r)/n_i]\sigma_y^2} = \frac{n_i h^2}{1 + (n_i - 1)/r}.$$

The prediction accuracy in this case, i.e., the correlation between an animal's estimated breeding

value using repeated records and its true breeding value is given by:

$$r_{\hat{a}_i,a_i} = r_{\bar{y}_i,a_i} = \frac{\mathrm{Cov}(\bar{y}_i, a_i)}{\sqrt{\mathrm{Var}(\bar{y}_i)\mathrm{Var}(a_i)}} = \frac{\sigma_a^2}{\sqrt{r + (1-r)/n_i}\sigma_y\sigma_a} = h\sqrt{\frac{n_i}{1+(n_i-1)r}} = \sqrt{b_{a_i\bullet\bar{y}_i}}.$$

Hence, it can be seen that compared with single record phenotypic selection, there is a gain in accuracy when predictions are based on repeated records and that the gain will depend on the values of r and n_i; higher gain in accuracy is obtained when r is low and when n_i is high.

Another alternative to predict breeding values is to use progeny performance, which is often employed for predicting breeding values of males for traits where records can be obtained only on females, such as milk yield. For example, let $\bar{y}_i$ be the average of single records on n_i progeny of sire i, and assume that the sire was mated to a random sample of females not related to him. In this case, each progeny record can be expressed as:

$$y_{ij} = \mu + \frac{1}{2}a_i + \frac{1}{2}d_{ij} + \delta_{ij} + \varepsilon_{ij},$$

where a_i is the breeding value of a specific sire i; d_{ij} is the breeding value of dam j ($j = 1, \ldots, n_i$) mated with sire i; and δ_{ij} and ε_{ij} refer to the Mendelian sampling and residual (environmental) components associated with the observation y_{ij}. Using this notation, the following model can be used to describe the progeny average of sire i:

$$\bar{y}_i = \mu + \frac{1}{2}a_i + \frac{1}{2}\bar{d}_i + \bar{\delta}_i + \bar{\varepsilon}_i \qquad (5)$$

where $\bar{y}_i = \frac{1}{n_i}\sum_{i=1}^{n_i} y_{ij}$, $\bar{d}_i = \frac{1}{n_i}\sum_{i=1}^{n_i} d_{ij}$, $\bar{\delta}_i = \frac{1}{n_i}\sum_{i=1}^{n_i} \delta_{ij}$, and $\bar{\varepsilon}_i = \frac{1}{n_i}\sum_{i=1}^{n_i} \varepsilon_{ij}$. Given that $E[\bar{d}_i] = 0$ and $E[\bar{\delta}_i] = 0$, the breeding value of sire i can be then predicted by $\hat{a}_i = b_{a_i\bullet\bar{y}_i}(\bar{y}_i - \mu)$, where $b_{a_i\bullet\bar{y}_i} = \mathrm{Cov}[a_i, \bar{y}_i]/\mathrm{Var}[\bar{y}_i]$. It is shown that:

$$\mathrm{Cov}(a_i, \bar{y}_i) = \mathrm{Cov}(a_i, a_i/2) = \sigma_a^2/2$$

and

$$\mathrm{Var}[\bar{y}_i] = \mathrm{Var}\left[\frac{1}{2}a_i + \frac{1}{2}\bar{d}_i + \bar{\delta}_i + \bar{\varepsilon}_i\right] = \frac{1}{4}\sigma_a^2 + \frac{1}{4}\frac{\sigma_a^2}{n_i} + \frac{\sigma_a^2}{2n_i} + \frac{\sigma_\varepsilon^2}{n_i} = \frac{(n_i+3)\sigma_a^2 + 4\sigma_\varepsilon^2}{4n_i} = \frac{(n_i+3)h^2 + 4(1-h^2)}{4n_i}\sigma_y^2 = \left[k + \frac{1-k}{n_i}\right]\sigma_y^2,$$

where $k = h^2/4$ is the intraclass correlation between half-sibs, such that the genetic regression coefficient is given by:

$$b_{a_i\bullet\bar{y}_i} = \frac{\sigma_a^2/2}{[k + (1-k)/n_i]\sigma_y^2} = \frac{h^2\sigma_y^2/2}{[h^2/4 + (1-h^2/4)/n_i]\sigma_y^2} = \frac{2n_ih^2}{4 + (n_i-1)h^2},$$

and the prediction accuracy by:

$$r_{a_i,\bar{y}_i} = \frac{\mathrm{Cov}[a_i, \bar{y}_i]}{\sqrt{\mathrm{Var}[a_i]\mathrm{Var}[\bar{y}_i]}} = \frac{h^2\sigma_y^2/2}{\sqrt{h^2\sigma_y^2[k+(1-k)/n_i]\sigma_y^2}} = \sqrt{\frac{n_ih^2/4}{1+(n_i-1)k}} = \sqrt{\frac{n_ih^2}{4+(n_i-1)h^2}} = \sqrt{b_{a_i\bullet\bar{y}_i}/2},$$

which approaches unity (one) as the number of progeny records increases.

Up to this point, it has been discussed how breeding values can be predicted using different sources of information, such as an animal's own performance (either a single record or multiple measurements) or progeny performance. Other sources of information that could also be used are the performance of parents, sibling, or other kinds of relatives. However, what generally happens is that multiple sources of information are available simultaneously, so the question becomes how to best combine all the information in order to improve prediction accuracy. Here, a classical approach will be discussed, the "selection index," and later on in this

chapter a more general and modern alternative, based on mixed model methodology, will be presented.

Consider, for example, that there are three sources of information available on animal i (represented here as y_{i1}, y_{i2}, and y_{i3}, and expressed as deviations from their means), so the goal is to predict the animal's breeding value with a linear combination of such information, i.e.,

$$\hat{a}_i = b_{i1}y_{i1} + b_{i2}y_{i2} + b_{i3}y_{i3},$$

such that the prediction accuracy (i.e., correlation between predicted and true breeding value) is maximized.

Maximization of $r_{\hat{a}_i,a_i}$ is equivalent to the maximization of $\log(r_{\hat{a}_i,a_i})$, which is generally easier to accomplish. The log correlation can be expressed as (here, to simplify the notation, the index i indicating the animal is dropped):

$$\log(r_{\hat{a},a}) = \log\left[\frac{\sigma_{\hat{a},a}}{\sqrt{\sigma^2_{\hat{a}}\sigma^2_a}}\right] = \log(\sigma_{\hat{a},a}) - \frac{1}{2}\sigma^2_{\hat{a}} - \frac{1}{2}\sigma^2_a,$$

where the covariance between $\hat{a}$ and a, and the variance of $\hat{a}$ are given respectively by:

$$\sigma_{\hat{a},a} = b_1\sigma_{y_1,a} + b_2\sigma_{y_2,a} + b_3\sigma_{y_3,a}$$

and

$$\begin{aligned}\sigma^2_{\hat{a}} =& b_1^2\sigma^2_{y_1} + 2b_1b_2\sigma_{y_1,y_2} + 2b_1b_3\sigma_{y_1,y_3} \\ &+ b_2^2\sigma^2_{y_2} + 2b_2b_3\sigma_{y_2,y_3} + b_3^2\sigma^2_{y_3}.\end{aligned}$$

Substituting these expressions into $\log(r_{\hat{a},a})$, taking the partial derivatives of $\log(r_{\hat{a},a})$ with respect to each of the regression coefficients b_j $(j = 1, 2, 3)$, and setting them to zero, gives the following set of equations:

$$\begin{cases} \dfrac{\partial\log(r_{\hat{a},a})}{\partial b_1} = \dfrac{\sigma_{y_1,a}}{\sigma_{\hat{a},a}} - \dfrac{b_1\sigma^2_{y_1} + b_2\sigma_{y_1,y_2} + b_3\sigma_{y_1,y_3}}{\sigma^2_{\hat{a}}} \\ \dfrac{\partial\log(r_{\hat{a},a})}{\partial b_2} = \dfrac{\sigma_{y_2,a}}{\sigma_{\hat{a},a}} - \dfrac{b_1\sigma_{y_1,y_2} + b_2\sigma^2_{y_2} + b_3\sigma_{y_2,y_3}}{\sigma^2_{\hat{a}}} \\ \dfrac{\partial\log(r_{\hat{a},a})}{\partial b_3} = \dfrac{\sigma_{y_3,a}}{\sigma_{\hat{a},a}} - \dfrac{b_1\sigma_{y_1,y_3} + b_2\sigma_{y_2,y_3} + b_3\sigma^2_{y_3}}{\sigma^2_{\hat{a}}} \end{cases}$$

which can be rearranged as:

$$\begin{cases} b_1\sigma^2_{y_1} + b_2\sigma_{y_1,y_2} + b_3\sigma_{y_1,y_3} = k\sigma_{y_1,a} \\ b_1\sigma_{y_1,y_2} + b_2\sigma^2_{y_2} + b_3\sigma_{y_2,y_3} = k\sigma_{y_2,a} \\ b_1\sigma_{y_1,y_3} + b_2\sigma_{y_2,y_3} + b_3\sigma^2_{y_3} = k\sigma_{y_3,a} \end{cases}$$

where $k = \sigma^2_{\hat{a}}/\sigma_{\hat{a},a}$.

Extending the system for any number m of components (i.e., sources of information), these equations can be expressed in matrix notation as:

$$\mathbf{Pb} = \mathrm{k}\mathbf{c},$$

where $\mathbf{P} = \begin{bmatrix} \sigma^2_{y_1} & \sigma_{y_1,y_2} & \cdots & \sigma_{y_1,y_m} \\ \sigma_{y_1,y_2} & \sigma^2_{y_2} & \cdots & \sigma_{y_2,y_m} \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_{y_1,y_m} & \sigma_{y_2,y_m} & \cdots & \sigma^2_{y_m} \end{bmatrix}$ is the covariance matrix of the vector $\mathbf{y} = [y_1, y_2, \ldots, y_m]'$, $\mathbf{b} = [b_1, b_2, \ldots, b_m]'$ is the vector of regression coefficients (weights) of each source of information, and $\mathbf{c} = [\sigma_{y_1,a}, \sigma_{y_2,a}, \ldots, \sigma_{y_m,a}]'$ is the vector of covariances between each piece of information and the breeding value of the animal, such that the weights $\mathbf{b}$ of the index $\hat{a} = \mathbf{b}'\mathbf{y}$ are given by $\mathbf{b} = k\mathbf{P}^{-1}\mathbf{c}$.

It should be noted that the constant k does not change the relative size of the regression coefficients $\mathbf{b}$ or the value of $r_{\hat{a},a}$, so it can be set to 1. In fact, if instead of maximizing $r_{\hat{a},a}$, the average square prediction error $E[\hat{a} - a]^2$ is minimized, then $\sigma^2_{\hat{a}} = \sigma_{\hat{a},a}$ and the system (usually called selection index equations) becomes:

$$\mathbf{b} = \mathbf{P}^{-1}\mathbf{c}.$$

The correlation between the index and the true breeding value is given by $r_{\hat{a},a} = \sigma_{\hat{a},a}/\sqrt{\sigma^2_{\hat{a}}\sigma^2_a} = \sqrt{\sigma_{\hat{a},a}/\sigma^2_a} = \sqrt{\frac{1}{\sigma^2_a}\sum_{j=1}^{m} b_j\sigma_{y_j,a}}$.

Multiple-Trait Selection

Usually more than one trait is considered in a selection program, as multiple traits may be economically important in a production system (e.g., [9]). There are many strategies for multi-trait selection, including the tandem approach (which selects rotationally one trait at a time) and the independent culling levels strategy (which sets minimum performance levels for each of the traits of interest), but they are generally suboptimal.

Here, the selection of a combination of multiple traits evaluated in economic terms will be discussed. Such a combination of traits is generally called "aggregate breeding value" or "breeding objective," and can be expressed as [3]:

$$T = \mathbf{w}'\mathbf{a} = w_1a_1 + w_2a_2 + \ldots + w_ka_k,$$

where $\mathbf{w} = (w_1, w_2, \ldots, w_k)'$ is the vector of economic weights (expressed as net economic value per unit of trait) for k traits of linear economic value, and $\mathbf{a} = (a_1, a_2, \ldots, a_k)'$ is a vector of breeding values relative to the k traits defining T. Here again, to simplify the notation, a subscript indexing the animal is suppressed.

Suppose records are available for m traits, which may or may not be included in the k traits describing the breeding objective. The goal then is to predict T based on the m traits observed, using the so-called economic selection index. The theory of selection index was introduced in the previous subsection as a means of combining multiple sources of information to predict breeding values for a specific trait. Here, similar methodology will be considered, but will be used to combine information from multiple traits to predict an overall economic merit for each animal, i.e.,

$$\hat{T} = I = \mathbf{v}'\mathbf{y} = v_1 y_1 + v_2 y_2 + \ldots + v_m y_m,$$

where $\hat{T}$ is the predicted overall economic merit of an animal, $\mathbf{v} = (v_1, v_2, \ldots, v_m)'$ is the vector of weighting factors, and $\mathbf{y} = (y_1, y_2, \ldots, y_m)'$ is the vector of phenotypic measurements.

An alternative for determining the weights $\mathbf{v} = (v_1, v_2, \ldots, v_m)'$ is to first predict separately the breeding values a_j, $j = 1, 2, \ldots, k$, for each trait involved in the breeding objective, using information from all the traits with measurements, $\mathbf{y} = (y_1, y_2, \ldots, y_m)'$. Afterward, such predictions are substituted for the true breeding values in the breeding objective equation, and then coefficients are grouped accordingly.

The breeding values a_j for each trait can be predicted by $\hat{a}_j = b_{j1} y_1 + b_{j2} y_2 + \ldots + b_{jm} y_m$, in which the weights are obtained as usual, to maximize $r_{\hat{a}_j, a_j}$ or minimize $E[\hat{a}_j - a_j]^2$. The equations which define the weights for the prediction of a_j are then given by:

$$\begin{cases} b_{j1}\sigma^2_{y_1} + b_{j2}\sigma_{y_1,y_2} + \ldots + b_{jm}\sigma_{y_1,y_m} = \sigma_{y_1,a_j} \\ b_{j1}\sigma_{y_1,y_2} + b_{j2}\sigma^2_{y_2} + \ldots + b_{jm}\sigma_{y_2,y_m} = \sigma_{y_2,a_j} \\ \quad\vdots \qquad\quad \vdots \qquad\qquad \vdots \qquad\quad \vdots \\ b_{j1}\sigma_{y_1,y_m} + b_{j2}\sigma_{y_2,y_m} + \ldots + b_{jm}\sigma^2_{y_m} = \sigma_{y_m,a_j} \end{cases}$$

This procedure is repeated for all k traits in the breeding objective, and the predictions $\hat{\mathbf{a}} = (\hat{a}_1, \hat{a}_2, \ldots, \hat{a}_k)'$ are then substituted for the true values $\mathbf{a} = (a_1, a_2, \ldots, a_k)'$ in the aggregate breeding value, i.e.,

$$\hat{T} = w_1\hat{a}_1 + w_2\hat{a}_2 + \ldots + w_k\hat{a}_k.$$

This overall index estimating T can be rewritten as $I = v_1 y_1 + v_2 y_2 + \ldots + v_m y_m$, by using appropriate multiplications and grouping of coefficients. It is shown that each coefficient v_i is given by $v_i = w_1 b_{1i} + w_2 b_{2i} + \ldots + w_k b_{ki}$, with $i = 1, 2, \ldots, m$.

Another way of deriving the weights $\mathbf{v} = (v_1, v_2, \ldots, v_m)'$ defining the economic selection index $I = \mathbf{v}'\mathbf{y}$ is to maximize the correlation $r_{T,I}$, which will generate the following equations:

$$\begin{cases} v_1\sigma^2_{y_1} + v_2\sigma_{y_1,y_2} + \ldots + v_m\sigma_{y_1,y_m} = \sigma_{y_1,T} \\ v_1\sigma_{y_1,y_2} + v_2\sigma^2_{y_2} + \ldots + v_m\sigma_{y_2,y_m} = \sigma_{y_2,T} \\ \quad\vdots \qquad\quad \vdots \qquad\qquad \vdots \qquad\quad \vdots \\ v_1\sigma_{y_1,y_m} + v_2\sigma_{y_2,y_m} + \ldots + v_m\sigma^2_{y_m} = \sigma_{y_m,T} \end{cases}$$

where $\sigma_{y_i,T}$ is the covariance between each measured trait i $(i = 1, 2, \ldots, m)$ and the linear function $T = \mathbf{w}'\mathbf{a}$, i.e., the aggregate breeding value. It can be shown that both approaches for determining the weights $\mathbf{v} = (v_1, v_2, \ldots, v_m)'$ are equivalent.

Mixed Model Methodology

Introduction

Many statistical methods for analysis of genetic data are specific (or more appropriate) for phenotypic measurements obtained from planned experimental designs with balanced data sets. While such situations may be possible within laboratory or greenhouse experimental settings, data from natural populations and agricultural species are generally highly unbalanced and fragmented by numerous kinds of relationships. Culling of data to accommodate conventional statistical techniques (such as those discussed to this point) may introduce bias and/or lead to a substantial loss of information. The mixed model methodology, on the other hand, allows efficient estimation of genetic parameters (such as variance components and heritability) and breeding values while accommodating extended pedigrees, unequal family sizes, overlapping generations, sex-limited traits, assortative mating, and natural or artificial selection.

The single trait prediction methods discussed in the previous section use only a single source of information or, when multiple sources of information are available, they require them to be split into independent subgroups, i.e., specific groups of relatives such as half-sibs, full-sibs, progeny, etc. However, in practice the data may be extremely complex due to the intricate pedigree structure commonly found in livestock species, e.g., beef and dairy cattle populations. Other drawbacks of the selection index include an inability to account for genetic trend over time and that the phenotypes must be pre-adjusted for environmental effects, which can be done, for example, using the average of contemporary groups of animals. However, contemporary group effects can be inferred only under the unrealistic assumption that they are genetically equal. Hence, a selection index can be reliably applied only to individual animals within same herd and born in same year.

In view of such limitations, linear mixed models (models including both fixed and random effects) and best linear unbiased predictions (BLUP) of breeding values were developed [10–12]. The BLUP methodology uses performance information from all known relatives to estimate breeding values, can be applied to whole herds or large populations using data from many years, and can also accommodate genetic differences between contemporary groups. Presently, mixed models are widely used in many fields of science as a flexible tool for the analysis of data where responses are clustered around some random effects, such that there is a natural dependence between observations in the same cluster. Examples of applications of mixed models in genetics and genomics include gene mapping and association analysis (e.g., [13, 14]), and gene expression assays using microarrays [15, 16] or RT-PCR [17], to name a few.

In some applications of mixed models the central objective is the estimation and hypothesis testing regarding fixed effects (e.g., treatment effects in an experimental study), in which case the random effects (e.g., block effects) are nuisance effects. In animal breeding, however, the main goal is the prediction of realized values of random effects (breeding values of animals), and the fixed effects are generally environmental factors that should be taken into account to adjust the observed phenotypic values. A third application or goal of mixed models is the estimation of variance components, such as genetic and environmental variances, or functions of them, such as heritability and repeatability.

In this section some basics regarding mixed models are briefly reviewed, with some emphasis toward the prediction of random effects, and subsequently some specific applications of the mixed model methodology in animal breeding and genetics are presented.

A linear mixed-effects model is defined as:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{u} + \boldsymbol{\varepsilon} \tag{6}$$

where $\mathbf{y}$ is the vector of responses (observations), $\boldsymbol{\beta}$ is a vector of fixed effects, $\mathbf{u}$ is a vector of random effects, $\mathbf{X}$ and $\mathbf{Z}$ are known incidence matrices relate $\mathbf{y}$ to the vectors $\boldsymbol{\beta}$ and $\mathbf{u}$, respectively, and ε is a vector of residual terms. Generally, it is assumed that $\mathbf{u}$ and ε are independent from each other and normally distributed with zero-mean vectors and variance–covariance matrices $\mathbf{G}$ and $\boldsymbol{\Sigma}$, respectively.

As mentioned before, in animal breeding a central goal refers to the prediction of random effects (breeding values). In linear (Gaussian) models as in (6) such predictions are given by the conditional expectation of $\mathbf{u}$ given the data, i.e., $E[\mathbf{u}|\mathbf{y}]$. Given the model specifications above, the joint distribution of $\mathbf{y}$ and $\mathbf{u}$ is:

$$\begin{bmatrix}\mathbf{y}\\ \mathbf{u}\end{bmatrix} \sim \mathrm{MVN}\left(\begin{bmatrix}\mathbf{X}\boldsymbol{\beta}\\ \mathbf{0}\end{bmatrix}, \begin{bmatrix}\mathbf{V} & \mathbf{ZG}\\ \mathbf{GZ}' & \mathbf{G}\end{bmatrix}\right),$$

where $\mathbf{V} = \mathbf{ZGZ}' + \boldsymbol{\Sigma}$.

From the properties of multivariate normal distributions, $E[\mathbf{u}|\mathbf{y}]$ is given by:

$$E[\mathbf{u}|\mathbf{y}] = E[\mathbf{u}] + \mathrm{Cov}[\mathbf{u}, \mathbf{y}']\mathrm{Var}^{-1}[\mathbf{y}](\mathbf{y} - E[\mathbf{y}]),$$

such that in this case:

$$\begin{aligned} E[\mathbf{u}|\mathbf{y}] &= \mathbf{GZ}'\mathbf{V}^{-1}(\mathbf{y} - \mathbf{X}\boldsymbol{\beta}) \\ &= \mathbf{GZ}'(\mathbf{ZGZ}' + \boldsymbol{\Sigma})^{-1}(\mathbf{y} - \mathbf{X}\boldsymbol{\beta}). \end{aligned}$$

This expression, however, depends on the fixed effects values $\boldsymbol{\beta}$, which also need to be inferred from the data. The fixed effects are then typically replaced by their estimates, such that predictions are made based on the following expression:

$$\hat{\mathbf{u}} = \mathbf{GZ}'\mathbf{V}^{-1}(\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}}).$$

To estimate the fixed effects $\boldsymbol{\beta}$, all random effects in Model (6) can be combined into a single vector,

$\boldsymbol{\xi} = \mathbf{Zu} + \boldsymbol{\varepsilon}$, such that the following fixed effects model is obtained: $\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \boldsymbol{\xi}$. It is shown that the expectation of the ξ term is $E[\boldsymbol{\xi}] = E[\mathbf{Zu} + \boldsymbol{\varepsilon}] = \mathbf{Z}E[\mathbf{u}] + E[\boldsymbol{\varepsilon}] = \mathbf{0}$, and that its variance is $\mathrm{Var}[\boldsymbol{\xi}] = \mathrm{Var}[\mathbf{Zu} + \boldsymbol{\varepsilon}] = \mathbf{Z}\mathrm{Var}[\mathbf{u}]\mathbf{Z}' + \mathrm{Var}[\boldsymbol{\varepsilon}] = \mathbf{ZGZ}' + \boldsymbol{\Sigma} = \mathbf{V}$. Under these settings, the distribution of $\mathbf{y}$ is multivariate normal with mean vector $\mathbf{X}\boldsymbol{\beta}$ and covariance matrix $\mathbf{V}$, i.e., $\mathbf{y} \sim MVN(\mathbf{X}\boldsymbol{\beta}, \mathbf{V})$, and the maximum likelihood estimator of β can be shown to be:

$$\hat{\boldsymbol{\beta}} = (\mathbf{X}'\mathbf{V}^{-1}\mathbf{X})^{-1}\mathbf{X}'\mathbf{V}^{-1}\mathbf{y},$$

which is distributed as $\hat{\boldsymbol{\beta}} \sim MVN(\boldsymbol{\beta}, (\mathbf{X}'\mathbf{V}^{-1}\mathbf{X})^{-1})$. If the design matrix $\mathbf{X}$ is not full column rank, a generalized inverse of $\mathbf{X}'\mathbf{V}^{-1}\mathbf{X}$ must be used to obtain a solution $\boldsymbol{\beta}^{\boldsymbol{0}} = (\mathbf{X}'\mathbf{V}^{-1}\mathbf{X})^{-}\mathbf{X}'\mathbf{V}^{-1}\mathbf{y}$ of the system, from which estimable functions $\boldsymbol{\theta} = \mathbf{L}\boldsymbol{\beta}$ are estimated as $\hat{\boldsymbol{\theta}} = \mathbf{L}\boldsymbol{\beta}^{\boldsymbol{0}}$.

The solutions $\hat{\boldsymbol{\beta}}$ and $\hat{\mathbf{u}}$ discussed before require $\mathbf{V}^{-1}$. As $\mathbf{V}$ can be of huge dimensions, especially in animal breeding applications, its inverse is generally computationally demanding if not unfeasible. However, Henderson [18] presented the mixed model equations (MME) to estimate $\boldsymbol{\beta}$ and $\mathbf{u}$ simultaneously, without the need for computing $\mathbf{V}^{-1}$. The MME were derived by maximizing (for $\boldsymbol{\beta}$ and $\mathbf{u}$) the joint density of $\mathbf{y}$ and $\mathbf{u}$, expressed as:

$$p(\mathbf{y}, \mathbf{u}) \propto |\boldsymbol{\Sigma}|^{-1/2}|\mathbf{G}|^{-1/2} \exp\left\{-\frac{1}{2}(\mathbf{y} - \mathbf{X}\boldsymbol{\beta} - \mathbf{Zu})' \boldsymbol{\Sigma}^{-1}(\mathbf{y} - \mathbf{X}\boldsymbol{\beta} - \mathbf{Zu}) - \frac{1}{2}\mathbf{u}'\mathbf{G}^{-1}\mathbf{u}\right\}.$$

The logarithm of this function is:

$$\begin{aligned}\ell &= \log[p(\mathbf{y}, \mathbf{u})] \propto |\boldsymbol{\Sigma}| + |\mathbf{G}| + (\mathbf{y} - \mathbf{X}\boldsymbol{\beta} - \mathbf{Zu})'\boldsymbol{\Sigma}^{-1} \\ &\quad (\mathbf{y} - \mathbf{X}\boldsymbol{\beta} - \mathbf{Zu}) + \mathbf{u}'\mathbf{G}^{-1}\mathbf{u} \\ &= |\boldsymbol{\Sigma}| + |\mathbf{G}| + \mathbf{y}'\boldsymbol{\Sigma}^{-1}\mathbf{y} - 2\mathbf{y}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\boldsymbol{\beta} - 2\mathbf{y}'\boldsymbol{\Sigma}^{-1}\mathbf{Zu} \\ &\quad + \boldsymbol{\beta}'\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\boldsymbol{\beta} + 2\boldsymbol{\beta}'\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{Zu} \\ &\quad + \mathbf{u}'\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Zu} + \mathbf{u}'\mathbf{G}^{-1}\mathbf{u}.\end{aligned}$$

The derivatives regarding $\boldsymbol{\beta}$ and $\mathbf{u}$ are:

$$\begin{bmatrix}\frac{\partial \ell}{\partial \boldsymbol{\beta}} \\ \frac{\partial \ell}{\partial \mathbf{u}}\end{bmatrix} = \begin{bmatrix}\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{y} - \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} - \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{Z}\hat{\mathbf{u}} \\ \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{y} - \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} - \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z}\hat{\mathbf{u}} - \mathbf{G}^{-1}\hat{\mathbf{u}}\end{bmatrix}.$$

Equating them to zero gives the following system:

$$\begin{bmatrix}\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} + \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{Z}\hat{\mathbf{u}} \\ \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} + \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z}\hat{\mathbf{u}} + \mathbf{G}^{-1}\hat{\mathbf{u}}\end{bmatrix} = \begin{bmatrix}\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{y} \\ \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{y}\end{bmatrix},$$

which can be expressed as:

$$\begin{bmatrix}\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{X} & \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} \\ \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{X} & \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} + \mathbf{G}^{-1}\end{bmatrix}\begin{bmatrix}\hat{\boldsymbol{\beta}} \\ \hat{\mathbf{u}}\end{bmatrix} = \begin{bmatrix}\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{y} \\ \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{y}\end{bmatrix},$$

known as the mixed model equations (MME).

Using the second part of the MME,

$$\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} + (\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} + \mathbf{G}^{-1})\hat{\mathbf{u}} = \mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{y},$$

such that

$$\hat{\mathbf{u}} = (\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} + \mathbf{G}^{-1})^{-1}\mathbf{Z}'\boldsymbol{\Sigma}^{-1}(\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}}).$$

It can be shown that this expression is equivalent to $\hat{\mathbf{u}} = \mathbf{GZ}'(\mathbf{ZGZ}' + \boldsymbol{\Sigma})^{-1}(\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}})$ and, more importantly, that $\hat{\mathbf{u}}$ is the best linear unbiased predictor (BLUP) of $\mathbf{u}$. Using this result into the first part of the MME,

$$\begin{aligned}&\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} + \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{Z}\hat{\mathbf{u}} = \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{y} \\ &\mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{X}\hat{\boldsymbol{\beta}} + \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{Z}(\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} + \mathbf{G}^{-1})^{-1} \\ &\qquad \mathbf{Z}'\boldsymbol{\Sigma}^{-1}(\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}}) = \mathbf{X}'\boldsymbol{\Sigma}^{-1}\mathbf{y} \\ &\hat{\boldsymbol{\beta}} = \{\mathbf{X}'[\boldsymbol{\Sigma}^{-1} - \boldsymbol{\Sigma}^{-1}\mathbf{Z}(\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} + \mathbf{G}^{-1})^{-1}\mathbf{Z}'\boldsymbol{\Sigma}^{-1}]\mathbf{X}\}^{-1} \\ &\qquad \mathbf{X}'[\boldsymbol{\Sigma}^{-1} - \boldsymbol{\Sigma}^{-1}\mathbf{Z}(\mathbf{Z}'\boldsymbol{\Sigma}^{-1}\mathbf{Z} + \mathbf{G}^{-1})^{-1}\mathbf{Z}'\boldsymbol{\Sigma}^{-1}]\mathbf{y}\end{aligned}.$$

Similarly, it is shown that this expression is equivalent to $\hat{\boldsymbol{\beta}} = (\mathbf{X}'\mathbf{V}^{-1}\mathbf{X})^{-1}\mathbf{X}'\mathbf{V}^{-1}\mathbf{y}$, which is the best linear unbiased estimator (BLUE) of $\boldsymbol{\beta}$.

It is important to note that $\hat{\boldsymbol{\beta}}$ and $\hat{\mathbf{u}}$ require knowledge of $\mathbf{G}$ and $\boldsymbol{\Sigma}$, or at least some function of them. As these matrices are rarely known, the practical approach is to replace $\mathbf{G}$ and $\boldsymbol{\Sigma}$ by some sort of point estimates $\hat{\mathbf{G}}$ and $\hat{\boldsymbol{\Sigma}}$ into the MME.

Many methods have been proposed to estimate variance components in mixed-effects models. The simplest is the analysis of variance (ANOVA) method, which works well for simple models (such as a one-way structure) or balanced data (such as data from designed experiments with no missing data), but they are not indicated for more complex models and data structures such as those generally found in the animal breeding context.

Alternative methods proposed for estimating variance components in more complex scenarios include

the expected mean squares approach of Henderson [19] and the minimum norm quadratic unbiased estimation [20]. However, maximum likelihood-based methods are currently the most popular (see, for example, [21]), especially the restricted (or residual) maximum likelihood (REML) approach [22], which attempts to correct for the well-known bias in the classical maximum likelihood (ML) estimation of variance components. Additional literature on variance component estimation and mixed model methodology can be found, for example, in [23–27].

The Animal Model

The advent of mixed-effects models has undoubtedly revolutionized the animal breeding field, and today they are widely used in the genetic improvement of many livestock and companion animal species. In this subsection some of the applications of mixed models for the genetic evaluation of populations using phenotypic and pedigree information will be presented. In the following section applications incorporating molecular marker information will be discussed as well.

As a first application of mixed models in animal breeding, the so-called "animal model" is considered here for the specific situation of a single trait and a single phenotypic observation (including missing values) per animal. The animal model can be described as:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a} + \boldsymbol{\varepsilon},$$

where $\mathbf{y}$ is an $(n \times 1)$ vector of observations (phenotypic scores), $\boldsymbol{\beta}$ is a $(p \times 1)$ vector of fixed effects (e.g., herd-year-season effects in cattle evaluations), and $\boldsymbol{\varepsilon}$ represents residual effects, assumed $\boldsymbol{\varepsilon} \sim N(\mathbf{0}, \boldsymbol{\Sigma})$ as before. In most applications of animal models, however, residuals are assumed independent across animals, such that the residual covariance structure can be expressed as $\mathbf{R} = \mathbf{I}\sigma^2_{\boldsymbol{\varepsilon}}$, where $\mathbf{I}$ is an identity matrix of appropriate order, and $\sigma^2_{\boldsymbol{\varepsilon}}$ is the residual variance. In the case of animal models, the random effects $\mathbf{u}$ represent the breeding values, i.e., $\mathbf{u} = \mathbf{a}$, assumed to be $\mathbf{a} \sim N(\mathbf{0}, \mathbf{G})$. The vector $\mathbf{a}$, of dimension $(q \times 1)$, may include breeding values of all animals with record or in the pedigree file, such that q is generally bigger than n.

The matrix $\mathbf{G}$, which in this case describes the covariances among the breeding values, follows from standard results for the covariances between relatives. It is seen that the additive genetic covariance between two relatives i and i' is given by $2\theta_{ii'}\sigma^2_a$, where $\theta_{ii'}$ is the coefficient of coancestry between individuals i and i', and σ^2_a is the additive genetic variance in the base population [28]. Hence, under the animal model, $\mathbf{G} = \mathbf{A}\sigma^2_a$, where $\mathbf{A}$ is the "additive genetic (or numerator) relationship matrix," having elements given by $a_{ii'} = 2\theta_{ii'}$.

As mentioned earlier, in animal breeding the usual main interest is prediction of breeding values – for selection of superior individuals – and on estimation of variance components. The fixed effects are, in some sense, nuisance factors with no central interest in terms of inferences, but which need to be taken into account (i.e., they need to be corrected for when inferring breeding values).

Because under the animal model $\mathbf{G}^{-1} = \mathbf{A}^{-1}\sigma^{-2}_a$ and $\mathbf{R}^{-1} = \mathbf{I}\sigma^{-2}_{\boldsymbol{\varepsilon}}$, the mixed model equations reduce to:

$$\begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \lambda\mathbf{A}^{-1} \end{bmatrix} \begin{bmatrix} \hat{\boldsymbol{\beta}} \\ \hat{\mathbf{a}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \end{bmatrix},$$

where $\lambda = \dfrac{\sigma^2_{\boldsymbol{\varepsilon}}}{\sigma^2_a} = \dfrac{1-h^2}{h^2}$, such that:

$$\begin{bmatrix} \hat{\boldsymbol{\beta}} \\ \hat{\mathbf{a}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \lambda\mathbf{A}^{-1} \end{bmatrix}^{-1} \begin{bmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \end{bmatrix}.$$

It is worth mentioning that $\mathbf{A}^{-1}$ can be obtained directly from the pedigree, without setting up $\mathbf{A}$ [29, 30], which is computationally very convenient.

Conditional on the variance components ratio λ, the BLUP of the breeding values are given then by $\hat{\mathbf{a}} = (\mathbf{Z}'\mathbf{Z} + \lambda\mathbf{A}^{-1})^{-1}\mathbf{Z}'(\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\beta}})$, which are the estimated breeding values (EBV). Alternatively, some breeders' associations express their results as predicted transmitting abilities (PTA) or expected progeny differences (EPD), which are equal to half the EBV, representing the portion of an animal's breeding values that is passed to its offspring.

The amount of information contained in an animal's genetic evaluation depends on the availability of its own record, and of phenotypic information from its relatives (including how many and how closely related to it). As a measure of amount of information in livestock genetic evaluations, EBV are typically reported with their associated accuracies, i.e. the correlation between true and estimated breeding values, $r_i = r_{\hat{a}_i, a_i}$. Instead of accuracy, some livestock species

genetic evaluations use reliability, which is the accuracy squared (r_i^2).

The calculation of r_i requires the diagonal elements of the inverse of the MME coefficient matrix, represented as:

$$\mathbf{C} = \begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \lambda\mathbf{A}^{-1} \end{bmatrix}^{-1} = \begin{bmatrix} \mathbf{C}^{\beta\beta} & \mathbf{C}^{\beta a} \\ \mathbf{C}^{a\beta} & \mathbf{C}^{aa} \end{bmatrix}.$$

It is shown that the prediction error variance (PEV) of EBV $\hat{a}_i$ is given by:

$$\text{PEV} = \text{Var}(\hat{a}_i - a_i) = c_i^{aa}\sigma_\varepsilon^2,$$

where c_i^{aa} is the ith diagonal element of $\mathbf{C}^{aa}$, relative to animal i. The PEV can be interpreted as the fraction of additive genetic variance not accounted for by the prediction. Therefore, PEV can also be expressed as:

$$\text{PEV} = (1 - r_i^2)\sigma_a^2,$$

such that $c_i^{aa}\sigma_\varepsilon^2 = (1 - r_i^2)\sigma_a^2$, from which the reliability is obtained as $r_i^2 = 1 - c_i^{aa}\sigma_\varepsilon^2/\sigma_a^2 = 1 - \lambda c_i^{aa}$.

Extensions and Variations of the Animal Model

The animal model discussed above can be extended also to multiple (correlated) traits [31, 32]. For instance, consider as an example the analysis of k traits, in which the model for each trait is expressed as:

$$\mathbf{y}_j = \mathbf{X}_j\boldsymbol{\beta}_j + \mathbf{Z}_j\mathbf{a}_j + \boldsymbol{\varepsilon}_j,$$

where $\mathbf{y}_j$, $\mathbf{X}_j$, $\boldsymbol{\beta}_j$, $\mathbf{Z}_j$, $\mathbf{a}_j$, and $\boldsymbol{\varepsilon}_j$ are defined as before, but here have an additional index to indicate the trait ($j = 1, 2, \ldots, k$).

For a joint analysis of the k traits, the single trait models can be combined as:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a} + \boldsymbol{\varepsilon},$$

where $\mathbf{y} = [\mathbf{y}_1'\ \mathbf{y}_2'\ \ldots\ \mathbf{y}_k']'$, $\boldsymbol{\beta} = [\boldsymbol{\beta}_1'\ \boldsymbol{\beta}_2'\ \ldots\ \boldsymbol{\beta}_k']'$, $\mathbf{a} = [\mathbf{a}_1'\ \mathbf{a}_2'\ \ldots\ \mathbf{a}_k']'$, and $\boldsymbol{\varepsilon} = [\boldsymbol{\varepsilon}_1'\ \boldsymbol{\varepsilon}_2'\ \ldots\ \boldsymbol{\varepsilon}_k']'$, and the design matrices in this case are:

$$\mathbf{X} = \begin{bmatrix} \mathbf{X}_1 & \mathbf{0} & \cdots & \mathbf{0} \\ \mathbf{0} & \mathbf{X}_2 & \cdots & \mathbf{0} \\ \vdots & \vdots & \ddots & \vdots \\ \mathbf{0} & \mathbf{0} & \cdots & \mathbf{X}_k \end{bmatrix} \text{ and } \mathbf{Z} = \begin{bmatrix} \mathbf{Z}_1 & \mathbf{0} & \cdots & \mathbf{0} \\ \mathbf{0} & \mathbf{Z}_2 & \cdots & \mathbf{0} \\ \vdots & \vdots & \ddots & \vdots \\ \mathbf{0} & \mathbf{0} & \cdots & \mathbf{Z}_k \end{bmatrix}.$$

It is assumed that $\text{Var}\begin{bmatrix} \mathbf{a} \\ \boldsymbol{\varepsilon} \end{bmatrix} = \begin{bmatrix} \mathbf{G} \otimes \mathbf{A} & \mathbf{0} \\ \mathbf{0} & \boldsymbol{\Sigma} \otimes \mathbf{I} \end{bmatrix}$,

where $\mathbf{G} = \begin{bmatrix} \sigma_{a_1}^2 & \sigma_{a_1a_2} & \cdots & \sigma_{a_1a_k} \\ \sigma_{a_1a_2} & \sigma_{a_2}^2 & \cdots & \sigma_{a_2a_2} \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_{a_1a_k} & \sigma_{a_2a_k} & \cdots & \sigma_{a_k}^2 \end{bmatrix}$ and $\boldsymbol{\Sigma} = \begin{bmatrix} \sigma_{\boldsymbol{\varepsilon}_1}^2 & \sigma_{\boldsymbol{\varepsilon}_1\boldsymbol{\varepsilon}_2} & \cdots & \sigma_{\boldsymbol{\varepsilon}_1\boldsymbol{\varepsilon}_k} \\ \sigma_{\boldsymbol{\varepsilon}_1\boldsymbol{\varepsilon}_2} & \sigma_{\boldsymbol{\varepsilon}_2}^2 & \cdots & \sigma_{\boldsymbol{\varepsilon}_2\boldsymbol{\varepsilon}_2} \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_{\boldsymbol{\varepsilon}_1\boldsymbol{\varepsilon}_k} & \sigma_{\boldsymbol{\varepsilon}_2\boldsymbol{\varepsilon}_2} & \cdots & \sigma_{\boldsymbol{\varepsilon}_k}^2 \end{bmatrix}$ are the genetic and residual variance–covariance matrices, respectively, $\mathbf{A}$ and $\mathbf{I}$ are the numerator relationship matrix and an identity matrix, and $\otimes$ represents the direct (Kronecker) product.

The MME for multi-trait analyses are of the same form as before, i.e.,

$$\begin{bmatrix} \mathbf{X}'(\boldsymbol{\Sigma}^{-1} \otimes \mathbf{I})\mathbf{X} & \mathbf{X}'(\boldsymbol{\Sigma}^{-1} \otimes \mathbf{I})\mathbf{Z} \\ \mathbf{Z}'(\boldsymbol{\Sigma}^{-1} \otimes \mathbf{I})\mathbf{X} & \mathbf{Z}'(\boldsymbol{\Sigma}^{-1} \otimes \mathbf{I})\mathbf{Z} + \mathbf{G}^{-1} \otimes \mathbf{A}^{-1} \end{bmatrix} \begin{bmatrix} \hat{\boldsymbol{\beta}} \\ \hat{\mathbf{a}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'(\boldsymbol{\Sigma}^{-1} \otimes \mathbf{I})\mathbf{y} \\ \mathbf{Z}'(\boldsymbol{\Sigma}^{-1} \otimes \mathbf{I})\mathbf{y} \end{bmatrix},$$

from which the BLUEs and BLUPs of $\boldsymbol{\beta}$ and $\mathbf{a}$ can be obtained, respectively.

The dimensionality of such multi-trait MME, however, can become a hurdle for solving it when more than two or three traits are considered. An alternative for the analysis of multiple traits is to use a canonical transformation of the traits [33–35], which consists of transforming the vectors of correlated traits into a new vector of uncorrelated variables. In such case, each transformed variable can be analyzed independently using standard single trait models, and subsequently the estimated breeding values are transformed back to the original scale of measurement.

Some other interesting applications of mixed models in animal breeding involve multiple random effects, as in the cases of repeated measurements of the same trait or traits with maternal effects. For the analysis of repeated measurements, as discussed in section "Selection Index" (Model 4), environmental effects can be partitioned into permanent and temporary effects. In this case, the mixed model, usually called "repeatability model," can be written as:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a} + \mathbf{W}\mathbf{p} + \boldsymbol{\varepsilon},$$

where all terms are as previously defined for a single trait animal model, and $\mathbf{p}$ is the vector of permanent

environmental effects, with each level pertaining to a common effect to all observations of each animal, and **W** is a known incidence matrix relating **y** to the vector **p**.

It is often assumed that $\mathbf{a} \sim N(\mathbf{0}, \mathbf{A}\sigma_a^2)$, $\mathbf{p} \sim N(\mathbf{0}, \mathbf{I}\sigma_p^2)$, and $\boldsymbol{\varepsilon} \sim N(\mathbf{0}, \mathbf{I}\sigma_{\boldsymbol{\varepsilon}}^2)$ are independent from each other. Under these assumptions, the MME becomes:

$$\begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} & \mathbf{X}'\mathbf{W} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z}+\lambda_a\mathbf{A}^{-1} & \mathbf{Z}'\mathbf{W} \\ \mathbf{W}'\mathbf{X} & \mathbf{W}'\mathbf{Z} & \mathbf{W}'\mathbf{W}+\lambda_p\mathbf{I} \end{bmatrix} \begin{bmatrix} \hat{\boldsymbol{\beta}} \\ \hat{\mathbf{a}} \\ \hat{\mathbf{p}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \\ \mathbf{W}'\mathbf{y} \end{bmatrix},$$

where $\lambda_a = \sigma_{\boldsymbol{\varepsilon}}^2/\sigma_a^2$ and $\lambda_p = \sigma_{\boldsymbol{\varepsilon}}^2/\sigma_p^2$.

There are some traits of interest in livestock, such as weaning weight in beef cattle, in which progeny performance is affected by the dam's ability to affect the calf's environment, such as in the form of nourishment through her milk production, the quantity and quality of which is in part genetically determined. In some cases, there can also be a paternally provided environmental component. In such cases, parents contribute to the performance of their progeny not only through the genes passed to the progeny (the "direct genetic effects") but also through their ability to provide a suitable environment (the "indirect genetic effects").

Here maternally influenced traits are considered, for which the mixed model can be written as [36]:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a} + \mathbf{K}\mathbf{m} + \mathbf{W}\mathbf{p} + \boldsymbol{\varepsilon},$$

where all terms are as before, except that the model now includes a vector **m** of random maternal genetic effects, and a vector **p** of random permanent environmental effects, with **K** and **W** as their respective incidence matrices. It is assumed that $\mathbf{a} \sim N(\mathbf{0}, \mathbf{A}\sigma_a^2)$, $\mathbf{m} \sim N(\mathbf{0}, \mathbf{A}\boldsymbol{\sigma}_{\boldsymbol{m}}^{\boldsymbol{2}})$, $\mathbf{p} \sim N(\mathbf{0}, \mathbf{I}\sigma_p^2)$, and $\boldsymbol{\varepsilon} \sim N(\mathbf{0}, \mathbf{I}\sigma_\varepsilon^2)$, and quite often a covariance structure between direct and maternal additive genetic effects is considered, assumed equal to $\mathbf{A}\sigma_{a,m}$.

Some other variations of the animal model, which are computationally convenient, include the "sire model" and the "reduced animal model" [37]. In the sire models, only sires are evaluated, using progeny records under the assumption of randomly selected mates. In the reduced animal model, instead of having equations set up for every animal (i.e., parents and progeny), it allows equations to be set up only for parents in the MME, making the dimensions of the system greatly reduced. The breeding values of the parents are estimated directly from the MME, and the progeny breeding values are then inferred by back solving from the predicted parental breeding values.

As a final note regarding the use of mixed models in animal breeding, it is important to mention that solving the MME does not necessary require the inversion of the coefficient matrix **C**. More computationally convenient alternatives for solving high dimensional systems of linear equations include methods based on iteration on the MME, such as the Jacobi or Gauss–Seidel iteration [38], and the "iteration on the data" strategy [39], which is a commonly used methodology in national genetic evaluations involving millions of records.

Marker-Assisted Selection

Introduction

The advent of molecular markers has created opportunities for a better understanding of genetic inheritance and for developing novel strategies for genetic improvement in agriculture. Molecular markers are used, for example, to study quantitative trait loci (QTL), which are defined as chromosomic regions contributing to variation in phenotypic traits. The location and effects of QTL can be inferred by combining information from marker genotypes and phenotypic scores of individuals and by exploring genetic linkage [40–43] and linkage disequilibrium [44, 45] information between marker loci and QTL, such as in experimental or mapping populations (e.g., backcross or F_2, or granddaughter designs) or in complex pedigrees in outbred populations. Information on markers associated with QTL can be used to enhance prediction of genetic merit of animals [46]. This is especially useful for low heritability traits, traits that are expensive or difficult to measure, or traits expressed in only one sex [47].

Classical Approaches with Few Markers

The application of molecular information for genetic improvement of animals and plants, or marker-assisted selection (MAS), requires that candidate-for-selection individuals are genotyped for specific markers. For MAS purposes, there are three types of genetic markers, and for each type there are specific statistical

approaches for incorporating their information into selection programs [47]. A first type of marker refers to situations in which the functional polymorphism itself can be genotyped. These markers are called "direct markers," as they indicate exactly the genotype an animal has at specific causative loci.

A second type of marker refers to those that are in population-wide linkage disequilibrium (LD) with the causative or functional mutations. In such cases, although the marker genotype of an animal does not unambiguously indicate the genotype at a specific functional locus, it still provide information regarding how likely an animal carries a specific allele or genotype at such a locus. Finally, a third kind of molecular marker refers to those loci that are in population linkage equilibrium with the functional mutations, which are often called "indirect markers." In such cases, although the marker information on a single animal in a population does not provide any information regarding the genetic merit of that animal, it still can be useful in exploring family (pedigree) structure when genotyped animals are related to each other.

While direct markers are the simplest and most efficient in MAS programs, their identification is much more difficult and generally involves a prescreening step using QTL mapping methods to identify promising chromosomic regions, followed by fine mapping (often using functional and positional candidate gene strategies), followed by validation (using some strategy such as a knock-out approach). On the other extreme, indirect markers are extensively available for most livestock species, but their use in MAS is more complex and the results are generally modest.

Statistical models to incorporate direct and/or LD markers in the genetic evaluations of animals are relatively straightforward. For example, a marker can be included into an animal model context with the following specification:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a}^* + \mathbf{M}\mathbf{g} + \boldsymbol{\varepsilon},$$

where all terms are as defined before, except that $\mathbf{a}^* \sim N(\mathbf{0}, \mathbf{A}\sigma^2_{a^*})$ represents now the random additive (nonmarked) polygenic effects, and $\mathbf{g}$ and $\mathbf{M}$ are the (fixed) QTL effects and an incidence matrix, respectively. In the case of direct markers, the matrix $\mathbf{M}$ represents the marker genotypes and is obtained directly from the genotyping of animals. In the case of LD markers, the incidence matrix $\mathbf{M}$ will represent genotype probabilities at each QTL locus, which can be derived using segregation analysis. The overall genetic merits of the animals are then given by the sum of their $\mathbf{a}^*$ and $\mathbf{g}$ components. Other strategies for combining the infinitesimal and the QTL components to increase long-term genetic gain have also been proposed (e.g., [48–50]); a review of MAS strategies can be found, for example, in [47].

In the case of indirect markers, however, the within-family LD between QTL and linked markers must be explored. One approach is to determine the marker effects or the marker-QTL linkage phases separately for each family. Alternatively, more general MAS models have been proposed to incorporate marker data in genetic evaluations for complex pedigrees [13, 51], which can be represented as:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{a}^* + \mathbf{M}\mathbf{q} + \boldsymbol{\varepsilon},$$

where the terms are as before, but here the QTL effects $\mathbf{q}$ are assumed random and normally distributed, such that:

$$\begin{bmatrix} \mathbf{a}^* \\ \mathbf{q} \end{bmatrix} \sim \mathrm{N}\left(\mathbf{0}, \begin{bmatrix} \mathbf{A}\sigma^2_{a^*} & \mathbf{0} \\ \mathbf{0} & \mathbf{G}_\lambda\sigma^2_q \end{bmatrix}\right),$$

where $\mathbf{G}_\lambda$ is the gametic relationship matrix for the QTL, and σ^2_q is the additive variance of the QTL allelic effects. The gametic relationship matrix gives the probabilities of identity between each of the two alleles in each individual, and it can be derived based on the QTL position λ and the marker information.

Genomic Selection

As most quantitative traits are influenced by many genes, tracking a small number of them using molecular markers (as in the MAS approaches discussed above) will explain only a small fraction of the total genetic variance. Moreover, individual genes are likely to have small effects and so a large amount of data is needed to accurately estimate their effects [52]. Genome-wide Marker-Assisted Selection (GWMAS), or simply Genomic Selection (GS), on the other hand, makes use of a very dense set of markers covering the entire genome, which potentially explain all genetic variance. In addition, given the LD between the dense markers and the QTL, estimated marker effects pertain across the population [53].

Meuwissen et al. [54] were the first to propose GS and suggested a model that can be described as:

$$\mathbf{y} = \mathbf{1}\mu + \sum_{j=1}^{p} \mathbf{M}_j \mathbf{q}_j + \boldsymbol{\varepsilon},$$

where μ is the overall mean, $\mathbf{q}_j$ represents the genetic effects of marked genes (j=1, 2, . . ., p), and $\mathbf{M}_j$ represents the design matrices (genotypes) relative to a large number (p) of biallelic markers (e.g., SNP loci), which present different levels of LD with QTL affecting the phenotypic trait of interest ($\mathbf{y}$). Here it is assumed that the QTL affecting the trait act additively, and that $\mathbf{q}_j$ refers to per-allele effects; nonadditive effects as well as effects relative to nonmarked QTL are lumped together into the residual term of the model.

Fitting such GS model using standard regression approaches is not trivial, as the number p of markers (and so the number of genetic effects to be estimated) may easily exceed the number n of individuals available. The "large p small n paradigm" is central in many applications of genomic technologies, including expression profiling and association analysis, and various statistical strategies have been proposed in the literature to overcome this problem, such as dimension-reduction techniques, stepwise fitting procedures, ridge regression [55], and least absolute selection and shrinkage operator – LASSO [56].

Specifically in GS, hierarchical modeling has become the methodology of choice, due to its flexibility and good statistical properties. Within this approach, the genetic effects $\mathbf{q}_j$ are assumed random effects and distributed according to some prespecified distribution [54]. For example, $\mathbf{q}_j$ may be assumed normally distributed with mean 0 and variance σ_j^2, and the hierarchy can be extended by assuming a prior distribution for the variances σ_j^2 [54, 57–59]. Alternative distributions can be adopted for the $\mathbf{q}_j$, such as double exponential or mixture distributions including a mass point at zero. It is interesting to note the connection between the ridge regression approach and a Bayesian model with normal priors with common variances $\sigma_j^2 = \sigma_0^2$, as well as the LASSO methodology and a Bayesian model with double exponential priors for the genetic effects [60].

The potential of GS to accelerate genetic progress has been demonstrated through many simulation studies (e.g., [54, 61, 62]), and confirmed by some real data applications. The first use of GS using thousands of markers in livestock has been in dairy cattle [63, 64], followed by some breeds of beef cattle and more recently in poultry. Table 1 shows some encouraging results on dairy cattle obtained by the USDA.

Animal Breeding, Foundations of. Table 1 Comparison of April 2010 genomic and traditional evaluations for bulls with an AI status of active or foreign

Trait	Average reliability (%)		
	Genomic	Traditional	Difference
Net merit	87	81	+6
Milk yield	93	91	+2
Fat yield	93	91	+2
Protein yield	93	91	+2
Productive life	81	71	+9
Somatic cell score	88	83	+5
Daughter pregnancy rate	79	69	+10
Final score	89	85	+4
Sire calving ease	90	84	+6
Daughter calving ease	80	67	+13

Source: AIPL – USDA; http://www.aipl.arsusda.gov/

Future Directions

As shown here, the mixed model methodology is extremely flexible and can be used in a wide variety of applications. Other extensions of the methods discussed here include models with nonadditive genetic effects (e.g., [65, 66]), mixed models for the analysis of non-Gaussian traits such as binary and categorical (e.g., [67, 68]) or counting data (e.g., [69]), robust models [70, 71], survival traits [72], nonlinear models to study, for example, growth curves (e.g., [73, 74]), among others. However, such models can get extremely complex and asymptotic statistical methods are generally required. Alternatively, Bayesian analysis employing Markov Chain Monte Carlo (MCMC) methods can be used, given their exceptional

flexibility and the possibility of incorporating prior information regarding the model parameters [75]. Bayesian analysis has been increasingly used in many applications of genetics and animal breeding, and for a review the reader can refer, for example, to [76–78]. A comprehensive treatment of Bayesian MCMC approaches in animal breeding is presented in [23].

Bayesian hierarchical modeling has also been extensively used in genomic selection [54, 79–81]. In addition, nonparametric and semiparametric methods, and machine learning techniques based on artificial intelligence have been proposed and used for the analysis of high density marker panels in the context of animal breeding, such as in [82–86]. Moreover, some other recent methods aim to combine all available phenotypic, pedigree, and genomic information for prediction of genetic merit of animals [87].

As indicated in the beginning of this chapter, the genetic improvement observed in many livestock and companion animal species is truly remarkable. Most of this genetic progress has been accomplished through selection, using the methods discussed here. Two technological and methodological developments however must be mentioned as turning points in the genetic trends observed in some species; these are the advent of artificial insemination and the mixed models. Seemingly, the development of high density SNP panels and, more recently, next generation sequencing technologies and their application in genomic selection strategies promise to be the next turning point. This new era for animal breeding and genetics will require a different profile of animal breeders, requiring not only knowledge of population and quantitative genetics, classical statistical and computational methods, but also some more modern statistical and computational methods based on hierarchical modeling [23, 88], non- and semiparametric methods, and machine learning techniques [60, 89]. It is indeed a very exciting time to work in animal breeding!

Bibliography

Primary Literature

1. Lush JL (1994) The genetics of populations. (Prepared for publication by Chapman AB, Shrode RR, with an addendum by Crow JF) Special report 94, College of Agriculture, Iowa State University, Ames
2. Bulmer MG (1985) The mathematical theory of quantitative genetics. Clarendon, Oxford
3. Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longmans Green, Harlow
4. Lynch M, Walsh B (1998) Genetic analysis of quantitative traits. Sinauer Associates, Sunderland
5. Hill WG (1969) On the theory of artificial selection in finite populations. Genet Res 13:143–163
6. Havenstein B, Ferket PR, Qureshi MA (2003) Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1509–1518
7. Bourdon RM (2000) Understanding animal breeding, 2nd edn. Prentice Hall, Upper Saddle River
8. Crow J, Kimura M (1970) An introduction to populations genetics theory. Haraper and Row, New York
9. Shook GE (2006) Major advances in determining appropriate selection goals. J Dairy Sci 89(4):1349–1361
10. Henderson CR (1949) Estimation of changes in herd environment. J Dairy Sci 32:709
11. Henderson CR (1975) Best linear unbiased estimation and prediction under a selection model. Biometrics 31:423–447
12. Henderson CR (1984) Applications of linear models in animal breeding. University of Guelph, Guelph
13. Fernando RL, Grossman M (1989) Marker-assisted selection using best linear unbiased prediction. Genet Sel Evol 21:467–477
14. Yu J et al (2006) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat Genet 38:203–208
15. Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS (2001) Assessing gene significance from cDNA microarray expression data via mixed models. J Comput Biol 8:625–637
16. Rosa GJM, Steibel JP, Tempelman RJ (2005) Reassessing design and analysis of two-color microarray experiments using mixed effects models. Comp Funct Genomics 6:123–131
17. Steibel JP, Poletto R, Coussens PM, Rosa GJM (2009) A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. Genomics 94:146–152
18. Henderson CR (1950) Estimation of genetic parameters. Ann Math Stat 21:309
19. Henderson CR (1953) Estimation of variance and covariance components. Biometrics 9:226
20. Rao CR (1971) Estimation of variance and covariance components MINQUE theory. J Multivar Anal 1:257–275
21. Harville DA (1977) Maximum likelihood approaches to variance component estimation and to related problems. J Am Stat Assoc 72(358):320–338
22. Patterson HD, Thompson R (1971) Recovery of inter-block information when block sizes are unequal. Biometrika 58(3):545–554
23. Sorensen D, Gianola D (2002) Likelihood, Bayesian, and MCMC methods in quantitative genetics. Springer, New York

24. Littell RC, Miliken GA, Stroup WW, Wolfinger RD (2006) SAS system for mixed models, 2nd edn. SAS Institute, Cary
25. Pinheiro JC, Bates DM (2000) Mixed-effects models in S and S-Plus. Springer, New York
26. Searle SR, Casella G, McCulloch CE (1992) Variance components. Wiley, New York
27. Verbeke G, Molenberghs G (1997) Linear mixed models in practice: A SAS-oriented approach, Lecture Notes in Statistics 126. Springer, New York
28. Wright S (1921) Systems of mating I. The biometric relations between parents and offspring. Genetics 6:111–123
29. Henderson CR (1976) A simple method for computing the inverse of a numerator relationship matrix used in prediction of breeding values. Biometrics 32:69–83
30. Quaas RL (1976) Computing the diagonal elements of a large numerator relationship matrix. Biometrics 32: 949–953
31. Henderson CR, Quaas RL (1976) Multiple trait evaluation using relatives' records. J Anim Sci 43:1188–1197
32. Schaeffer LR (1984) Sire and cow evaluation under multiple trait models. J Dairy Sci 67:1567–1580
33. Thompson R (1977) Estimation of quantitative genetic parameters. In: Pollak E, Kempthorne O, Bailey TB (eds) Proceedings of the international conference on quantitative genetics, Iowa State University Press, Ames, pp. 639–657
34. Meyer K (1985) Maximum-likelihood estimation of variance-components for a multivariate mixed model with equal design matrices. Biometrics 41:153
35. Ducrocq V, Besbes B (1993) Solution of multiple trait animal models with missing data on some traits. J Anim Breed Genet 110:81–92
36. Quaas RL, Pollak EJ (1981) Modified equations for sire models with groups. J Dairy Sci 64:1868–1872
37. Quaas RL, Pollak EJ (1980) Mixed model methodology for farm and ranch beef cattle testing programs. J Anim Sci 51:1277–1287
38. Misztal I, Gianola D (1988) Indirect solution of mixed model equations. J Dairy Sci 77(Suppl 2):99–106
39. Schaeffer LR, Kennedy BW (1986) Computing solutions to mixed model equations. In: 3rd World congress on genetic applied livestock production, Lincoln, Nebraska, 16–22 July 1986, vol XII. pp 382–393
40. Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199
41. Haley CS, Knott SA (1992) A simple regression method to for mapping quantitative trait loci in line crosses using flanking markers. Heredity 69:315–324
42. Haley CS, Knott SA, Elsen J-M (1994) Mapping quantitative trait loci in crosses between outbred lines using least squares. Genetics 136:1195–1207
43. Pérez-Enciso M, Misztal I (2004) Qxpak: a versatile mixed model application for genetical genomics and QTL analyses. Bioinformatics 20(16):2792–2798
44. Meuwissen THE, Goddard ME (2000) Fine mapping of quantitative trait loci using linkage disequilibria with closely linked marker loci. Genetics 155:421–430
45. Pérez-Enciso M (2003) Fine mapping of complex trait genes combining pedigree and linkage disequilibrium information: a Bayesian unified framework. Genetics 163: 1497–1510
46. Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743–756
47. Dekkers JCM, Hospital F (2002) The use of molecular genetics in the improvement of agricultural populations. Nat Rev Genet 3(1):22–32
48. Dekkers JCM, van Arendonk JAM (1998) Optimizing selection for quantitative traits with information on an identified locus in outbred populations. Genet Res 71(3):257–275
49. Manfredi E, Barbieri M, Fournet F, Elsen JM (1998) A dynamic deterministic model to evaluate breeding strategies under mixed inheritance. Genet Sel Evol 30:127–148
50. Chakraborty R, Moreau L, Dekkers JCM (2002) A method to optimize selection on multiple identified quantitative trait loci. Genet Sel Evol 34(2):145–170
51. Goddard ME (1992) A mixed model for analyses of data on multiple genetic-markers. Theor Appl Genet 83:878–886
52. Goddard ME, Hayes BJ (2007) Genomic selection. J Anim Breed Gen 124(6):323–330
53. Schaeffer LR (2006) Strategy for applying genome-wide selection in dairy cattle. J Anim Breed Genet 123:218–223
54. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
55. Whittaker JC, Thompson R, Visscher PM (2000) Marker-assisted selection using ridge regression. Genet Res 75:249–252
56. Tibshirani R (1996) Regression shrinkage and selection via the Lasso. J R Stat Soc B 58:267–288
57. Gianola D, Perez-Enciso M, Toro MA (2003) On marker-assisted prediction of genetic value: beyond the ridge. Genetics 163:347–365
58. Xu S (2003) Estimating polygenic effects using markers of the entire genome. Genetics 163(2):789–801
59. ter Braak CJF, Boer MP, Bink MCAM (2005) Extending Xu's Bayesian model for estimating polygenic effects using markers of the entire genome. Genetics 170(3): 1435–1438
60. Hastie T, Tibshirani R, Friedman JH (2001) The elements of statistical learning: data mining, inference, and predictions. Springer, New York
61. Calus MPL, Veerkamp RF (2007) Accuracy of breeding values when using and ignoring the polygenic effect in genomic breeding value estimation with a marker density of one SNP per cM. J Anim Breed Genet 124:362–368
62. Muir WM (2007) Comparison of genomic and traditional BLUP-estimated breeding value accuracy and selection response under alternative trait and genomic parameters. J Anim Breed Genet 124:342–355

63. VanRaden PM, Van Tassell CP, Wiggans GR, Sonstegard TS, Schnabel RD, Taylor J, Schenkel FS (2009) Reliability of genomic predictions for North American dairy bulls. J Dairy Sci 92:16–24
64. Weigel KA, de Los Campos G, González-Recio O, Naya H, Wu XL, Long N, Long N, Rosa GJM, Gianola D (2009) Predictive ability of direct genomic values for lifetime net merit of Holstein sires using selected subsets of single nucleotide polymorphism markers. J Dairy Sci 92:5248–5257
65. Henderson CR (1985) Best linear unbiased prediction of nonadditive genetic merits in non-inbred populations. J Anim Sci 60:111–117
66. Hoeschele I, VanRaden PM (1991) Rapid inverse of dominance relationship matrices for noninbred populations by including sire and dam subclass effects. J Dairy Sci 74:557–569
67. Gianola D (1982) Theory and analysis of threshold characters. J Anim Sci 54:1079–1096
68. Gianola D, Foulley JL (1983) Sire evaluation for ordered categorical-data with a threshold-model. Genet Sel Evol 15(2):201–223
69. Tempelman RJ, Gianola D (1996) A mixed effects model for overdispersed count data in animal breeding. Biometrics 52:265–279
70. Strandén I, Gianola D (1998) Attenuating effects of preferential treatment with student-t mixed linear models: a simulation study. Genet Sel Evol 31:25–42
71. Rosa GJM, Padovani CR, Gianola D (2003) Robust linear mixed models with normal/independent distributions and Bayesian MCMC implementation. Biom J 45(5):573–590
72. Ducrocq V, Casella G (1996) A Bayesian analysis of mixed survival models. Genet Sel Evol 28(6):505–529
73. Varona L (1997) Multiple trait genetic analysis of underlying biological variables of production functions. Livest Prod Sci 47:201–209
74. Forni S, Piles M, Blasco A et al (2009) Comparison of different nonlinear functions to describe Nelore cattle growth. J Anim Sci 87(2):496–506
75. Gianola D, Fernando RL (1986) Bayesian methods in animal breeding theory. J Anim Sci 63:217–244
76. Shoemaker JS, Painter IS, Weir BS (1999) Bayesian statistics in genetics - a guide for the uninitiated. Trends Genet 15:354–358
77. Blasco A (2001) The Bayesian controversy in animal breeding. J Anim Sci 79(8):2023–2046
78. Beaumont MA, Rannala B (2004) The Bayesian revolution in genetics. Nat Rev Genet 5:251–261
79. Yi N, Xu S (2008) Bayesian Lasso for quantitative trait loci mapping. Genetics 179:1045–1055
80. Gianola D, de Los Campos G, Hill WG et al (2009) Additive genetic variability and the Bayesian alphabet. Genetics 183(1):347–363
81. de Los Campos G, Naya H, Gianola D, Crossa J, Legarra A, Manfredi E, Weigel K, Cotes J (2009) Predicting quantitative traits with regression models for dense molecular markers and pedigrees. Genetics 182:375–385
82. Gianola D, Fernando RL, Stella A (2006) Genomic-assisted prediction of genetic value with semiparametric procedures. Genetics 173:1761–1776
83. Gianola D, van Kaam JBCHM (2008) Reproducing kernel Hilbert spaces regression methods for genomic assisted prediction of quantitative traits. Genetics 178:2289–2303
84. Long N, Gianola D, Rosa GJM, Weigel KA, Avendaño S (2007) Machine learning procedure for selecting SNPs in genomic selection: application to early mortality in broilers. J Anim Breed Genet 124(6):377–389
85. González-Recio O, Gianola D, Long N, Weigel KA, Rosa GJM, Avendano S (2008) Nonparametric methods for incorporating genomic information into genetic evaluations: an application to mortality in broilers. Genetics 178(4):2305–2313
86. Campos G, Gianola D, Rosa GJM (2009) The linear model of quantitative genetics is a reproducing kernel Hilbert spaces regression. J Anim Sci 87:1883–1887
87. Misztal I, Legarra A, Aguilar I (2009) Computing procedures for genetic evaluation including phenotypic, full pedigree, and genomic information. J Dairy Sci 92:4648–4655
88. Gelman A, Carlin JB, Stern HS, Rubin DB (2004) Bayesian data analysis, 2nd edn. Chapman and Hall, London
89. Bishop CM (2006) Pattern recognition and machine learning. Springer, New York

Books and Reviews

Chapman AB (1980) General and quantitative genetics. World animal science series. Elsevier, Amsterdam
Lange K (1997) Mathematical and statistical methods for genetic analysis. Springer, New York
Liu BH (1998) Statistical genomics. CRC Press, Boca Raton
Mrode R (2005) Linear models for the prediction of animal breeding values, 2nd edn. CAB International, New York
Ott J (1991) Analysis of Human Genetic Linkage. Johns Hopkins, Baltimore
Sham PC (1998) Statistics in human genetics. Arnold, London
Van Vleck LD (1993) Selection index and introduction to mixed model methods for genetic improvement of animals. CRC Press, Boca Raton

Animal Breeding, Long-Term Challenges

Peer Berg
Faculty of Agricultural Sciences, Aarhus University, Tjele, Denmark

Article Outline

Glossary

Animal breeding Intentional breeding for certain traits, or combination of traits, by selecting animals for breeding with superior genotypes in growth rate, egg, meat, milk, or wool production, or in other desirable traits.

Animal genetic resources Genetic diversity, either characterized or as yet uncharacterized, that is found in economically important animals.

Animal welfare Animal welfare is the viewpoint that some or all animals, especially those under human care, should be treated in such a way that they do not suffer unnecessarily.

Effective population size Is the size of an idealized population that would behave the same as an actual population. The ideal population is one in which there is random mating and no selection. The effective size of a population is typically smaller than its actual size.

Genetic trends Changes in the mean breeding value of a population over time for one or more traits.

Stakeholders Any party that has an interest ("stake") in a project or activity, which in the context of this chapter is animal breeding.

Definition of the Subject

A major long-term challenge of animal breeding is to ensure that animal breeding is sustainable, in order to contribute to a stable long-term contribution of food for the globe. The need to address sustainability in animal breeding schemes has increased as the development in the last decades has been toward larger demands for food, fewer breeds contributing to the production of animal products, low effective population sizes despite the actual populations being large, and a decreasing number of breeding schemes providing the majority of the genetics underlying production of animal products.

The FAO report on *The State of the World's Animal Genetic Resources for Food and Agriculture* [1] indicates that the vast majority of developing countries have not been successful in sustaining genetic improvement in their livestock populations. Among the breeds considered to be in active use, 77% are located in developing countries. Animal genetic resources for food and agriculture (AnGR) provide the biological capital on which livestock production systems and food security are built. Planning for sustainable livestock development should, from the outset, take account of genetic differences among the species, the breeds, and the animals considered for use, along with their adaptive fitness to the production environments in which they will be kept. The different ways in which animals are used in different production systems and communities should also be recognized [2].

Introduction

Animal production has significantly increased its productivity during the last 50 years. This has been a combined effect of improvements in the environment provided to the animals, for example, improved feeding and management, as well as efficient breeding schemes resulting in significant and cumulating genetic progress. These improvements are synergistic, as genetic improvements and management and feeding improvements stimulate each other. Examples of drastic changes in productivity include

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

- Manyfold increases in milk production of dairy cattle
- Improvements of growth, leanness, and litter size in pigs
- Manyfold increases of growth and egg production in broiler and layer hens, respectively

This trend in productivity is expected to continue for several reasons. In a competitive market for food production, genetic improvements are an essential parameter to ensure a profitable production. However, the demand for food is also expected to increase, and thus stimulates a larger and more efficient production. The World Bank has estimated that it will be necessary to increase meat production by about 80% between 2000 and 2030.

However, the improvements in productivity have also contributed to a global concentration on fewer breeds for the largest part of animal production. In dairy cattle, Holstein-Friesian is now the dominating breed of the world, with exchange of semen and embryos across countries resulting in a relatively low effective population size (∼40–70, [3, 4]). In poultry breeding, a few multinational companies now control the commercial genetic improvement and thus the genetics of the vast majority of eggs and poultry meat produced globally.

Recently, the effect of animal breeding has come under critical evaluations from an ethical point of view, reflecting public concerns on the effects of current and future animal breeding. Developments in biology, neuroscience, and genetics have resulted in changes in our perception of the world, and there is an increasing focus on the effects of animal breeding and the ethics of changing animals by selective breeding for our own good (e.g., [5, 6]). Ethical problems in animal production have historically focused on housing and husbandry, but more emphasis is now focused on the effect of animal breeding [7]. It is argued that the fact that selective breeding can introduce welfare problems places an ethical responsibility on the animal breeding industry [7].

The European animal breeders have realized that these powerful technologies to create genetic progress have generated public concern about the impact of animal breeding and the use of new technologies [8].

According to FAO [2]: *A strategic and logistical approach to sustainable livestock development is required. To appropriately address the use of available AnGR and the role of genetic improvement in sustainable development, from the outset, all policies, plans, and programs for the livestock sector must:*

- *Be based on soundly established and agreed livestock development objectives (LDOs) and well-integrated and realistic livestock development strategies (LDS) that are able to achieve the LDOs.*
- *Account for major environmental, structural, and socioeconomic differences among the production systems concerned.*
- *Ensure participation of the end users (the livestock keepers themselves). Both men and women should have access to relevant information, be involved in the formulation of policies and plans, and have ample opportunities to give their opinions.*
- *Be appropriately funded.*
- *Promote step-by-step development and the sustainability of the actions undertaken.*
- *Be based on well-documented approaches that are understood and agreed by all the stakeholders involved at each stage.*
- *Take fully into account the fundamental principles of genetic improvement and their technical implications.*

Thus, a major future challenge of animal breeding is to ensure a sustainable use of the animal genetic resources available and to implement sustainable breeding schemes in active populations.

The focus of this entry is the long-term challenges of animal breeding focusing on sustainability of animal breeding schemes. This is done by discussing the concept of sustainability in the context of animal breeding and then describing risk factors affecting sustainability. Then guidelines for developing sustainable and future directions to ensure sustainable are discussed.

Sustainability

Sustainability is a term that has a widespread use, and thus is often used in different meanings. It is often used in the sense of human sustainability on planet Earth and this has resulted in the most widely quoted definition of sustainability and sustainable development, that of the Brundtland Commission of the United Nations on March 20, 1987: "sustainable development is development that meets the needs of the present without

compromising the ability of future generations to meet their own needs" [9]. This definition is also relevant to animal breeding; it is, however, not very operational.

In order to define and address sustainability in the context of animal breeding, it is first necessary to identify the stakeholders of animal breeding schemes. These stakeholders all have interests in animal breeding, and some of their interests might be conflicting. Understanding the diverse interest in animal breeding is a prerequisite for developing sustainable breeding schemes.

Obvious stakeholders are the animal breeders, breeding companies, and breeding associations, which have the major responsibility for defining and running the breeding scheme. Additional stakeholders include producers, veterinarians, manufacturers and retail enterprises, and consumers. For some aspects, the society as a whole may be considered a stakeholder in itself. These stakeholders have different perceptions of the desired outcome of animal breeding schemes and thus sustainability, influenced by their role in production and utilization of animal products and their time horizon.

In the short term, objectives of different stakeholders can be very different. Animal breeders will typically focus on the factors limiting the profitability of the primary production. The interest of manufacturers and retail enterprises is on product quality, quantity, and possibilities of adding value to the primary product to ensure profitability of manufacturing and retail. Consumers will typically focus on product quality and price, product safety in addition to potentially cultural and political issues, such as environmental impact and animal welfare.

However, it can be expected that stakeholders will share many long-term interests in animal breeding schemes. Long-term interests of producers and manufacturers are likely to be more similar to the interests of consumers in the long-term, as they are the main actors determining quantity and quality demands. A thorough discussion of sustainability in the context of animal breeding is provided in [10], focusing on the need to address the interests of all stakeholders.

Interests of stakeholders include the following areas, which should be considered for defining sustainable animal breeding schemes for the future [11]:

- Food security, including
  - Quantity produced
  - Product quality
  - Food safety from both a biological and technical perspective
- Socioeconomic effects, including
  - Impacts of breeding schemes on rural economy, employment, and trade
  - Subsidies and their effects on objectives and methods used in animal breeding
  - Public (ethical) perception of the methods and technologies applied in animal breeding
- Environmental impact, including
  - The relationship between animal production and measures of environmental quality
  - Landscape management where animal production plays a major role
  - Interactions with biodiversity and ecosystems
- Health, welfare, and ethics including
  - Health management and biosecurity related to both the risk of zoonoses and contamination of products
  - Ethics of breeding and production

Sustainable Animal Breeding

Animal breeding is based on selecting animals with a superior genotype relative to the desired direction of selection, as reflected in the breeding objective. However, breeding values cannot be observed directly in animals, and they have to be predicted based on phenotypic records on the individuals themselves and their relatives and/or genetic markers. Thus, animal breeding is based on selecting the seemingly superior individuals given their predicted breeding values. Secondly, selected parents pass half of their genes to a given offspring, and each individual is thus a genetically unique individual. A direct result of this is that planning of animal breeding schemes is not an exact science. Genetic progress can be predicted but the realized response to selection can vary depending on a number of factors reflecting biological and economic uncertainty that influences the outcome of a breeding scheme. The breeding objective represents the desired direction of change of a population, most often expressed as the marginal economic value of changing a trait. The breeding objective should address

all aspects of animal production and the characteristics of the product.

Ignoring the interests of some stakeholders when defining breeding objectives might negatively affect the success of a breeding scheme. This can be addressed by considering the interests of all stakeholders. The large potential of animal breeding is the contribution of additive improvements toward a long-term objective. However, there are factors that are not necessarily stable over time, factors that can potentially also change the interests of stakeholders. Examples of such factors include market structure, fluctuations in prices, regulations of production systems, disease outbreaks, and variation in the response to selection, both in the traits directly selected for and in traits indirectly affected as a correlated effect of selection. In addition to these factors, uncertainty on the assumptions underlying the design of a breeding scheme contributes to the risk of this breeding scheme. The risk of a breeding scheme is related to the deviations of the realized effects of a breeding scheme relative to those planned. These factors have two effects: some contribute to a reduction of the realized outcomes of a breeding scheme (e.g., diseases) and others contribute to an increased variability of the outcome of a breeding scheme.

Thus, a sustainable animal breeding scheme should address the interests of all stakeholders and actively reduce the risks of the breeding scheme. This is in line with the SEFABAR project that produced a "Code of Good Practise for Farm Animal Breeding and Reproduction Organisations" [10, 12]. The code addresses issues of food safety and public health, product quality, genetic diversity, efficiency, environmental impact, animal health, animal welfare, and breeding and reproduction technologies. The code is based on the following six general statements:

- Breeding organizations must follow zootechnical, animal welfare, and animal health legislations and relevant regulations and practices.
- Breeding organizations must consult and collaborate with international, national, and regional authorities for the development and implementation of policies, practices, and regulations. These policies should assist the achievement of economic, environmental, and social sustainability of the animal breeding sector.
- Breeding organizations must use modern biosecurity methods to minimize disease transmission.
- Breeding organizations must ensure the health and welfare of the animals under their care.
- Breeding organizations must treat the animals under their care with respect.
- Breeding organizations must ensure that selection for production traits is balanced by appropriate attention to reproduction traits and health- and welfare-related traits.

The intention is that this should result in sustainable breeding programs by an economically viable balance of (a) food safety and public health, (b) product quality, (c) genetic diversity, (d) efficiency, (e) environmental impact, and (f) animal health and welfare.

Risks in Animal Breeding Schemes

The expected outcome of a breeding scheme might not be the same as the realized outcome for several reasons. These include

- Uncertainty on the genetic model assumed
- Uncertainty on the assumed genetic parameters (heritabilities, genetic correlations, etc.)
- Genetic drift resulting from finite population size
- Suboptimal decisions and implementation of the breeding scheme
- Breeding objectives that do not include all relevant costs and benefits of the production system targeted
- Changes in markets and consumer preferences

Some of these can contribute to both positive and negative deviations from the expected outcome, for example, deviations in genetic parameters from those assumed might either increase or decrease genetic gain relative to that expected.

However, most deviations are expected to result in a realized outcome of selection being lower than that expected, as the predictions assume that parameters are known and selection decisions are optimal. If the breeding objective does not reflect the true breeding objective, this will always result in selection decisions being suboptimal and response to selection being lower than expected. Likewise, changes in markets, consumer

preferences, or political regulations of the industry will result in suboptimal goals being targeted by the breeding scheme, and thus the realized outcome being inferior to that expected.

Thus, two groups of factors contributing to the risk of animal breeding schemes can be identified.

The first group is risks intrinsic to the breeding scheme. The response of a breeding scheme might deviate from expectations due to variability of response to selection for the traits considered in the breeding objective and in the selection index. This can be due to uncertainties about genetic parameters used for predicting breeding values, uncertainties in the breeding values predicted from phenotypic records and/or genetic markers, and genetic drift.

Genetic drift can also increase the frequency of deleterious (recessive) alleles. Examples include BLAD [13] and CVM [14] in dairy cattle and the halothane sensitivity locus in pigs [15]. These alleles increased in frequency either by directly having a positive impact on some of the traits selected for, or being linked to alleles with positive effects on the traits selected for. In general, all traits not directly selected for and not correlated with the traits in the selection criterion are most affected by genetic drift. In a closed population, genetic drift is highly related to the rate of inbreeding.

Unfavorable correlated responses to selection can appear if important traits are not included in the breeding objective and selection criterion. There is evidence of general negative effects of selection for increased production resulting in increased occurrence of behavioral, physiological, and immunological disorders [16]. A theory (resource allocation theory) has been proposed to explain the general negative correlated changes in fitness and health-related traits when selecting for production traits. The theory predicts that with limited resources available, selection for increased production results in less resources being allocated to, for example, health and reproductive traits [17]. In a mice model it was shown that a line selected for large litter size allocated more resources to lactation and mobilized body energy for a longer time period than a control line [18]. However, results suggest that not all aspects of immune response are decreased by selection for production. In a comparison of chicken lines from 1957 to 2001, it was concluded that the 2001 chickens selected for growth have a decreased adaptive immune response but a better cell-mediated immune response [19]. These examples highlight the need to consider the direct response to selection not only in the traits selected for, but also in other traits of relevance for production, animal health, and animal welfare.

Effective population size (which in a closed population is inversely proportional to rate of inbreeding) is the most important indicator of genetic risk in a breeding scheme, being inversely proportional to both genetic drift in a closed population and variation in selection response. Larger effective population size results in less genetic drift and less variability in the response to selection. Selection intensity, population size, and selection and mating criteria all influence the effective population size. It has been argued that the effective population size should be at least 50–100 individuals in order to avoid negative effects of genetic drift [20], but should be significantly larger in order to maintain mutations contributing to maintaining genetic variability.

The second group of risks is external to the breeding scheme. These include environmental risks due to changes in environmental factors unforeseen when planning the breeding scheme. Such risks are diseases, particularly epidemic diseases and diseases under national and international regulations (e.g., foot-and-mouth disease), and changed market and/or production regulations. These changes will most often result in the realized outcome of a breeding scheme being less than expected.

Guidelines for Developing Sustainable Breeding Schemes

Animal breeding schemes produce additive genetic improvement, and are thus most powerful when applied for a long-term improvement of a population. Thus, breeding objectives should reflect the future market for which production is aimed. The development of breeding objectives includes an assessment of the breed and its characteristics relative to the future market preferences. Traditionally, this includes an economic analysis aiming at estimating the marginal value of improving a trait. These marginal values are often the economic value of changing a trait given all other traits are constant. The breeding objective is then defined by the marginal values of the traits. However, this is not

necessarily sufficient as a breeding objective should also consider the risk and uncertainty of aiming at a particular market, including status and trends in consumer preferences, social attitude, political and economic regulations, and infrastructure.

The breeding objective should reflect the most profitable improvement of the population, conditional on the current and future market conditions. The derivation should include traits beyond those currently recorded or included in a selection criterion.

The expected genetic trends should also be evaluated to ensure the breeding objective is sustainable. Ethical evaluations and public perception of animal welfare might alter the relative weights used in the breeding objective. Including a trait in the breeding objective does not guarantee that that particular trait is improved. Traits with low heritability, traits negatively correlated to important traits in the breeding objective, and traits measured late in life are at risk of not being improved despite being in the breeding objective. Traits that fulfill all the above-mentioned criteria include health-related traits, such as mastitis and fertility in dairy cattle. It has been suggested that such traits should have a larger weight in the breeding objective, the larger weight reflecting a nonmarket value [21]. Such nonmarket values could reflect public concern on, for example, increased disease susceptibility resulting from the current breeding objective.

Ignoring important traits in deriving marginal (economic) values to be used in the breeding objective will bias the prediction of the economic outcome of the breeding scheme. For example, ignoring quality measures that are not considered in current market conditions but are important in distinguishing the product from other competing products might be harmful to the long-term competitive ability of the product.

Breeding objectives should not only be defined in terms of production, but should also include traits related to animal health and welfare resulting in robustness being part of the breeding objective [16].

Derivation of the marginal value of all important traits is crucial, in order to predict the effects of selection, not only on traits selected on, but also traits correlated to those traits. This is necessary to avoid unfavorable genetic changes in traits not selected for.

The expected outcome for a given breeding objective also depends on the recording scheme. Recording is often one of the most costly elements of a breeding scheme and should thus be an integral part of planning a breeding scheme. Generally, the objective of recording should be to allow for favorable genetic trends in the traits included in the breeding objective. This might be complicated by biological constraints of, for example, when a trait is realized and the number of individuals it can be recorded on. Examples are traits measured late in life (e.g., longevity), measured after slaughter (e.g., meat quality), or measured on one sex (e.g., female fertility), which poses challenges in defining efficient recording schemes. Modern technology allows for some remedies to this challenge. Genetic markers can be measured early in life, and in addition they can be used to predict the breeding value for traits not yet recorded [22].

In addition to the traits directly selected for, other important traits should also be recorded, in order to document the trend in these traits. Such traits include traits that are economically or ethically important, but they are not necessarily included in the breeding objective if they are at acceptable levels. However, correlated effects of selection might change these traits in undesired directions.

Two aspects of sensitivity to environmental factors should be considered. Chance events, such as disease epidemics have the potential to critically damage a breeding scheme, particularly if breeding animals are kept in a limited region or in a few herds. The foot-and-mouth disease outbreak is an example of a disease outbreak that has threatened breeding nuclei. Backup and safety procedures are necessary for safeguarding a breeding scheme. This can be done by implementation of techniques such as cryopreservation of embryos, eggs and semen, and segmentation of the breeding nucleus. This will naturally be very species specific, depending on the biological possibilities in the given species.

The second aspect of environmental sensitivity relates to environmental sensitivity, whether an individual's performance is sensitive to the environment in which it is kept. More specifically, this is an issue if genotypes and environment interact, such that an individual would be ranked differently in two environments relative to other individuals. A more realistic

example is offspring from two bulls having different relative merits in two environments. The implications of an interaction are multiple. First, prediction of the performance in an environment that differs from the environment in which selection was performed might be impossible. Second, genetic improvement in one environment might not result in similar genetic changes in another environment. As an example, in some breeding schemes, candidates for selection are housed individually in order to allow for individual recording of the traits of interest, whereas animals in commercial production are housed in groups, providing a significantly different environment to that selected in. In such cases, where the environment of the breeding nucleus and the production herds differ, then genetic trend should be recorded in the production environment to ensure that selection is in the direction desired. If genotype–environment interaction exists, genetic evaluations should take this into account. Also the breeding objective should be specific on which environment(s) genetic changes are optimized for. A specific type of genotype–environment interaction is the situation where environments describe a continuum of environmental effects, and thus cannot be grouped in classes of environmental effects. Reaction norm models describe such interactions, and a feature of these models is that they can model the sensitivity to the environment, and that this sensitivity is partly genetically determined. This has two implications. First, it is expected that environmental sensitivity will change as a correlated effect of selection on the mean, assuming nonzero correlations between mean performance and sensitivity. Second, it implies that environmental sensitivity can be selected for or against. The second implication also means that genetic changes will require changes in the environment to be fully expressed in all environments. This is an important outcome of a breeding scheme, and it has to be taken into consideration whether such environmental changes can be accommodated in commercial environments. There are many examples of highly improved breeds, selected in environments with a high level of management, that fail to express their genetic potential when transferred to environments characterized by lower management levels, for example, when highly selected breeds are transferred to tropical countries with lower input levels and other environmental characteristics. Thus, potential changes in environmental sensitivity have to be taken into account when predicting and evaluating the expected response to selection.

The optimum selection environment is not always equal to the environment in which the response is to be realized, but depends on the degree of genotype–environment interaction (determined by the ratio of variances in slope and level of a linear reaction norm), the correlation between level and slope, and the heritability of the trait [23].

In relation to breeding objectives, it was stressed that the market for which production is aiming should be defined. The reasoning is that this is a prerequisite to ensure that consumers will accept the product and its characteristics and that the product(s) addresses the preferences of the consumers. Consumer responses have been instrumental in changing egg production systems in European countries, and the use of gene transfer worldwide.

Measuring the success of a breeding scheme involves two components. First, expected response to selection should be predicted, as the measure against which to compare the operational and strategic outcome of the breeding scheme. To predict the expected response to selection estimates of genetic parameters, covariances between traits, generation intervals, recording scheme, and resulting accuracies of the predicted breeding values, selection intensities and the weights used in the breeding objective are required. Uncertainty about one or more of these should be taken into account when predicting response to selection. These predictions are based on a genetic model, on which there may also be uncertainty. Second, realized genetic trends should be compared to the predicted response to selection, both in order to evaluate the success of the breeding scheme, but also to modify it if required to better fulfill the overall objectives of the breeding scheme.

Most breeding schemes rely on genetic progress accumulating in a nucleus, representing the animals active in the breeding scheme. The genetic progress is then disseminated to production herds. This might involve several tiers, such as a breeding and multiplier level, in order to efficiently disseminate genetically superior individuals or semen to production herds. The time from genetic progress in the breeding nucleus

to the dissemination of this progress in production herds is termed genetic lag, and is a major indicator of the efficiency of utilizing genetic progress in production.

The outcome of a breeding scheme is realized in the years following the selection decisions and the majority of the costs. An economic evaluation of a breeding scheme thus requires that costs and revenues are discounted back to a common base. This should take into account that returns more distant in time are more uncertain than those occurring in the near future. This involves defining the time horizon for which revenues are discounted, reflecting the uncertainties about the market and production conditions. This reflects an economic time horizon of the breeding scheme.

Expected and realized response to selection should be compared at regular interval as an integral part of controlling and optimizing the breeding scheme. This should be done at intervals shorter than the economic time horizon.

Future Directions

Following is a discussion including potential impacts on the development of certain areas of science.

Animal breeding is a competitive business, and producers pursue genetic material that best suits their needs. This naturally means purchasing animals that are economically optimal in the specific production. This potentially limits the focus on animal welfare–related traits not directly related to productivity.

However, since animal breeding decisions for most species are made centrally and, increasingly, internationally it has been argued that international agreements are needed [7] to ensure sustainable breeding schemes that address all relevant traits.

There is evidence that breeding companies are addressing these challenges. Survival in poultry has been improved by, among other initiatives, selective breeding and current focus is on selection against feather pecking, cannibalism, leg disorders, and heart/lung disorders [24]. And more generally, breeding objectives now contain more traits, also related to animal health and costs of production. In many cases, inclusion of health- and welfare-related traits results in more profitable breeding schemes than selection on production alone [25].

It should, however, also be acknowledged that despite the well-documented negative effects that selection for production traits has had on welfare-related traits, modern genetic technologies also give possibilities of understanding the biology underlying animal welfare and actively select for improved welfare [26]. Welfare-related traits are likely to show genetic variation, and it is thus also possible to select for increased animal welfare [27].

New technologies have the potential to affect animal welfare in both positive and negative directions [28]. Positive examples are the use of sexed semen in dairy cattle, which can decrease the number of unwanted male offspring that are killed after birth in some breeds and result in easier birth of female offspring. An overall positive effect is conditional on no adverse effects of the technology on the calves and/or the mothers.

Genomic selection [22] is currently under implementation in the large dairy, pig, and poultry breeding schemes. Genomic selection is fundamentally different to previous selection strategies, which relied on phenotypic records being recorded on the candidates for selection and/or their relatives. With genomic selection, this is replaced by a two-step procedure where the association being genome wide, markers and phenotypes are established in a part of the population where both phenotypic recording and genotyping are performed. Secondly, breeding values can be predicted for individuals based on their genotype. This allows for accurate breeding values being available earlier in life. This is particularly useful for traits recorded late in life, traits with low heritability, and traits recorded in one sex, which are largely the characteristics of many traits related to animal health and welfare. Genomic selection thus improves efficiency of selection for traits that are currently difficult to improve genetically, and thus has the potential to contribute to more sustainable animal breeding schemes [29].

Effective population size is a measure of the risk of a breeding scheme. Constraining the rate of inbreeding has the potential to reduce the variance of response to selection, reduce the loss of genetic variance, and reduce genetic drift. Quadratic selection indices jointly maximizing response to selection while constraining rate of inbreeding have been developed [30]. This quadratic index optimizes the genetic contributions of

parents to the next generation, and the contribution of any parent depends on which other parents that are selected.

Acceptable rates of inbreeding are often described as being in the range of 0.5–1% per generation, equivalent to an effective population size of 50–100. More specifically, the acceptable level of inbreeding depends on the extent of inbreeding depression on fitness [20], and thus a sustainable breeding scheme ought to validate the acceptable level of inbreeding.

The implementation of sustainable breeding schemes requires, as described above, a thorough analysis based on an understanding of genetics, economics, and market characteristics. Training and education is a prerequisite for qualified personnel to contribute to the development and maintenance of sustainable breeding schemes.

Sustainability is a new measure in the context of animal breeding, but many elements contributing to sustainability are well known and have been taken into account previously. The aim of developing sustainable animal breeding schemes puts these elements into a coherent context, highlighting the need to take interests of all stakeholders into account, as well as the risks and uncertainties of assumptions and outcomes.

Bibliography

Primary Literature

1. FAO (2007) The state of the world's animal genetic resources for food and agriculture. In: Rischkowsky B, Pilling D (eds) Rome (Available at www.fao.org/docrep/010/a1250e/a1250e00.htm)
2. FAO (2010) Breeding strategies for sustainable management of animal genetic resources: FAO animal production and health guidelines, vol 3. FAO, Rome, 160 pp
3. Kim ES, Kirkpatrick BW (2009) Linkage disequilibrium in the North American Holstein population. Anim Genet 40:279–288. doi:10.1111/j.1365-2052.2008.01831.x
4. Sørensen AC, Sørensen MK, Berg P (2005) Inbreeding in Danish dairy cattle breeds. J Dairy Sci 88:1865–1872
5. Hastedt H (2000) Beats, humans and the etics of animal breeding. Arch Tierz 43:218–225
6. Larrere R (2003) The issue of modern breeding a social request, consumer request or responsible inquiries? Prod Anim 16:329–332
7. Olsson IAS, Gamborg C, Sandoe P (2006) Taking ethics into account in farm animal breeding What can the breeding companies achieve? J Agric Environ Ethics 19:37–46
8. Neeteson-van Nieuwenhouven A, Merks J, Bagnato A, Liinamo A (2006) Sustainable transparent animal breeding and reproduction. Livest Prod Sci 103(3):282–291
9. United Nations General Assembly (1987) Report of the World Commission on Environment and Development: Our Common Future. Transmitted to the General Assembly as an Annex to document A/42/427 – Development and International Co-operation: Environment. Retrieved on: 2010-06-30
10. Gamborg C, Sandoe P (2005) Sustainability in farm animal breeding a review. Livest Prod Sci 92:221–231
11. Woolliams J, Berg P, Mäki-Tanila A, Meuwissen T, Fimland E (2005) Sustainable management of animal genetic ressources. Nordic Gene Bank Farm Animals, Nordisk Genbank Husdyr. ISBN 92-893-1089-8
12. CODE-EFABAR (2010). http://www.effab.org/CODEEFABAR.aspx. Assessed 30-6-2010
13. Shuster DE, Kehrli ME, Ackermann MR, Gilbert RO (1992) Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc Natl Acad Sci USA 89:9225–9229
14. Agerholm JS, Bendixen C, Andersen O, Arnbjerg J (2001) Complex vertebral malformation in Holstein calves. J Vet Diagn Invest 13:283–289
15. Carden AE, Hill WG, Webb AJ (1983) The inheritance of halothane susceptibility in pigs. Genet Sel Evol 15:65–82
16. Star L, Ellen E, Uitdehaag K, Brom FWA (2007) A plea to implement robustness into a breeding goal: poultry as an example. J Agric Environ Ethics 21:109–125
17. van der Waaij EH (2004) A resource allocation model describing consequences of artificial selection under metabolic stress. J Anim Sci 82:973–981
18. Rauw WM, Luiting P, Beilharz RG, Verstegen MWA, Vangen O (1999) Selection for litter size and its consequences for the allocation of feed ressources a concept and its implications illustarted by mice selection experiments. Livest Prod Sci 60:329–342
19. Cheema MA, Qureshi MA, Havenstein GB (2005) A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1519–1529
20. Bijma P (2000) Long-term genetic contributions. Prediction of rates of inbreeding and genetic gain in selected populations. PhD thesis. ISBN 90-5808-241-5
21. Olesen I, Groen AF, Gjerde B (2000) Definition of animal breeding goals for sustainable production systems. J Anim Sci 78:570–582
22. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
23. Kolmodin R, Bijma P (2004) Response to mass selection when the genotype by environment interaction is modeled as a linear reaction norm. Genet Sel Evol 36:435–454
24. Flock DK, Laughlin KF, Bentley J (2005) Minimizing losses in poultry breeding and production: how breeding companies contribute to poultry welfare. Worlds Poult Sci J 61:227–237

25. Lawrence AB, Conington J, Simm G (2004) Breeding and animal welfare: practical and theoretical advantages of multi-trait selection. Anim Welfare 13:S191–S196
26. Jensen P, Buitenhuis B, Kjaer J, Zanella A, Mormede P, Pizzari T (2008) Genetics and genomics of animal behaviour and welfare – Challenges and possibilities. Appl Anim Behav Sci 113:383–403
27. Kanis E, van den Belt H, Groen AF, Schakel J, de Greef KH (2004) Breeding for improved welfare in pigs: a conceptual framework and it suse in practise. Anim Sci 78:315–329
28. Clark JAM, Potter M, Harding E (2006) The welfare implications of animal breeding and breeding technologies in commercial agriculture. Livest Sci 103:270–281
29. Mark T, Sandøe P (2010) Genomic dairy cattle breeding: risks and opportunities for cow welfare. Anim Welfare 19(1):113–121
30. Meuwissen THE (1997) Maximising the response of selection with a predefined rate of inbreeding. J Anim Sci 75: 934–940

Books and Reviews

FAO (2010) Breeding strategies for sustainable management of animal genetic resources: FAO animal production and health guidelines, vol 3. FAO, Rome, 160 pp

Animal Breeding, Modeling in

Lawrence R. Schaeffer
Department of Animal and Poultry Science, Centre for Genetic Improvement of Livestock, University of Guelph, Guelph, ON, Canada

Article Outline

Glossary

Contemporary groups A group of animals of approximately the same age living in the same environment and being treated by the same management practices during the same interval of time.

Estimated breeding value An estimate of the total additive genetic merit of an individual, the effects that are directly passed to offspring.

Genotypes The particular set of alleles at all gene loci that influence the phenotypes.

Inbreeding coefficients The proportion of alleles at gene loci that are identical due to being inherited from a common ancestor.

Infinitesimal genetic model A genetic model that assumes there are an infinite number of gene loci affecting a trait each with a small and equal effect.

Mixed model equations Proposed by Henderson in 1949 for the estimation of breeding values and other nongenetic effects from phenotypes.

Phenotypes The observable characteristics of an animal that can be measured, scored, or recorded.

Definition of the Subject

Modeling in animal breeding involves describing the major factors that influence the performance ability or production level of animals in order to predict the genetic merit of future progeny for that ability. Successful modeling depends on good record collection systems, accurate pedigree records, and sophisticated statistical models. Models have evolved over time as computer technology has advanced. Genetic evaluation of dairy bulls began in the early 1930s using simple daughter averages for milk production in selection index procedures of Lush and his students [1]. Genetic evaluation systems spread to all livestock and to many countries due to Lush. Henderson [2] introduced best linear unbiased prediction (BLUP) around 1950, and this methodology is still widely used in animal breeding except that the models are more detailed and complex. Gianola and others [3, 4] taught animal breeders how to use Bayesian methods which are especially useful for non-normally distributed data.

All methods consider the total additive genetic merit of animals. Additive genetic effects are directly transmitted to progeny, assuming infinitely many gene loci, each with a small and equal effect on the trait of interest. Examples of traits are milk yields of dairy animals, growth rates of meat-producing animals, occurrence of health problems, ability to reproduce, and behavior.

Models to analyze traits differ depending on the nature of the traits being evaluated. Traits can be broadly grouped into production traits, growth traits, reproductive traits, health traits, and survival, and the models for each group are very specialized. In the 1930s, dairy bulls were selected almost solely for the ability of their daughters to produce milk. Over time, the success of that selection pressure for one trait caused negative correlated responses in reproduction potential and disease susceptibility. Genetic evaluation systems consider many traits simultaneously with a primary focus on efficiency of economic production and cost savings.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Introduction

Modeling in animal breeding began with the expression

$$\mathbf{p} = \mathbf{g} + \mathbf{e},$$

where **p** is the phenotype of an individual, **g** is the genetic source of variation or genotype in **p**, and **e** is the environmental source of variation, presented by Wilhelm Ludwig Johannsen in 1909 [5]. A phenotype is a measurement of an animal's performance for an economically important trait, such as daily milk yield of a dairy cow, goat, or sheep; a growth record on a beef animal, pig, or rabbit; or wool production of a sheep or llama. Current models are extensions of this basic equation.

Genetic Component

The **g** term of the model is partitioned as

$$\mathbf{g} = \mathbf{g}_a + \mathbf{g}_d + \mathbf{g}_i,$$

where

- $\mathbf{g}_a$ are additive genetic effects, the collective effects generated by individual alleles at every loci in the genome.
- $\mathbf{g}_d$ are the dominance genetic effects, the collective effects generated by combinations of alleles at every locus in the genome.
- $\mathbf{g}_i$ are the many interactions among loci throughout the genome, in a collective sense.

Animal breeders are primarily concerned with additive genetic effects because those effects are passed directly from parents to progeny. Dominance and interaction genetic effects occur depending on the combinations of alleles from the paternal and maternal sides of the pedigree, and cannot be predicted prior to making a mating of specific individuals.

Further, $\mathbf{g}_a$ is assumed to be the combined effects of an infinite number of loci, each with a small and equal effect, i.e.,

$$\mathbf{g}_a = \sum_{k=1}^{\infty} g_k.$$

The population is assumed to be very large and mating randomly. This is known as the *infinitesimal genetic model* upon which genetic evaluation systems are based. This is a reasonable model if you consider that there are roughly 30,000 loci (this estimate is continually changing), and if one assumes only two alleles at each locus, then there are three possible genotypes per locus, or $3^{30,000}$ possible genotype outcomes. This number is large enough to be nearly infinite for all practical purposes. The number of possible genotype outcomes is actually much bigger if you allow that there are more than two alleles for many loci.

Environmental Component

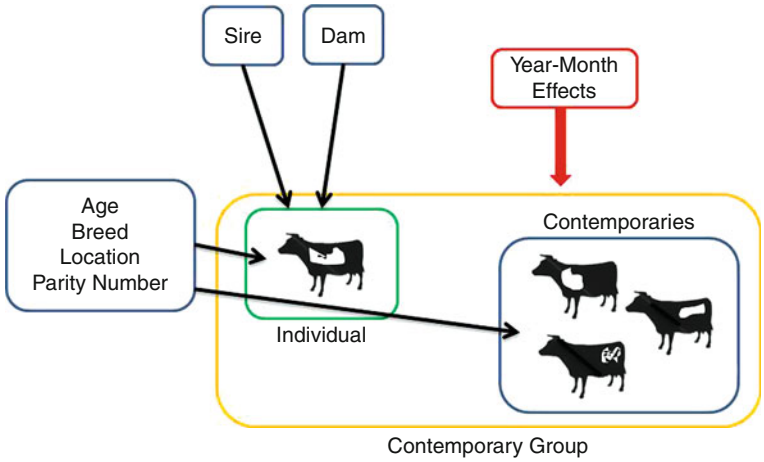
The **e** term can be split into identifiable factors that are known to have effects on the phenotypes. Some effects are shared with other animals in the same location at the same time, and some effects are specific to individuals.

Animal Model

The common model in animal breeding is called an *animal model*, based on the underlying infinitesimal genetic model, containing factors to account for time trends, contemporary groups, additive genetic effects, residual, and other factors, that depend on the species and trait of interest. Figure 1 depicts a typical animal model in a diagrammatic format. Each individual is affected by the additive genetic merits of its sire and dam, as well as year and month of birth or calving. Contemporaries are animals that share the same year and month effects as the individual because they exist in the same space and time. These effects include weather, location, herd owner, and herd management practices of feeding, breeding, and health care. Individual production is affected by breed, age, and parity number, and these effects are common to all animals of the same breed, age, and parity number.

Depending on the species and trait, other factors could be included in the diagram.

Models in animal breeding are conveniently presented in matrix notation. Models consist of three parts which are the equation, the expectations and distributions of random variables, and finally a list of assumptions and limitations. The three parts are briefly described.



Animal Breeding, Modeling in. Figure 1
Diagram of genetic and environmental factors affecting dairy cow milk production

The Equation

A linear statistical model is written generally (in matrix notation) as

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e},$$

where

y is a vector of observations on the trait of interest from individual animals, either one or more per animal.

b is a vector of levels of factors that are known to affect the magnitude of the observations. These factors are fixed factors, in that they are constants and not random variables drawn from a particular distribution or population of effects. One factor that should always be included in an animal model is one that accounts for time, either year, or year-season. Seasons may be groups of months, individual months or weeks of the year. This time factor is meant to account for changes in average production over time due to changes in production technology, global warming, nutritional improvements, or financial impacts that affect the entire population. Other factors are effects of gender, breed, age of dam, and diet.

u is a vector of levels of random factors, which are random samples from a large population of levels of that effect. Animal additive genetic effects are one such random factor contained in **u**. Two random factors that should always be included in an animal model are the animal additive genetic effect, **a**, and the contemporary group effect, **c**, $(\mathbf{u}' = (\mathbf{a}' \ \mathbf{c}'))$. Contemporaries are animals that coexist in the same location and time space, and thus share the same management care and treatment during the time they are observed for a trait. Contemporary groups are commonly called herds, flocks, or tanks, and are nested within year or year-season effects. Contemporary groups are random because there are a large number of them, each contemporary group contains different individuals, and the effects during that one occurrence are random in nature.

e is a vector of random residual (environmental) effects specific to each observation, that cannot be accounted for by other factors in the model. The residual effects may be samples from different populations having different variances.

Expectations and Covariances

The model also describes the distributions of the random factors and indicates their expected values or means and covariance structures. That is,

$$\begin{aligned} \mathrm{Var}(\mathbf{u}) &= \mathbf{G} \\ \mathrm{Var}(\mathbf{e}) &= \mathbf{R}, \text{ and} \\ \mathrm{Cov}(\mathrm{u}, \mathbf{e}') &= 0, \end{aligned}$$

where Var() is a variance-covariance matrix, and Cov() is a covariance matrix.

Further, **G** can be partitioned into other matrices for each factor contained within **u**. Because **u** will contain animal additive genetic effects, **a**, and contemporary group effects, **c**, then both **a** and **c** are assumed to follow normal distributions with null means. The variance-covariance matrix of **a** is

$$Var(\mathbf{a}) = \mathbf{A}\sigma_a^2,$$

where **A** is the numerator additive genetic relationship matrix of Wright (2), and σ_a^2 is the additive genetic variance of the trait of interest. More will be given on the additive relationship matrix shortly.

Similarly,

$$\mathrm{Var}(\mathbf{c}) = \mathbf{I}\sigma_c^2,$$

where σ_c^2 is the contemporary group variance (variance of all contemporary group effects), and **I** is an identity matrix, which means the contemporary group effects are independent of each other. Finally,

$$\mathrm{Cov}(\mathbf{a}, \mathbf{c}') = 0,$$

additive genetic and contemporary group effects are independent.

The residual effects are assumed to be independent of all other random factors, meaning their covariances with other random factors are 0. If known, the ratios of residual variance to the additive genetic variance and to the contemporary group variance should be provided, or at least the values that are intended to be used.

Assumptions and Limitations

Limitations occur because of a lack of information in the data records. A factor that could be important is not included in the model because there is no information about that factor in the data files on animals. For example, an animal's record may or may not be affected by its health status. If the record was affected, then health status should be a factor in the linear model. However, health information may not be a part of the data files, and therefore, it cannot be included in the model. Consequently, an assumption is needed that animals were healthy when observations were taken. Depending on the trait and species, this assumption may or may not be critical.

A complete list of the explicit or implied assumptions should be a part of every model description, but often they are omitted. Readers of a scientific report may not be as familiar with the data files and production system as the authors, and may not be able to assess the assumptions that need to be made. The presence of this part of the model helps readers to judge the quality of an analysis.

Genetic Relationships

The animal model works best when pedigree information is complete and accurate. Preparing pedigree files can be tedious, depending on the species. Animals can be registered in different organizations with different identifications in each organization, both of which may be found in the data files. Efforts are needed to make sure that each animal has only one unique identification in both the data files and the pedigree file. Good identification systems include codes for breed of the animal and year of birth.

Next, pedigrees of animals need to be arranged in chronological order. Parents should appear in a list before (ahead of) their progeny. Ordering a pedigree is most easily accomplished by sorting animals by birthdate. Birthdates, however, can be incorrectly recorded, or for many individuals may not be available. One approach is to assume that all birthdates are incorrect. Assign all animals a generation number of 1. Then cycle through the pedigree file and modify the generation numbers so that the generation numbers of the sire and dam of an animal are at least one unit greater than the generation number of that animal. Iterate through the pedigree file as many times as needed until no further modifications are made to any generation numbers.

Inbreeding Coefficients

Genetic relationships among individuals were worked out by Wright [6] as correlation coefficients. Later, only the numerators of these correlation coefficients were needed in genetic evaluation, and were called Wright's numerator, additive genetic relationships. The dimensions of the additive genetic relationship matrix, **A**, equal the number of animals (N) in the pedigree. The pedigree file usually contains more individuals than are represented with records in the data file. If constructed,

the **A** matrix would be 95% full (nonzero numbers). For one million animals, the storage of these numbers would be an overwhelming problem computationally. Fortunately, during genetic evaluation, only the inverse of this matrix is needed. The inverse is typically very sparse by comparison, and the elements in the matrix can be generated as needed and do not ever need to be stored. For this to work, the inbreeding coefficients of every animal need to be determined.

Henderson [7] showed that the additive relationship matrix could be written as

$$\mathbf{A} = \mathbf{TBT}',$$

where **T** is a lower triangular matrix and **B** is a diagonal matrix. The diagonals of **B**, say b_{ii}, were shown to equal

$$b_{ii} = (0.5 - 0.25(F_s + F_d)),$$

where F_s and F_d are the inbreeding coefficients of the sire and dam, respectively, of the ith individual. If one parent is unknown, then

$$b_{ii} = (0.75 - 0.25F_p),$$

where F_p is the inbreeding coefficient of the parent that is known. Lastly, if neither parent is known, then $b_{ii} = 1$. An inbreeding coefficient indicates the proportion of alleles that are in common within an individual due to being inherited from common ancestors some generations back in the pedigree. This happens when related animals are mated together.

The key discovery of Henderson [7] was that

$$\mathbf{A}^{-1} = \mathbf{T}'^{-1}\mathbf{B}^{-1}\mathbf{T}^{-1},$$

and that each row of $\mathbf{T}^{-1}$ had a one on the diagonal and two negative one-halves on the off-diagonals corresponding to the locations of the sire and dam of that individual. Only two nonzero numbers that are always equal to 1 or $-\frac{1}{2}$. The elements of $\mathbf{B}^{-1}$ are equal to b_{ii}^{-1}. Every animal could have a different b_{ii}, so these would need to be stored.

Meuwissen and Luo [8] developed a very efficient algorithm to compute inbreeding coefficients, and from these come b_{ii}. The algorithm requires animals to be chronologically ordered and processed, so that inbreeding coefficients of an animal's ancestors are known before that animal is processed. Once the inbreeding coefficients are known, then b_{ii} are easily obtained. Having b_{ii} for each individual, then elements of $\mathbf{A}^{-1}$ can be calculated readily, as needed. Consult Meuwissen and Luo [8] for details of their algorithm.

Missing Parents

Animals with unknown parents are assumed to be animals from a large randomly mating population of unrelated, unselected individuals. This group of animals is known as the *base population*. In dairy cattle, the base population might be animals that were born in the 1950s. However, even today, there are animals in the data files and pedigree files that have unknown parentage. Clearly, these animals are genetically different from animals born 60 years earlier, and they should belong to a population different from the base population. For this reason, unknown parents are assigned to genetic groups based on the year of birth of their progeny, and whether the progeny was male or female [9, 10]. Suppose an animal was born in 2009 and was a male. If the male parent of this animal was unknown, then it would be assigned to a Sire of Males group for 2009, and if the female parent was unknown assignment would be to a Dam of Males group for 2009. The assumption made for assignments is that the selection intensity of each pathway differs so that the genetic means of these groups would be different. The other two pathways are Sires of Females and Dams of Females. These groups may be further subdivided, based on breeds or countries of origin.

Computationally, genetic groups are treated as though they were a separate individual, which requires simple modifications in the computation of elements of $\mathbf{A}^{-1}$. Genetic groups are essential in the animal model in order to obtain unbiased estimates of genetic trends, and accurate evaluations of all animals. There are always animals with unknown parents resulting from movement of animals between countries, but also between herds or flocks within a country.

Genetic Evaluation

Best linear unbiased prediction (or BLUP) has been used since 1970 when Henderson applied the method to genetic evaluation of dairy bulls in the northeastern United States even though the theory

had been available since 1950. BLUP requires setting up and solving a linear system of equations that is equal in size to the number of animals and number of levels of other effects that are in the model. The equations are known as the mixed model equations (MME). The solutions to these equations are usually obtained by iteration rather than through the direct inverse of the large coefficient matrix. Many various strategies have been devised to iterate solutions quickly.

Iteration is a technique where initial solutions begin at zero, and by going through one equation at a time, each solution is updated based on the values of the other solutions at that time. An updated solution causes all subsequent solutions to change during the updating process. Iterations continue until the changes are less than a certain value (like 1×10^{-9}), at which point the solutions have reached convergence.

Mixed Model Equations

The mixed model equations (MME) of Henderson [2] yield the BLUP predictors of the random effects of the linear model and generalized least squares estimators of the fixed effects. Following the notation for the linear model, the MME, generally, are written as

$$\begin{pmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z} \\ \mathbf{Z}'^{-1}\mathbf{X} & \mathbf{Z}'\mathbf{R}^{-1}\mathbf{Z} + \mathbf{G}^{-1} \end{pmatrix} \begin{pmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{u}} \end{pmatrix} = \begin{pmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}'\mathbf{R}^{-1}\mathbf{y} \end{pmatrix}.$$

From one model to the next, **X**, **Z**, **R**, and **G** change in how they may be constructed, but the MME always have the same form. Computations also differ and may simplify in certain cases.

Estimation of Variances

Two methods of estimation of **G** and **R** are commonly used in animal breeding. First is Restricted (or Residual) Maximum Likelihood (REML) [11–13], of which there are four computational versions, and second is Bayesian Estimation using Gibbs Sampling as a tool to maximize the joint posterior distribution [14]. Both methods require major computing time, and consequently, smaller subsamples of the complete data file are chosen for estimating covariance matrices.

The main advantage of these two methods is that the estimated covariance matrices are positive definite matrices that can be used directly in MME. A covariance matrix must be positive definite like a variance must always be positive.

Another advantage of the methods is that the REML and Bayesian estimators are more accurate than other methods. The disadvantage is the increased computing demands necessitating the need to subsample the data into smaller sets.

Reliabilities

All estimates of breeding values of animals require information about the accuracy or reliability of that estimate. Theoretically, standard errors of prediction are derived from the inverse of the coefficient matrix of the MME. Given that solutions result from iteration techniques rather than inversion, the standard errors of prediction must be approximated. Standard errors of prediction are often converted into a percent reliability that goes from 0 to 100. There are many approximation methods in use.

Reliabilities depend on the number of progeny, the number of contemporaries of those progeny, the completeness of pedigree information, and the variance and covariance parameters. Depending on the reliability of an animal's Estimated Breeding Value, (EBV), the EBV may or may not be made officially public. Minimum standards are agreed upon by industry committees.

Genetic Trends

Genetic trends are estimated by averaging animal EBVs by year of birth, or by years in which they make records. In dairy cattle, for example, the EBVs of all cows that were born in a given year and which completed at least one lactation can be averaged. By plotting these averages by year of birth, the genetic trend in milk production can be quantified. Plotting the average EBVs of dairy bulls by their year of birth would give a different trend, reflecting how bulls were chosen to be in artificial insemination. The cow averages would reflect how those bulls were used in breeding programs, and would likely lag a couple of years behind the sire birth years. Graphs of genetic trends should have details of what the averages represent.

Single Record per Animal

The models presented in this and the following sections become progressively more complicated, due to the type of traits that are considered. First, a simple animal model is illustrated which covers traits that are observed only once in an animal's lifetime, such as age at first breeding. Next, the situation where animals can be observed more than once per lifetime, such as annual antler production in elk, wool production in sheep, or race results in horses. Then traits influenced by maternal effects, such as birth weights, weaning weights, and calving ease, are considered. Longitudinal data, traits observed over time, such as milk production or egg production, provide models that analyze the shapes of curves. Multiple trait models are applied to two or more traits at a time, and include genetic and environmental correlations among the traits. Threshold models for categorical data are described, and finally, a model for the analysis of survival data is presented.

Data

The case of a single record per animal is presented in some detail because this is the simplest model and illustrates the process of constructing mixed model equations, and what happens to the solutions to those equations. The models are presented in a generic fashion without reference to particular species.

Table 1 contains the pedigree information and data on 16 animals. The first four animals are base population animals without records and without known parents. The inbreeding coefficients are shown and

Animal Breeding, Modeling in. Table 1 Example data for simple animal model

Animal	Sire	Dam	F_i	b_{ii}	Year	CG	Record
1	0	0	0.00000	1.00000			
2	0	0	0.00000	1.00000			
3	0	0	0.00000	1.00000			
4	0	0	0.00000	1.00000			
5	1	2	0.00000	0.50000	1	1	78
6	3	4	0.00000	0.50000	1	1	26
7	3	2	0.00000	0.50000	1	1	111
8	1	4	0.00000	0.50000	1	1	122
9	5	6	0.00000	0.50000	1	1	98
10	7	8	0.00000	0.50000	1	2	48
11	5	8	0.12500	0.50000	1	2	109
12	7	6	0.12500	0.50000	1	2	94
13	1	6	0.00000	0.50000	1	2	103
14	3	8	0.00000	0.50000	2	3	78
15	5	4	0.00000	0.50000	2	3	69
16	7	8	0.00000	0.50000	2	3	44
17	9	6	0.25000	0.50000	2	3	12
18	11	10	0.18750	0.46875	2	4	54
19	11	12	0.06250	0.43750	2	4	89
20	9	8	0.12500	0.50000	2	4	82

the values of b_{ii} which come from the inbreeding coefficients. Records were made over 2 years, and there were two contemporary groups within each year. Assume that the residual variance is 600, the genetic variance is 300, and the variance of contemporary group effects is 100. Heritability (denoted as h^2) is defined as the genetic variance divided by the total phenotypic variance, which is the sum of the genetic, contemporary group, and residual variances. In this case, $h^2 = 0.3$.

Mixed Model Equations

The equation of the model is (in scalar form)

$$y_{ijk} = (YR)_i + (CG)_j + a_k + e_{ijk},$$

where

y_{ijk} are the observations on animal k, belonging to contemporary group, $(CG)_j$, born in year, $(YR)_i$,

$$\mathbf{y} = \{y_{ijk}\}.$$

$(YR)_i$ are the fixed, year effects,

$$\mathbf{b} = \{(YR)_i\}.$$

$(CG)_j$ are the random, contemporary group effects,

$$\mathbf{c} = \{(CG)_j\}.$$

a_k are the random, additive genetic effects of individual animals,

$$\mathbf{a} = \{a_k\}$$

e_{ijk} are random, residual effects.

Also,

$$\mathbf{u} = \begin{pmatrix} \mathbf{c} \\ \mathbf{a} \end{pmatrix},$$

and

$$\mathrm{Var}\begin{pmatrix} \mathbf{c} \\ \mathbf{a} \end{pmatrix} = \begin{pmatrix} \mathbf{I}_4\sigma_c^2 & 0 \\ 0 & \mathbf{A}\sigma_a^2 \end{pmatrix} = \mathbf{G}.$$

Finally,

$$\mathbf{R} = \mathbf{I}_{16}\sigma_e^2,$$

and $\mathbf{Z}$ can be partitioned into one matrix for contemporary groups, and one for animal additive genetic values, i.e.,

$$\mathbf{Z} = \begin{pmatrix} \mathbf{Z}_{cg} & \mathbf{Z}_a \end{pmatrix}.$$

Inverse of Relationship Matrix

Notice that the MME contain $\mathbf{G}^{-1}$, which equals

$$\mathbf{G}^{-1} = \begin{pmatrix} \mathbf{I}\frac{1}{\sigma_{cg}^2} & 0 \\ 0 & \mathbf{A}^{-1}\frac{1}{\sigma_a^2} \end{pmatrix}.$$

Thus, the inverse of the relationship matrix is needed. This matrix can be constructed readily following simple rules that Henderson [7] provided. For each animal with both parents known, 9 numbers are added into the inverse matrix. For an animal with only one parent known, 4 numbers are added, and for an animal with both parents unknown, 1 number is added. Start with a matrix of order 20 that is completely null. Then process the pedigree file, one animal at a time until all animals are included.

Step 1: For animal i, let $\delta = b_{ii}^{-1}$. For animal 1, $\delta = 1$; for animal 6, $\delta = 2$, and for animal 19, $\delta = 2.2857$.

Step 2: Add δ to the diagonal element for that animal. For animal 19, add *delta* to element (19,19) of $\mathbf{A}^{-1}$.

Step 3: If the male parent is known, subtract -0.5δ from elements (i, s) and (s, i), where i is the animal's number and s is the sire's number. For example, for animal 19, $i = 19$ and $s = 11$. Also add 0.25δ to element (s, s).

Step 4: If the female parent (denoted by d) is known, subtract -0.5δ from elements (i, d) and (d, i), and add 0.25δ to element (d, d).

Step 5: If both parents are known add 0.25δ to elements (s, d) and (d, s).

Numbers are accumulative as the pedigrees are processed. Many of the elements will stay null values, especially for animals that do not have progeny.

Solutions to Equations

The resulting mixed model equations have 26 rows and columns with 26 unknowns to be estimated (2 year effects, 4 contemporary groups, and 20 animal effects).

Even for this small example, to display the equations fully would take up too much space. The solutions to the MME were as follows:

Year 1	=	88.8
Year 2	=	61.6
CG 1	=	.015
CG 2	=	−.015
CG 3	=	−3.303
CG 4	=	3.303

Animal		EBV	Reliability
1	=	8.32	.13
2	=	1.06	.11
3	=	−5.68	.14
4	=	−3.69	.10
5	=	4.38	.23
6	=	−15.50	.25
7	=	−.95	.25
8	=	7.37	.23
9	=	−4.28	.20
10	=	−6.58	.18
11	=	9.49	.24
12	=	−3.79	.24
13	=	−.02	.17
14	=	4.61	.17
15	=	2.41	.18
16	=	−.30	.16
17	=	−17.17	.25
18	=	−.90	.21
19	=	6.66	.16
20	=	4.65	.18

The solutions for the animal additive genetic effects are known as *Estimated Breeding Values* or EBVs. Progeny are expected to inherit an average of the EBVs of its parents, on average. EBVs are sorted from highest to lowest, or best animal to poorest animal. Every animal in the pedigree file obtains a solution due to genetic relationships to progeny and other individuals. The EBV of an animal consists of combined information from the animal's parents, its progeny, and its own performance record. The BLUP methodology combines the information in an optimal manner to maximize the correlation of the EBV with the animal's true genetic merit. The reliability is obtained from the inverse of the MME coefficient matrix, and expressed as a correlation coefficient. The greater is the reliability, the more certainty in the ranking of the animals. The highest reliability was 0.25, for animals 6, 7, and 17. Note that animal 6 had 4 progeny, plus a record on itself, and both parents were known. Animal 7 had 3 progeny. Animal 17 had no progeny, but was an offspring of animal 6 and was inbred the most. Reliability also reflects the number of contemporaries that each animal has, but the differences in this example were not great. The reliabilities of all animals in this example are very small, but there were only 16 observations in total. Dairy bulls, for example, can have hundreds or thousands of progeny giving them reliabilities above 0.99. A minimum reliability level is chosen before EBVs are released to the public.

The solutions for the year effects were 88.8 and 61.6, respectively. Thus, performances were lower in year 2 compared to year 1. There is likely a reason for this difference, such as year 2 being hotter, or a shortage of good feed, or feed prices may have caused many animals to be removed from farms. The difference between years is not genetic.

Contemporary group effects were random in the model, and the solutions have an average of zero as a consequence of how they were included in the model. The CG solutions average zero within the year effects, due to the fact they were a factor nested within year effects.

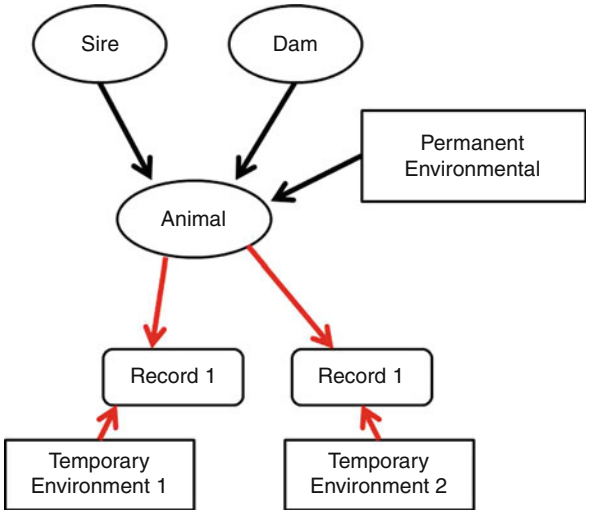
Genetic trends can be computed from the EBVs. Because animals have only one record each, the genetic averages for years 1 and 2 are the average EBVs of animals 5–13 for year 1, and animals 14–20 for year 2. These give −1.10 for year 1 and −0.01 for year 2. Thus, the animals in year 2 were slightly better than in year 1, genetically.

Repeated Records Animal Model

Animals are often observed more than once for some traits, such as

- Fleece weight of sheep in different years
- Calf records of a beef cow over time
- Test day records within a lactation for a dairy cow
- Litter size of sows over time
- Antler size of deer in different seasons
- Racing results of horses from several races

Animals are influenced by their environments, such as an athlete changes due to training and practice. These effects are called permanent environmental effects, and they accompany the animal every time the animal is observed for that trait as in Fig. 2. Permanent environmental effects are not genetic, in the sense that the animal does not transmit these effects to any of its



Animal Breeding, Modeling in. Figure 2
Diagram for an animal with repeated records including a permanent environmental effect

offspring. The following figure illustrates a model to describe permanent environmental effects. Only with repeated records can the permanent environmental and genetic effects be separated and estimated.

Data

In the example data of Table 2, animals have either one, two, or three records made in different years. The contemporaries for each year differ, but are confounded with the year effect in this example. If the animals could be assigned to different herds or management groups, then contemporary groups and years would not be confounded.

Normally performance of animals either improves or declines with the age of the animal, so that ages of animals should be known. The age of the animal in year 1 is given in the table, and so ages range from 1 to 4 years.

Mixed Model Equations

The equation of the model is (in scalar form)

$$y_{ijkl} = (YR)_i + (\text{Age})_j + a_k + p_k + e_{ijkl},$$

where

y_{ijkl} is observation l on animal k, belonging to age group j, born in year i.

Animal Breeding, Modeling in. Table 2 Example data for repeated records animal model

Animal	Sire	Dam	Age	Year 1	Year 2	Year 3
			Yr 1			
1			3	22	35	
2			3	31	46	
3			3	44	24	
4			3	53		
5			2	61	57	42
6			2	32		39
7	1	2	2	39	51	62
8	3	4	1	48	72	
9	5	6	1	71		96
10	1	4	1	37	56	47
11	3	6	1	66		86
12	1	2	1		46	38

$(YR)_i$ are fixed, year effects.
$(\text{Age})_j$ are fixed, age group effects.
a_k are random, additive genetic effects of individual animals.
p_k are random, permanent environmental effects of individual animals.
e_{ijk} are random, residual effects.

The covariance matrices of the random variables (animals, permanent environmental, and residual effects, respectively) are

$$\text{Var}\begin{pmatrix}\mathbf{a}\\ \mathbf{p}\\ \mathbf{e}\end{pmatrix} = \begin{pmatrix}\mathbf{A}\sigma_a^2 & 0 & 0\\ 0 & \mathbf{I}\sigma_p^2 & 0\\ 0 & 0 & \mathbf{I}\sigma_e^2\end{pmatrix}.$$

The total variance is

$$\sigma_y^2 = \sigma_a^2 + \sigma_p^2 + \sigma_e^2,$$

and the heritability is

$$h^2 = \frac{\sigma_a^2}{\sigma_y^2}.$$

Repeatability is a measure of the average similarity of repeated records on animals across the population (part genetic and part environmental), and is defined as a ratio of variances as

$$r = \frac{\sigma_a^2 + \sigma_p^2}{\sigma_y^2},$$

which is always going to be greater than or equal to heritability.

Let

Xb represent the fixed factors of years and ages
Za represent the random animal additive genetic effects
Wp represent the random animal permanent environmental effects

then the mixed model equations may be written as

$$\begin{pmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} & \mathbf{X}'\mathbf{W} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \mathbf{A}^{-1}k_a & \mathbf{Z}'\mathbf{W} \\ \mathbf{W}'\mathbf{X} & \mathbf{W}'\mathbf{Z} & \mathbf{W}'\mathbf{W} + \mathbf{I}k_p \end{pmatrix} \begin{pmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}} \\ \hat{\mathbf{p}} \end{pmatrix} = \begin{pmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \\ \mathbf{W}'\mathbf{y} \end{pmatrix},$$

and let

$$k_a = \sigma_e^2 / \sigma_a^2 = 1.33333, \quad \text{and} \quad k_p = \sigma_e^2 / \sigma_p^2 = 3.$$

There are 26 observations in the example of Table 2, with 3 years and 4 age groups represented, plus 12 animal additive genetic effects and 12 permanent environmental effects giving a total of 31 equations. The **A** matrix is simple to construct because none of the animals are inbred. The resulting solutions are given in Table 3.

There are two possible uses of these solutions. First is to rank the animals for their genetic ability in order to plan future matings using the Estimated Breeding Values. Secondly, there is the decision about which animals to keep to make another record. The criterion for making this decision is the Most Probable Producing Ability, [1] which is the sum of the genetic and permanent environmental solutions (shown in the last column of Table 3). Thus, Animal 9 would likely make the best future performance of those 12 animals and would also likely generate the best future progeny.

Animal Breeding, Modeling in. Table 3 A set of solutions to the example data on repeated records model

Years		Ages		Animal			MPPA
				Genetic		Perm. Env.	
1	0.00	1	50.92	1	−8.37	−0.27	8.64
2	18.34	2	46.01	2	−1.50	0.98	−0.52
3	29.08	3	37.24	3	1.06	−1.84	−0.78
		4	19.50	4	3.14	3.16	6.30
				5	4.96	−0.84	4.12
				6	0.71	−5.00	−4.29
				7	−2.94	1.77	−1.17
				8	2.18	0.07	2.25
				9	9.68	6.08	15.76
				10	−6.66	−3.60	−10.26
				11	6.00	4.55	10.55
				12	−10.64	−5.07	−15.71

Comments

One could ask if permanent environmental effects are permanent. The answer is yes, but as an animal ages, it encounters new permanent environmental effects which accumulate with the previous effects. Hence, permanent environmental effects are cumulative over the life of an animal. This means permanent environmental effects are not constant throughout an animal's life. With the model as described in this section, the assumption is that permanent environmental effects are constant. Some of the cumulative parts, therefore, flow into the temporary environmental effects, and some are averaged with the previous permanent environmental effects.

Another assumption is that the genetic component of each record on an animal is the same. Genes are known to change in activity as an animal ages due to age, but also due to epigenetics (environmental effects that cause change to an animal's DNA). Thus, repeated records on one animal could have different genetic components. That means the genetic correlation between records is less than unity. A better model would be to assume that all records are genetically different (but correlated) traits. This would take into account both the different genetic effects associated with each record, and also the accumulation of permanent environmental effects with time.

Maternal Effects Animal Model

In mammalian species of livestock, such as beef cattle, sheep, or swine, the female provides an environment for its offspring to survive and grow in terms of protection and nourishment. Figure 3 illustrates how maternal effects can affect offspring records. Females vary in their ability to provide a suitable environment for their offspring, and this variability has a genetic basis. Offspring directly inherit an ability to grow (or survive) from both parents, and environmentally do better or poorer depending on their dam's genetic maternal ability. Maternal ability is a genetic trait expressed by the dam in the offsprings' performance, and is transmitted, like all genetic traits, from both parents. Maternal ability is only expressed by females when they have offspring (i.e., much like milk yield in dairy cows) [15, 16].

Data

The example data of Table 4 are weights on animals at an early age.

A model to account for maternal ability is

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_1\mathbf{a} + \mathbf{Z}_2\mathbf{m} + \mathbf{Z}_3\mathbf{p} + \mathbf{e},$$

where $\mathbf{y}$ is the growth trait of a young animal, $\mathbf{b}$ is a vector of fixed factors influencing growth, in this case contemporary group effects, $\mathbf{a}$ is a vector of random animal additive genetic effects (i.e., direct genetic effects), $\mathbf{m}$ is a vector of random maternal genetic (dam) effects, and $\mathbf{p}$, in this model, is a vector of maternal permanent environmental effects (because dams may have more than one offspring in the data – repeated records).

The expectations of the random vectors, $\mathbf{a}$, $\mathbf{m}$, $\mathbf{p}$, and $\mathbf{e}$ are all null vectors in a model without selection, and the variance-covariance structure is

$$\mathrm{Var}\begin{pmatrix}\mathbf{a}\\ \mathbf{m}\\ \mathbf{p}\\ \mathbf{e}\end{pmatrix} = \begin{pmatrix}\mathbf{A}\sigma_a^2 & \mathbf{A}\sigma_{am} & 0 & 0\\ \mathbf{A}\sigma_{am} & \mathbf{A}\sigma_m^2 & 0 & 0\\ 0 & 0 & \mathbf{I}\sigma_p^2 & 0\\ 0 & 0 & 0 & \mathbf{I}\sigma_e^2\end{pmatrix},$$

where σ_a^2 is the additive genetic variance, σ_m^2 is the maternal genetic variance, σ_{am} is the covariance between additive and maternal genetic effects, and σ_p^2 is the maternal permanent environmental variance. Also,

$$\begin{pmatrix}\mathbf{a}\\ \mathbf{m}\end{pmatrix}\Big|\ \mathbf{A},\ \mathbf{G}\Big) \sim N\left(\begin{pmatrix}0\\ 0\end{pmatrix},\quad \mathbf{G}\otimes\mathbf{A}\right),$$

where

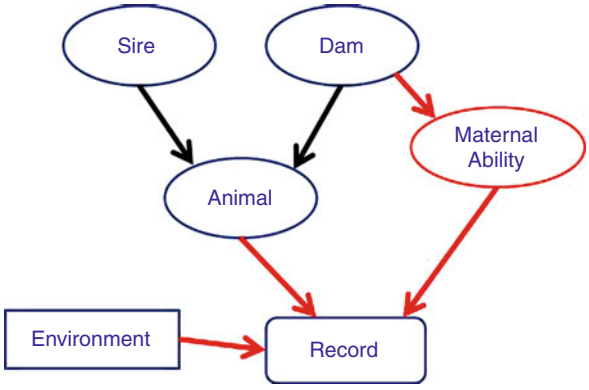
$$\mathbf{G} = \begin{pmatrix}\sigma_a^2 & \sigma_{am}\\ \sigma_{am} & \sigma_m^2\end{pmatrix},$$

and

$$\mathbf{p}|\mathbf{I}, \sigma_p^2 \sim N(0, \mathbf{I}\sigma_p^2),$$

and

$$\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2).$$



Animal Breeding, Modeling in. Figure 3 Diagram illustrating maternal genetic effects

Animal Breeding, Modeling in. Table 4 Example data for maternal genetic effects model

Animal	Sire	Dam	CG	Weight
5	1	3	1	156
6	2	3	1	124
7	1	4	1	135
8	2	4	2	163
9	1	3	2	149
10	2	4	2	138

In this model, a female animal, i, could have its own growth record for estimating $\hat{a}_i$. The same female could later have offspring for estimating $\hat{m}_i$ and $\hat{p}_i$, and the offspring would also contribute toward $\hat{a}_i$. The maternal effects model can be more complicated if, for example, embryo transfer is practiced. Recipient dams would have maternal effects, but would not have direct genetic effects on that calf [17].

Mixed Model Equations

The MME are represented as

$$\begin{pmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z}_1 & \mathbf{X}'\mathbf{Z}_2 & \mathbf{X}'\mathbf{Z}_3 \\ \mathbf{Z}'_1\mathbf{X} & \mathbf{Z}'_1\mathbf{Z}_1 + \mathbf{A}^{-1}k_{11} & \mathbf{Z}'_1\mathbf{Z}_2 + \mathbf{A}^{-1}k_{12} & \mathbf{Z}'_1\mathbf{Z}_3 \\ \mathbf{Z}'_2\mathbf{X} & \mathbf{Z}'_2\mathbf{Z}_1 + \mathbf{A}^{-1}k_{12} & \mathbf{Z}'_2\mathbf{Z}_2 + \mathbf{A}^{-1}k_{22} & \mathbf{Z}'_2\mathbf{Z}_3 \\ \mathbf{Z}'_3\mathbf{X} & \mathbf{Z}'_3\mathbf{Z}_1 & \mathbf{Z}'_3\mathbf{Z}_2 & \mathbf{Z}'_3\mathbf{Z}_3 + \mathbf{I}k_{33} \end{pmatrix} \begin{pmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}} \\ \hat{\mathbf{m}} \\ \hat{\mathbf{p}} \end{pmatrix} = \begin{pmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'_1\mathbf{y} \\ \mathbf{Z}'_2\mathbf{y} \\ \mathbf{Z}'_3\mathbf{y} \end{pmatrix},$$

where

$$\begin{aligned} \begin{pmatrix} k_{11} & k_{12} \\ k_{12} & k_{22} \end{pmatrix} &= \begin{pmatrix} \sigma_a^2 & \sigma_{am} \\ \sigma_{am} & \sigma_m^2 \end{pmatrix}^{-1} \sigma_e^2, \\ &= \begin{pmatrix} 49 & -7 \\ -7 & 26 \end{pmatrix}^{-1} (81), \\ &= \begin{pmatrix} 1.7192 & 0.4628 \\ 0.4628 & 3.2400 \end{pmatrix}. \end{aligned}$$

The solutions to the MME are

$$\hat{\mathbf{b}} = \begin{pmatrix} 137.8469 \\ 150.4864 \end{pmatrix}, \quad \hat{\mathbf{p}} = \begin{pmatrix} 0.0658 \\ -0.0658 \end{pmatrix},$$

$$\hat{\mathbf{a}} = \begin{pmatrix} 2.3295 \\ -2.3295 \\ 0.1280 \\ -0.1280 \\ 5.1055 \\ -4.1143 \\ 0.2375 \\ 2.0161 \\ 0.5447 \\ -3.7896 \end{pmatrix}, \quad \text{and} \quad \hat{\mathbf{m}} = \begin{pmatrix} -0.3328 \\ 0.3328 \\ 0.1646 \\ -0.1646 \\ -0.6379 \\ 0.6792 \\ -0.1254 \\ -0.3795 \\ 0.0136 \\ 0.4499 \end{pmatrix}.$$

Comments

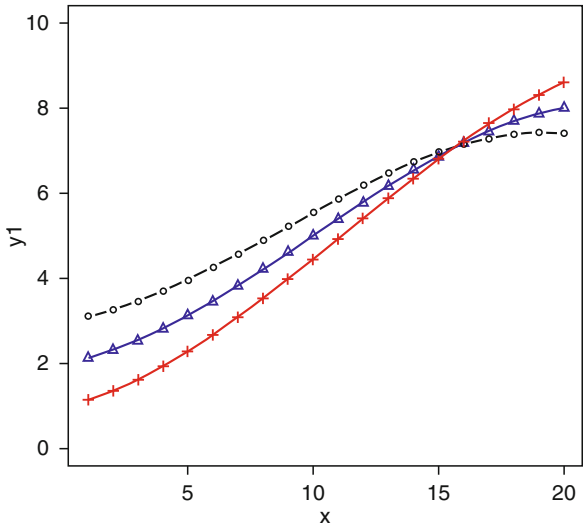
Maternal genetic models require a good data structure to be successful [18]. That means that there should be many females with weight records in the data who also have several progeny with weight records in the data. In this way direct genetic and maternal genetic effects can be efficiently separated during estimation. In the example data, the structure is not good because the females that were dams did not have any weight records on themselves. Hence the strong negative relationship between direct and maternal genetic estimates, which means the maternal genetic estimates are based mostly on the direct genetic estimates and the prior genetic correlation that was assumed, which was negative.

Sire structure is also important in that sires should have many daughters that have also had their own progeny. The maternal genetic ability of a sire's daughters cannot be accurately estimated without those daughters displaying their maternal ability on their own progeny. In many studies or application of maternal genetic effects models, the data structure is too poor from which to estimate variances and covariances of direct and maternal genetic effects.

Random Regression Animal Model

All biological creatures grow and perform over their lifetime. Traits that are measured at various times during that life are known as *longitudinal* data. Examples are body weights [19], body lengths, milk production [20], feed intake, fat deposition, and egg production [21]. On a biological basis, there could be different genes that turn on or turn off as an animal ages causing changes in physiology and performance. The time variable (or age) can be recorded in years, months, weeks, days, hours, minutes, or seconds, so that, in effect, there could be a continuum or continuous range of points in time when an animal could be observed for a trait. These traits have also been called *infinitely dimensional* traits.

If observations were plotted on a graph where the x-axis is time and the y-axis is the magnitude of the observations, then a *trajectory* is obtained for a group of animals. However, not every individual will follow



Animal Breeding, Modeling in. Figure 4
Trajectories of different animals for a trait measured over time

the average trajectory, see Fig. 4. There are variations in the shape of their trajectory, and the breeder may wish to change the shape for animals in his(her) production system in order to be more profitable.

The average trajectory, in black, is given by the equation

$$f(t_i) = A + Bt_i + Ct_i^2 + Dt_i^3,$$

where $A = 2$, $B = 0.1$, $C = 0.03$, and $D = -0.001$. The red and blue trajectories are extremes for animals in the population. The assumption is that $f(t_i)$ is the average trajectory and every animal will have its own trajectory, that means every animal will have different A, B, C, and D values. Because these parameters are regression coefficients, the model becomes the *random regression* model, and every animal will have four regression coefficients to be estimated, as deviations from the average trajectory regression coefficients.

Regression Functions

A problem in using time covariates to various powers is that the numbers can become very large, very quickly. For example, if the time variable, t, goes from 1 to 20, then t^3 would range from 1 to 8,000. In least squares like equations, the diagonal element for that variable would be $(8,000)^2$. Then there could be many thousands of observations. The large numbers can lead to serious rounding errors and may cause problems in solving the equations.

Another problem is that there will be high correlations among the time variables in the function, because they are all based on the same t value. This may also lead to near singularity and to problems in solving the equations. A solution for the above problems is to use Legendre polynomials which convert the time variable, t, into covariates that are firstly scaled to be between -1 and $+1$, and then converted to be independent of each other.

Scaling Time Variables Time variables have to be standardized to the interval between -1 and $+1$. The formula to standardize t_ℓ is

$$x_\ell = -1 + 2\left(\frac{t_\ell - t_{\min}}{t_{\max} - t_{\min}}\right).$$

Legendre Polynomials The first two Legendre polynomials are defined as

$$P_0(x) = 1, \quad \text{and}$$
$$P_1(x) = x,$$

then, in general, the $n+1$ polynomial is described by the following recursive equation:

$$P_{n+1}(x) = \frac{1}{n+1}((2n+1)xP_n(x) - nP_{n-1}(x)).$$

These quantities are "normalized" using

$$\phi_n(x) = \left(\frac{2n+1}{2}\right)^{0.5} P_n(x).$$

This gives the following series:

$$\phi_0(x) = \left(\frac{1}{2}\right)^{0.5} P_0(x) = 0.7071$$
$$\phi_1(x) = \left(\frac{3}{2}\right)^{0.5} P_1(x)$$
$$= 1.2247x$$
$$P_2(x) = \frac{1}{2}(3xP_1(x) - 1P_0(x))$$
$$\phi_2(x) = \left(\frac{5}{2}\right)^{0.5} (\frac{3}{2}x^2 - \frac{1}{2})$$
$$= -0.7906 + 2.3717x^2,$$

and so on. The first six can be put into a matrix, Λ, as

$$\Lambda' = \begin{pmatrix} 0.7071 & 0 & 0 \\ 0 & 1.2247 & 0 \\ -0.7906 & 0 & 2.3717 \\ 0 & -2.8062 & 0 \\ 0.7955 & 0 & -7.9550 \\ 0 & 4.3973 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 4.6771 & 0 & 0 \\ 0 & 9.2808 & 0 \\ -20.5206 & 0 & 18.4685 \end{pmatrix}.$$

Now define a vector, **m**, containing the polynomials of standardized time values,

$$\mathbf{m}' = \begin{pmatrix} 1 & x & x^2 & x^3 & x^4 & x^5 \end{pmatrix}.$$

The covariates to use in the model are equal to

$$\Lambda'\mathbf{m}.$$

To illustrate, suppose time goes from 10 to 60 days over which animals are observed for their growth, $t_{\min} = 10$ and $t_{\max} = 60$. An animal is observed on day 43. The standardized time variable is

$$x = -1 + 2\frac{(43 - 10)}{(60 - 10)} = -0.32,$$

and

$$\mathbf{m}' = \begin{pmatrix} 1 & -0.32 & 0.1024 & -0.0328 & 0.01048 \\ & -0.00336 \end{pmatrix}.$$

Finally,

$$\Lambda'\mathbf{m} = \begin{pmatrix} 0.7071 \\ -0.3919 \\ -0.5477 \\ 0.7446 \\ 0.0782 \\ -0.7961 \end{pmatrix}.$$

The order of Legendre polynomials is equal to the highest power of x. Research is needed to determine the best order of fit for any given situation.

Random Regression Model

Like other animal models, the random regression model (RRM) accounts for years, contemporary groups, and animal additive genetic effects, but in addition, it needs to account for the following:

- Curves for different groups of animals, such as age groups, month of calving groups, and breeds which could have different shapes of curves. These factors would be fixed effects in the model. The curves may be fit using Legendre polynomials, or other functions of time, or as classification variables with many levels.
- Curves for each individual animal, using Legendre polynomials, which are random factors in the model. Each animal has a number of regression coefficients to be estimated. For each animal, there are additive genetic parameters as well as permanent environmental parameters.
- The possibility of residual variances changing over the observable time period. For example, as animals grow, their mean weight increases and so does the variance of weights at a given age.

A simplified RRM for a single trait can be written as

$$y_{ijkn:t} = F_i + g(t)_j + r(a, x, m1)_k + r(pe, x, m2)_k + e_{ijkn:t},$$

where

$y_{ijkn:t}$ is the nth observation on the kth animal at time t belonging to the ith fixed factor and the jth group.

F_i is a fixed effect that is independent of the time scale for the observations, such as a cage effect, a location effect, or a herd-test date effect.

$g(t)_j$ is a function or functions that account for the phenotypic trajectory of the average observations across all animals belonging to the jth group.

$r(a, x, m1)_k = \sum_{\ell=0}^{m_1} a_{k\ell} x_{ijk:\ell}$ is the notation adopted for a random regression function. In this case, a denotes the additive genetic effects of the kth animal, x is the vector of time covariates, and $m1$ is the order of the regression function. So that $x_{ijk:\ell}$ are the covariables related to time t, and $a_{k\ell}$ are the animal additive genetic regression coefficients to be estimated.

$r(pe, x, m2)_k = \sum_{\ell=0}^{m_2} p_{k\ell} x_{ijk:\ell}$ is a similar random regression function for the permanent environmental (pe) effects of the kth animal.

$e_{ijkn:t}$ is a random residual effect with mean null and with possibly different variances for each t or functions of t.

The function, $g(t)_j$, can be either linear or nonlinear in t. Such a function is necessary in a RRM to account for the phenotypic relationship between y and the time covariables (or other types of covariables that could be used in a RRM). In a test day model, $g(t)_j$ accounts for different lactation curve shapes for groups of animals defined by years of birth, parity number, and age and season of calving within parities, for example. With growth data, $g(t)_j$ accounts for the growth curve of males or females of breed X or breed Y from young or old dams.

If the shape of the phenotypic relationship is not known or is nonlinear, then $g(t)_j$ could be a set of classification variables. Classification variables take up more degrees of freedom and require a large number of observations per level, but they do not force the user to explicitly define the shape of the trajectory. A mathematical function, on the other hand, does not use many degrees of freedom and gives a smooth trajectory over time regardless of the number of observations. The choice of classification variables or mathematical function is up to the researcher. If data are very numerous, and the mathematical function fits the data well, then either approach will generally lead to the same results. The phenotypic relationships, $g(t)_j$, are important to a RRM analysis and deserve care and effort in their correct specification.

Mixed Model Equations In matrix notation, the RRM is

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_1\mathbf{a} + \mathbf{Z}_2\mathbf{p} + \mathbf{e},$$

where $\mathbf{b}$ contains F_i and $g(t)_j$ effects, $\mathbf{a}$ contains $m_1 + 1$ additive genetic regression coefficients for each animal, $\mathbf{p}$ contains $m_2 + 1$ permanent environmental regression coefficients for each animal with data, and $\mathbf{e}$ contains the temporary environmental effects. Also,

$$\mathrm{Var}\begin{pmatrix}\mathbf{a}\\ \mathbf{p}\\ \mathbf{e}\end{pmatrix} = \begin{pmatrix}\mathbf{A}\otimes\mathbf{G} & 0 & 0\\ 0 & \mathbf{I}\otimes\mathbf{P} & 0\\ 0 & 0 & \mathbf{R}\end{pmatrix},$$

where $\mathbf{G}$ is the variance-covariance matrix of the additive genetic random regression coefficients of order $m_1 + 1$; $\otimes$ if the direct product operator which multiplies every element of $\mathbf{A}$ by the matrix $\mathbf{G}$; $\mathbf{P}$ is the variance-covariance matrix of the permanent environmental random regression coefficients of order $m_2 + 1$; and $\mathbf{R}$ is a diagonal matrix of temporary environmental variances which could vary depending on t, or $\mathbf{R}$ could be block diagonal with an autocorrelation structure for

Animal Breeding, Modeling in. Table 5 Example data for random regression model

Cow	Sire	Dam	Visit 1		Visit 2		Visit 3		Visit 4	
			Age(m)	Obs.	Age(m)	Obs.	Age(m)	Obs.	Age(m)	Obs.
1	7	5	22	224	34	236	47	239		
2	7	6	30	244	42	247	55	241	66	244
3	8	5	28	224	40	242				
4	8	1			20	220	33	234	44	228

each animal's records. The mixed model equations (MME) are represented as

$$\begin{pmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z}_1 & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z}_2 \\ \mathbf{Z}_1'\mathbf{R}^{-1}\mathbf{X} & \mathbf{Z}_1'\mathbf{R}^{-1}\mathbf{Z}_1 + \mathbf{A}^{-1}\otimes\mathbf{G}^{-1} & \mathbf{Z}_1'\mathbf{R}^{-1}\mathbf{Z}_2 \\ \mathbf{Z}_2'\mathbf{R}^{-1}\mathbf{X} & \mathbf{Z}_2'\mathbf{R}^{-1}\mathbf{Z}_1 & \mathbf{Z}_2'\mathbf{R}^{-1}\mathbf{Z}_2 + \mathbf{I}\otimes\mathbf{P}^{-1} \end{pmatrix} \begin{pmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}} \\ \hat{\mathbf{p}} \end{pmatrix} = \begin{pmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}_1'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}_2'\mathbf{R}^{-1}\mathbf{y} \end{pmatrix}.$$

Example Data Analysis by RRM

A very simplified example is given below to illustrate the degree of complexity of RRM. Four animals were observed multiple times at different ages for a trait, as shown in Table 5.

The model equation might be

$$\begin{aligned} y_{jik:t} = {} & V_j + b_0 + b_1(A) + b_2(A)^2 \\ & + (a_{i0}z_0 + a_{i1}z_1 + a_{i2}z_2) \\ & + (p_{i0}z_0 + p_{i1}z_1 + p_{i2}z_2) + e_{jik:t} \end{aligned}$$

where

V_j is a random contemporary group (visit) effect which is assumed to follow a normal distribution with mean 0 and variance, $\sigma_c^2 = 4$.

b_0, b_1, and b_2 are fixed regression coefficients on $(A) =$ age and age squared which describes the general relationship between age and the observations.

a_{i0}, a_{i1}, and a_{i2} are random regression coefficients for animal i additive genetic effects, assumed to follow a multivariate normal distribution with mean vector null and variance-covariance matrix, **G**.

p_{i0}, p_{i1}, and p_{i2} are random regression coefficients for animal i permanent environmental effects, assumed to follow a multivariate normal distribution with mean vector null and variance-covariance matrix, **P**.

z_0, z_1, and z_2 are the Legendre polynomials based on standardized ages and derived as indicated earlier. The minimum age was set at 18 and the maximum age was set at 68 for calculating the Legendre polynomials.

and e_{jik} is a temporary residual error term assumed to follow a normal distribution with mean 0 and variance, $\sigma_e^2 = 9$. In this example, the residual variance is assumed to be constant across ages.

The model in matrix notation is

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Wv} + \mathbf{Za} + \mathbf{Zp} + \mathbf{e},$$

where

$$\mathbf{X} = \begin{pmatrix} 1 & 22 & 484 \\ 1 & 30 & 900 \\ 1 & 28 & 784 \\ 1 & 34 & 1156 \\ 1 & 42 & 1764 \\ 1 & 40 & 1600 \\ 1 & 20 & 400 \\ 1 & 47 & 2209 \\ 1 & 55 & 3025 \\ 1 & 33 & 1089 \\ 1 & 66 & 4356 \\ 1 & 44 & 1936 \end{pmatrix}, \quad \mathbf{y} = \begin{pmatrix} 224 \\ 244 \\ 224 \\ 236 \\ 247 \\ 242 \\ 220 \\ 239 \\ 241 \\ 234 \\ 244 \\ 228 \end{pmatrix},$$

$$\mathbf{W} = \begin{pmatrix} 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 1 \end{pmatrix},$$

and for animal 1,

$$\mathbf{Z} = \begin{pmatrix} 0.7071 & -1.0288 & 0.8829 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0.7071 & -0.4409 & -0.4832 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0.7071 & 0.1960 & -0.7299 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}$$

In order to reduce rounding errors, the covariates of age for the fixed regressions can be forced to have

a mean of approximately zero by subtracting 38 from all ages and 1642 from all ages squared.

The entire MME cannot be presented, but parts of the MME are given below.

$$\mathbf{W}'\mathbf{W} = \begin{pmatrix} 3 & 0 & 0 & 0 \\ 0 & 4 & 0 & 0 \\ 0 & 0 & 3 & 0 \\ 0 & 0 & 0 & 2 \end{pmatrix},$$

$$\mathbf{W}'\mathbf{X} = \begin{pmatrix} 3 & -34 & -2,758 \\ 4 & -16 & -1,648 \\ 3 & 21 & 1,397 \\ 2 & 34 & 3,008 \end{pmatrix},$$

$$\mathbf{X}'\mathbf{X} = \begin{pmatrix} 12 & 5 & -1 \\ 5 & 1,995 & 166,883 \\ -1 & 166,883 & 14,415,319 \end{pmatrix},$$

$\mathbf{Z}'\mathbf{Z}$ is composed of the following four blocks of order 3, for the four animals with records;

$$\text{Animal1} \quad \begin{pmatrix} 1.5 & -0.9006 & -0.2335 \\ -0.9006 & 1.2912 & -0.8383 \\ -0.2335 & -0.8383 & 1.5457 \end{pmatrix},$$

$$\text{Animal2} \quad \begin{pmatrix} 2 & 0.7275 & 0.0259 \\ 0.7275 & 2.0233 & 1.3612 \\ 0.0259 & 1.3612 & 2.1815 \end{pmatrix},$$

$$\text{Animal3} \quad \begin{pmatrix} 1 & -0.6235 & -0.4902 \\ -0.6235 & 0.5615 & 0.0648 \\ -0.4902 & 0.0648 & 0.5761 \end{pmatrix},$$

$$\text{Animal4} \quad \begin{pmatrix} 1.5 & -1.1085 & 0.0134 \\ -1.1085 & 1.5121 & -1.2082 \\ 0.0134 & -1.2082 & 2.2687 \end{pmatrix}.$$

and $\mathbf{Z}'\mathbf{X}$ for all animal is

$$\mathbf{Z}'\mathbf{X} = \begin{pmatrix} 2.12 & -7.78 & -761.55 \\ -1.27 & 19.99 & 1516.76 \\ -.33 & -18.76 & -1201.42 \\ 2.83 & 28.99 & 2458.59 \\ 1.03 & 46.44 & 4337.80 \\ 0.04 & 27.97 & 2979.60 \\ 1.41 & -5.66 & -636.39 \\ -.88 & 7.05 & 636.63 \\ -.69 & -2.14 & -22.46 \\ 2.12 & -12.02 & -1061.36 \\ -1.57 & 23.03 & 1684.81 \\ .02 & -24.57 & -1515.25 \end{pmatrix}.$$

The right hand sides of the MME are

$$\mathbf{X}'\mathbf{y} = \begin{pmatrix} 2,823 \\ 2,070 \\ 68,064 \end{pmatrix},$$

$$\mathbf{W}'\mathbf{y} = \begin{pmatrix} 692 \\ 945 \\ 714 \\ 472 \end{pmatrix},$$

and

$$\mathbf{Z}'\mathbf{y} = \begin{pmatrix} 494.2629 \\ -287.6596 \\ -90.7117 \\ 690.1296 \\ 249.1165 \\ 7.3023 \\ 329.5086 \\ -200.1692 \\ -168.8920 \\ 482.2422 \\ -351.3606 \\ -7.8918 \end{pmatrix}.$$

The variance-covariance matrices of the additive and permanent environmental effects need to be known for BLUP. Normally, these are not well known and must be estimated simultaneously with the other effects of the model. Let

$$\mathbf{G} = \begin{pmatrix} 94.0000 & -3.8500 & 0.03098 \\ -3.8500 & 1.5000 & -0.0144 \\ 0.03098 & -0.0144 & 0.0014 \end{pmatrix},$$

and

$$\mathbf{P} = \begin{pmatrix} 63.0000 & -2.1263 & 0.0447 \\ -2.1263 & 0.5058 & -0.00486 \\ 0.0447 & -0.00486 & 0.0005 \end{pmatrix}.$$

The solutions to MME are

$$\hat{\mathbf{b}}' = \begin{pmatrix} 234.9797 & 1.4670 & -0.01399 \end{pmatrix},$$

$$\hat{\mathbf{c}}' = \begin{pmatrix} -0.8630 \\ 1.2885 \\ 0.1443 \\ -0.5698 \end{pmatrix}.$$

Let the solutions for the animal additive genetic random regression coefficients be presented in tabular form as follows.

Animal	a_0	a_1	a_2
1	−2.021529	0.175532	−0.002696
2	5.751601	−2.139115	0.025848
3	−2.474456	2.554412	−0.029269
4	−5.376687	−0.370873	0.002174
5	−1.886714	1.464975	−0.016963
6	3.333268	−1.065525	0.013047
7	1.503398	−1.081654	0.012555
8	−2.948511	0.681643	−0.008633

Similarly, the solutions for the animal permanent environmental random regression coefficients can be given in tabular form.

Animal	p_0	p_1	p_2
1	−0.296786	0.246946	−0.002521
2	3.968256	−730659	0.009430
3	−0.834765	0.925329	−0.008164
4	−4.505439	−441805	0.001257

Ranking Animals The problem is to rank the animals for selection purposes. If animals are ranked on the basis of a_0, then animal 2 would be the highest (if that was desirable). If ranked on the basis of a_1, then animal 3 would be the highest, and if ranked on the basis of a_2, then animal 2 would be the highest. To properly rank the animals, an EBV at different ages could be calculated, and then these could be combined with appropriate economic weights. EBVs were calculated for 24, 36, and 48 mo of age, and economic weights of 2, 1, and 0.5, respectively, for the three EBVs were used to compute a Total Economic Value (TEV), as

$$2 * \mathrm{EBV}(24) + 1 * \mathrm{EBV}(36) + .5 * \mathrm{EBV}(48).$$

The Legendre polynomials for ages 24, 36, and 48 mo are given in the rows of the following matrix **L**,

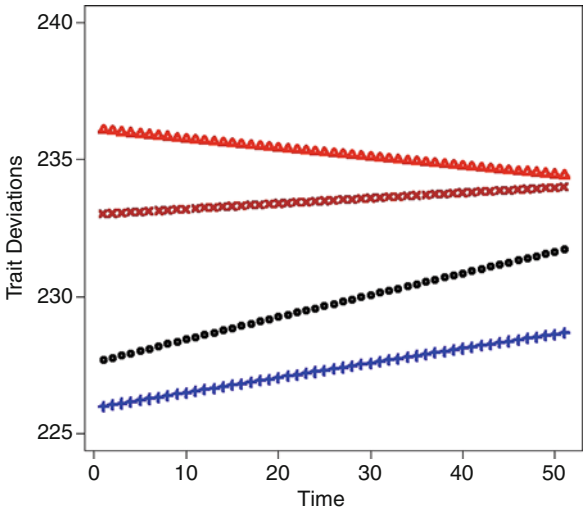
$$\mathbf{L} = \begin{pmatrix} 0.7071 & -0.8328 & 0.3061 \\ 0.7071 & -0.3429 & -0.6046 \\ 0.7071 & 0.2449 & -0.6957 \end{pmatrix}.$$

The results are shown in the following table.

Animal	EBV(24)	EBV(36)	EBV(48)	TEV
1	−1.58	−1.49	−1.38	−5.33
2	5.86	4.78	3.53	18.26
3	−3.89	−2.61	−1.10	−10.93
4	−3.49	−3.68	−3.89	−12.61
5	−2.56	−1.83	−96	−7.43
6	3.25	2.71	2.09	10.25
7	1.97	1.43	.79	5.76
8	−2.66	−2.31	−1.91	−8.58

The animal with the highest TEV was animal 2. All animals ranked rather similarly at each age on their EBVs. Rankings of animals could change with age. Thus, the pattern of growth could be changed.

Plotting Curves The shapes of curves of individual animals could also be plotted from the solutions, and shown as deviations from the average curves. Figure 5 contains animals 1 (black), 2 (red), 4 (blue), and 6 (brown).



Animal Breeding, Modeling in. Figure 5
Curves for animals 1 (*black*), 2 (*red*), 4 (*blue*), and 6 (*brown*), in the example data

Animal 2 had the highest TEV, but that ranking was achieved by Animal 2 having the highest initial deviation. However, the curve declines very rapidly after time 18. The curves of the other animals do not decline as rapidly, and the curve for Animal 1 actually increases slightly. Depending on the biology of the trait and the welfare of the animal, one may decide to select for animals with increasing curves rather than declining curves. The weights in the TEV could be adjusted accordingly.

Comments

The orders of the Legendre polynomials do not need to be equal for both the animal additive genetic and animal permanent environmental effects. The order of the fixed regressions could also be different, and as seen in this example can be based on a different function of time covariates.

There are other kinds of orthogonal functions that could be used in place of Legendre polynomials, and some of these have been tried without much benefit to the analyses (Yazdi?). Another alternative has been spline (split polynomial) functions, [19, 22] in which a curve is broken down into sections. Within each section, a simple linear or quadratic function is sufficient to fit the data within that section. The sections are joined together by "knots," the locations of which need to be estimated. Spline functions have gained some popularity lately.

Because random regression models were new to animal breeding in 1994, [20] they were applied to many different types of research problems. RRM have been most successful in the analysis of dairy cattle test day production within lactations, and in growth of pigs, sheep, rainbow trout, and beef cattle. One limitation with growth data is that animals are not usually weighed more than three or four times in their life due to the amount of labor involved in collecting weights and the stress induced on the animals during the weighing process. The orders of the regression coefficients for growth traits are usually limited to 2 or 3.

Multiple Trait Models

Animals are observed for many traits relating to production, reproduction, conformation, longevity or fitness, and health such that knowing the total economic merit of an animal helps to keep costs of production to a minimum. Most traits are genetically correlated to each other, meaning that some genes affect more than one trait. Because contemporary animals make their records in the same environment, environmental correlations due to management, feed, and temperature also exist to affect observations on all traits. Thus, a sensible approach is to analyze groups of traits using *multiple trait models* [23–26]. In this way, information from correlated traits can be used to improve the accuracy of all trait evaluations. A multiple trait (MT) model is one in which two or more traits are analyzed simultaneously in order to take advantage of genetic and environmental correlations between traits.

Low Heritability Traits: MT models are useful for traits where the differences between genetic and residual correlations are large (e.g., greater than 0.5 difference) or where one trait has a much higher heritability than the other traits. EBVs for traits with low heritability tend to gain more in accuracy than EBVs for traits with high heritability, although all traits benefit to some degree from the simultaneous analysis(24).

Culling: Another use of MT models is for traits that occur at different times in the life of the animal such that culling of animals results in fewer observations on animals for traits that occur later in life compared to those at the start. Consequently, animals which have observations later in life tend to have been selected based on their performance for earlier traits. Thus, analysis of later life traits by themselves could suffer from the effects of culling bias, and the resulting EBV could lead to errors in selecting future parents. MT analyses have been shown to partially account, to some degree, for the selection that has taken place [25, 26]. If the percentage of missing trait observations is high and the missing observations are not due to random chance, then biases could be very large in EBVs for that trait and maybe others.

The success of an MT analysis relies on the accuracy of the genetic and residual correlations that are assumed. Computations for MT models are more complicated than for single trait analyses. If m is the number of traits and N is the number of animals in the pedigree file, then there are at least mN equations to be

solved rather than just N, and the same relative difference in memory space to hold solutions. However, computer hardware advances (with gigabytes of memory) have been very rapid in the last decade. Consequently, many genetic evaluation systems are now multiple trait systems.

Models and Traits

Usually, traits are grouped together for MT analyses because they are observed at approximately the same point in time. For example, milk, fat, and protein yields of dairy animals are observed on the same day, per animal. Thus, for a group of similar traits, the linear models associated with those traits include the same major factors like years, ages, contemporary groups, and animal additive genetic effects. Another grouping of traits would be those for reproductive traits, observed primarily on females, such as conception rate, litter size, birthing ease, and offspring losses at birth. The linear models used for production traits are quite different from those used for reproductive traits, or health traits.

Even within a group, the linear models for the traits within a group could be different from each other. Consider two traits with a single observation per trait on animals. Let the model equation for trait 1 be

$$y_{1ij} = B_{1i} + a_{1j} + e_{1ij},$$

where B_{1i} is a fixed effect with p_B levels, a_{1j} is a random, animal additive genetic effect for trait 1, and e_{1ij} is a random residual environmental effect for trait 1.

The model equation for trait 2 might be

$$y_{2ij} = C_{2i} + a_{2j} + e_{2ij},$$

where C_{2i} is a fixed effect (different from B_{1i} for trait 1) with p_C levels, a_{2j} is a random, animal additive genetic effect for trait 2, and e_{2ij} is a random residual environmental effect for trait 2.

For example, y_{1ij} could be birth weight, so that B_{1i} could identify animals born in the same season. Trait 2 could be yearling weights and C_{2i} could identify contemporary groups of animals of the same sex, same herd, and same rearing unit within herd.

Because the two traits will be analyzed simultaneously, variances and covariances need to be specified for the traits together. For example, the additive genetic variance-covariance (VCV) matrix (between traits) could be written as

$$\mathbf{G} = \begin{pmatrix} g_{11} & g_{12} \\ g_{12} & g_{22} \end{pmatrix} = \begin{pmatrix} 1 & 2 \\ 2 & 15 \end{pmatrix},$$

and the residual environmental VCV matrix as

$$\mathbf{E} = \begin{pmatrix} e_{11} & e_{12} \\ e_{12} & e_{22} \end{pmatrix} = \begin{pmatrix} 10 & 5 \\ 5 & 100 \end{pmatrix}.$$

The genetic and residual correlations are, respectively,

$$\rho_g = 2/(15)^{0.5} = 0.516,$$
$$\rho_e = 5/(1000)^{0.5} = 0.158$$

with heritabilities specified as

$$h_1^2 = \frac{1}{11} = 0.0909,$$

and

$$h_2^2 = \frac{15}{115} = 0.1304.$$

For all data, using $\mathbf{A}$ as the additive numerator relationship matrix, then

$$\mathrm{Var}\begin{pmatrix} \mathbf{a}_1 \\ \mathbf{a}_2 \end{pmatrix} = \begin{pmatrix} \mathbf{A}g_{11} & \mathbf{A}g_{12} \\ \mathbf{A}g_{12} & \mathbf{A}g_{22} \end{pmatrix}.$$

Data Example

A two trait example with three factors is given in Table 6. Note that animals whose trait 1 observation was below 3.0 were not allowed to make a trait 2 observation. If the true variances and covariances are known, then this selection bias will be lessened through the multiple trait analysis, as long as the analysis includes the trait 1 records of animals with missing trait 2 observations.

There were two levels of factor B associated with trait 1, and 3 levels of factor C associated with trait 2. The models assumed are those given in the previous section including the covariance matrices that were given.

Animal Breeding, Modeling in. Table 6 Example data for multiple trait models

Animal	Sire	Dam	B-level	C-level	Trait 1	Trait 2
1	0	0	1	1	2.3	
2	0	0	1	2	2.6	
3	0	0	1	3	9.8	53
4	0	0	1	1	4.7	4
5	0	0	1	2	5.5	63
6	1	3	2	3	2.5	
7	1	4	2	2	8.4	35
8	1	5	2	3	8.2	41
9	2	3	2	1	9.0	27
10	2	4	2	1	7.8	32
11	2	5	2	2	2.8	
12	6	10	2	3	7.4	67

Mixed Model Equations

The models are written in matrix notation below.

$$\begin{pmatrix} \mathbf{y}_1 \\ \mathbf{y}_2 \end{pmatrix} = \begin{pmatrix} \mathbf{X}_1 & \mathbf{0} \\ \mathbf{0} & \mathbf{X}_2 \end{pmatrix} \begin{pmatrix} \mathbf{b}_1 \\ \mathbf{b}_2 \end{pmatrix} + \begin{pmatrix} \mathbf{Z}_1 & \mathbf{0} \\ \mathbf{0} & \mathbf{Z}_2 \end{pmatrix} \begin{pmatrix} \mathbf{a}_1 \\ \mathbf{a}_2 \end{pmatrix} + \begin{pmatrix} \mathbf{e}_1 \\ \mathbf{e}_2 \end{pmatrix}$$

Let $\mathbf{R}$ be the covariance matrix of residual effects. If observations are ordered by traits within animals, then $\mathbf{R}$ is the direct sum of submatrices, one for each animal. The submatrices depend on which traits are observed for a given animal. If an animal has been observed for both (all) traits, then the diagonal block is $\mathbf{E}$ as given earlier. If an animal has been observed only for trait 1, then the block is

$$\mathbf{E}_1 = \begin{pmatrix} e_{11} & 0 \\ 0 & 0 \end{pmatrix},$$

and if the animal has been observed only for trait 2, then

$$\mathbf{E}_2 = \begin{pmatrix} 0 & 0 \\ 0 & e_{22} \end{pmatrix}.$$

With more than two traits, the number of combinations of missing traits increases. The covariance matrix of residual effects for the example data is a block diagonal matrix,

$$\mathbf{R} = diag(\mathbf{E}_1\ \mathbf{E}_1\ \mathbf{E}\ \mathbf{E}\ \mathbf{E}\ \mathbf{E}_1\ \mathbf{E}\ \mathbf{E}\ \mathbf{E}\ \mathbf{E}\ \mathbf{E}_1\ \mathbf{E}).$$

The inverse of $\mathbf{R}$ is needed for the mixed model equations, and this can be obtained by inverting the block diagonal matrices (ignoring the zero rows and columns when there are missing observations).

The resulting mixed model equations were of order 29 by 29. The solutions, for this example, were

$$B_{11} = 5.0209$$
$$B_{12} = 6.5592$$
$$C_{21} = 20.0882$$
$$C_{22} = 49.0575$$
$$C_{23} = 51.9553$$

Animal	Sire	Dam	Trait 1	Trait 2
1	0	0	−0.3573	−1.6772
2	0	0	−0.0730	1.0418
3	0	0	0.4105	1.1707
4	0	0	−0.0449	−1.4922
5	0	0	0.0646	0.9570
6	1	3	−0.1033	−1,410
7	1	4	−0.1975	−2.2983
8	1	5	−0.1410	−9,633
9	2	3	0.3079	1.6227
10	2	4	0.1426	1.1273
11	2	5	−0.1830	0.6418
12	6	10	0.1554	1.5089

Notice that every animal has an EBV for both traits, even though trait 2 was missing for some animals. The EBV for an animal with a missing trait observation is constructed based on the genetic and environmental correlations between traits, and based on their genetic relationships to other animals that were observed for both traits.

Economic Indexes

Many producers are so overwhelmed by the numerous EBV available for so many traits that they are unable to

visualize the relative contributions of different traits to their overall productivity. Thus, economic indexes were developed where trait EBVs are scaled to unit variances, and then relative economic values are applied to the scaled EBVs. Sometimes different types of indexes are derived depending on the market objectives of the producers. A beef producer, for example, may be producing animals of high carcass merit, and would use an index that puts more weight on carcass traits of marbling score, tenderness, and dressing percentage. Another producer may be producing calves and may want more weight on the reproductive ability of cows and calf survival from birth to weaning. A particular breed may wish to be known as the "Have Everything" breed and would utilize a different set of economic weights where many traits are emphasized.

Economic weights are dynamic in that they change over time, and can often change very dramatically and quickly, usually more quickly than a breeding program can be changed due to the long generation intervals of the species involved. Producers must therefore continually reassess their goals and objectives.

Economic indexes based on multiple trait EBVs tend to be more stable over time compared to the same EBVs computed on each trait separately.

Models for Categorical Data

Categorical data arise in animal breeding in situations where a trait is subjectively scored by the producer or a trained individual. For example, calves can be born with absolutely no assistance of the producer or to the other extreme where the producer must ask for veterinarian assistance to perform a caesarian section to deliver the calf and save the cow. Between those two extremes are different levels of difficulty of calving. Recording programs provide four or five categories of difficulty, and the producer must decide to which category a calving belongs. The assumption is that one producer assigns all calvings based on the same criteria, but a different producer may have slightly different criteria.

The number of calvings falling into each category depends on the subjectivity of the producers. In most breeds, the category for unassisted or easy births is usually very high (from 50% to 90% of all calvings), while the other categories are often much smaller and the category for caesarian births being the smallest. The categories are ordered from one extreme to the other.

In dairy cattle, cows are scored for 30 or more conformation traits (i.e., style points) by trained judges, and each trait has 9 or more categories. Judges are trained and updated annually, but judges can differ in their abilities to score traits, particularly when they are trained to score a cow in a few minutes.

Theory

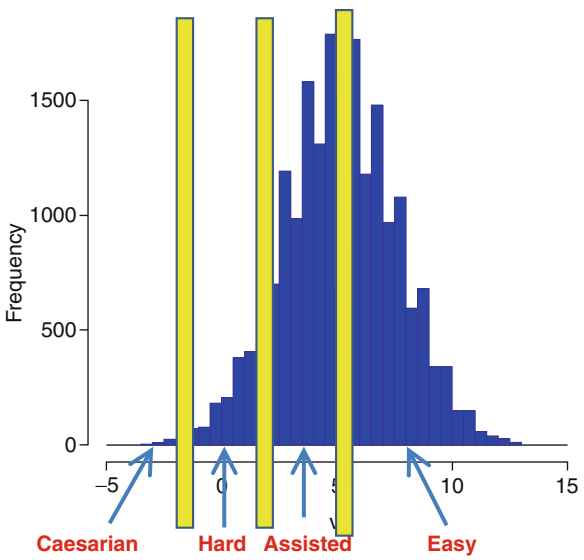
Although a trait may be scored into one of a limited number of categories, an underlying non-observable, normally distributed trait could be hypothesized [27–29]. Then thresholds along the scale are where the categories of the observed scores are defined, as shown in Fig. 6.

Thus, when an animal's underlying scale trait exceeds a threshold value, then it belongs to the next higher category. The threshold model is ideal for analyzing categorical data. The analysis follows Bayesian concepts and is nonlinear in the solutions to this model.

Write a model for the underlying scale variable.

$$\ell_{ijkm} = t_i + b_j + a_k + e_{ijkm},$$

where ℓ_{ijkm} is an unobserved value on the underlying scale for the trait of interest; t_i is one of the threshold



Animal Breeding, Modeling in. Figure 6
Normal distribution with thresholds for calving ease

points; b_j is a fixed factor such as age of the dam, breed, year, or season; a_k is an animal additive genetic effect; and e_{ijkm} is a residual error effect. The model could be much more complex than that described here, depending on the trait and situation.

Note that, instead of observations on the underlying scale, only the category to which an animal belongs is known. There are various quantities which need to be computed repeatedly in the analysis, and these are based on normal distribution functions.

1. $\Phi(x)$ is known as the cumulative distribution function of the normal distribution. This function gives the area under the normal curve up to the value of x, for x going from minus infinity to plus infinity (the range for the normal distribution). For example, if $x = 0.4568$, then $\Phi(x) = 0.6761$, or if $x = -0.4568$, then $\Phi(x) = 0.3239$. Note that if there are m categories and $k = m$, then $\Phi_k = 1$.
2. $\phi(x)$ is a function that gives the height of the normal curve at the value x, for a normal distribution with mean zero and variance 1. That is,

$$\phi(x) = (2\pi)^{-0.5} \exp -0.5x^2.$$

For example, if $x = 1.0929$, then $\phi(x) = 0.21955$.

3. $P(k)$ is the probability that x from a $N(0, 1)$ distribution is between two threshold points, or is in category k. That is,

$$P(k) = \Phi_k - \Phi_{k-1}.$$

If $k = 1$, then $\Phi_{k-1} = 0$.

Begin with phenotypic values for t_i based on a normal distribution. From the t_i, observations on the underlying scale can be "created." Each one would have a different weight in the analysis due to the frequency of a category being observed. Then new values of b_j and a_k are calculated. Finally, new values of t_i are determined, and the process is repeated. Eventually, the process converges until "solutions" for all variables do not change. This is an overly simplified explanation. For more details, see [30].

Solutions

Using the estimates from the nonlinear system of equations, the probability of a animal's offspring falling into each category can be calculated. Let $\hat{a}_k = 0.123$, and let there be a 3 category trait. The solutions for the two thresholds were $\hat{t}_1 = 0.376$ and $\hat{t}_2 = 1.012$. Then the value on the underlying scale for the first category would be

$$\begin{aligned} x &= \hat{t}_1 + \hat{a}_k, \\ &= 0.376 + 0.123, \\ &= 0.499. \end{aligned}$$

Then

$$\Phi(x) = \Phi(0.499) = 0.691.$$

Similarly, the probability of the animal's offspring to be in categories 1 or 2 would be based on the second threshold,

$$x = 1.012 + 0.123 = 1.135,$$

or $\Phi(x) = 0.872$. Thus, the proportion of offspring that would fall in category 2 would be $0.872 - 0.691 = 0.181$. The proportion that would be in category 3 would be $1.0 - 0.872 = 0.128$.

Animals could be ranked on their $\hat{a}$, or the result could be expressed as a probability of being in a particular category. For a trait like calving ease, for example, one might want to maximize the probability of having an easy birth.

Comments

While a threshold model is a theoretically best approach to the analysis of categorical data, research has found small differences in accuracy of ranking animals from procedures that treat the category numbers as any continuously distributed trait [31]. As the number of categories increases, the differences between a threshold model analysis and simple linear model analysis become smaller.

Computational problems may arise with threshold models due to small numbers of observations in one or more categories. This often causes two categories to be merged into one to bypass the problem.

Categorical traits are often standardized to a normal scale, and then analyzed with a usual linear model [32]. If the threshold values on a normal scale were 0.376 and 1.012 for a three category trait, then all observations in category 1 would receive a score of $(0.376 - 0)/2 + 0 = 0.188$. Category 2 observations would be scored $(1.012 - 0.376)/2 + 0.376 = 0.694$,

and category 3 would be scored $(3 - 1.012)/2 + 1.012 = 2.006$. Standardization is often conducted within years, or time periods in which the subjective scoring was applied consistently.

Because categorical traits are subjectively recorded, the subjectiveness can fluctuate over time. An animal that was assessed to be category 1 20 years ago, may now (if the animal were alive today at the same age) belong to category 2. The standards of the observer(s) have changed over those 20 years. The change is similar to the change in the value of the US dollar between now and 20 years ago. Today $1 does not buy as much as $1 back in 1990. The consequence of shifting standards (in animal breeding) is that genetic trends cannot be properly estimated. Comparing animals many years apart is not statistically accurate for categorical data. This is not a major problem because producers mainly want to compare animals that are alive today, and for this purpose, the assumption is that subjective standards change rather slowly and not by very much in a short time span of about 5 years.

Methods have also been developed for multiple trait models involving traits that are categorical and other traits that are continuous [33, 34]. These have primarily been cases of binomial traits, such as disease traits (yes or no situations). Binomial traits have only one threshold to be estimated, and binomial traits seem to benefit from a threshold model.

Models for Survival Data

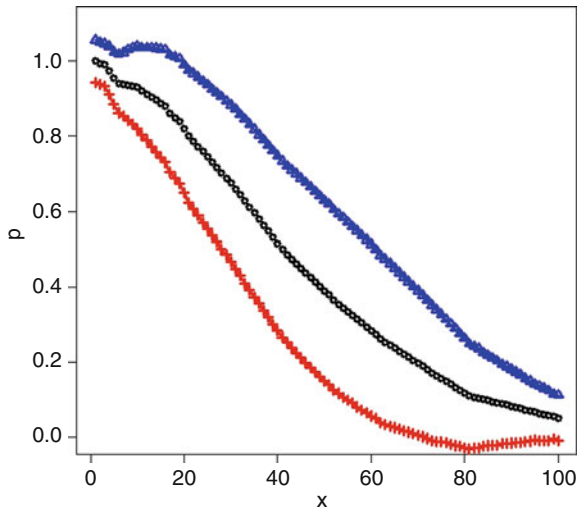
In animal production systems, animals remain in the herd or flock as long as they are productive and generate more income than expenses. Eventually, animals leave the production unit due to natural death or injury, or due to lowered production levels or reproductive problems such that the animal is deemed unprofitable. The latter factors are determined by the owner, and owners differ widely in their management skills, accounting, and decision-making abilities. There seems to be a small amount of genetic variability among animals in survival rates that do not depend on productive abilities, and genetic evaluation systems for survivability or longevity have been developed. The heritability of survival is usually less than 0.02. Dairy sires, for example, need thousands of progeny in order to have an accurate Estimated Breeding Value for survival ability of their progeny.

Survival has been defined in many different ways. In a binary sense, alive is equal to 1, every day until the animal dies and thereafter becomes 0 for the remainder of the observable time period. For example, in dairy cattle, the observable period is from first calving until 100 months later (approximately 10 years of age) when the majority of cows have died. The time intervals in that range can be hours, days, weeks, months, or years. Define a survival vector for the ith cow as $\mathbf{s}_i$, in terms of months (from 1 to 100), such that the values of $\mathbf{s}_i$ are equal to 1 for every month the cow was alive, and equal to 0 for the month in which it dies and every month thereafter to the limit of 100 months after first calving.

A cow at 44 months after first calving today is still alive, and its future death time is unknown, then the values for months 45–100 need to be considered missing or not observed. For a cow that is still alive 100 months after first calving, then $\mathbf{s}_i$ contains all ones. If a cow has gone missing from the data, in the sense that it was sold to another owner and that owner is not on a milk recording program, then the cow has not died, but the exact time of its death is not known. In this case, $\mathbf{s}_i$ will contain 1s up until the time it was sold, and all remaining values in $\mathbf{s}$ are unknown (neither 1 nor 0).

Having defined $\mathbf{s}_i$ for cow i, then let $\mathbf{S}$ be the average of all $\mathbf{s}_i$ for cows that do not have any missing values. The elements of $\mathbf{S}$ are equal to the probabilities of being alive at each month after first calving, and can be plotted as shown in Fig. 7. The value at a given time (month) is a probability, p_t. The black points refer to the average $\mathbf{S}$ for all cows, while the blue and red lines indicate 2 standard deviations above and below the average, respectively. Note that the variation is less around p_1 and p_{100} than in the middle of the range.

Because a curve is involved, a random regression model could be applied [35, 36] such that a separate set of curve parameters could be estimated for each animal. The model would include the year-season of first calving (with separate curves for each year-season), random herd within year-season of first calving, a variable that indicates whether the herd was increasing, decreasing, or being stable in size at a particular instance of time, a regression on the genetic EBV of an animal for production, random animal additive genetic regression coefficients, and random animal permanent environmental regression coefficients. The model would also need to account for different residual



Animal Breeding, Modeling in. Figure 7
Survival curve for Jersey cattle after first calving, in months

variances for each month after first calving. Meuwissen et al. [36] have shown links between various models for survival analyses. Jamrozik et al. [37] did a comparison of the effectiveness of different models for survival.

Another model is the proportional hazard model (PHM) [38],

$$\lambda(t) = \lambda_{0,s}(t)\exp[\mathbf{x}'_m\beta + \mathbf{z}'_m u],$$

where $\lambda(t)$ is the probability of a cow being culled at time t given she was alive before time t; $\lambda_{0,s}(t)$ is the Weibull baseline hazard function; β contains time-dependent covariates affecting the hazard function with $\mathbf{x}'_m$ being the corresponding design vectors; $\mathbf{u}$ is a vector of random factors including herd, year-seasons, and animal additive genetic effects; and $\mathbf{z}'_m$ is the associated incidence vector. Thus, a nonlinear system of equations needs to be solved.

Lastly, survival could be defined as a discrete trait, such as the survival of an animal to the end of first, second, or third lactation. The three "traits" can be analyzed as a multiple trait system, in which cows need not be observed for all traits. This is similar to the random regression approach except the number of months after first calving is reduced to just 3 or 4 broader periods of time instead of monthly.

Because the heritability of survival is so low, and analyses are affected by the animals that are still alive at the time of analysis, perhaps a better indicator of survivability would be a measure of profitability of the animal. The traits that are part of profitability would tend to have higher heritabilities, and would contribute toward an animal remaining in the herd. Further research on this area is needed.

Added Complexities

Heterogeneous Variances

In dairy cattle, the within contemporary group variation of records was deemed to differ between contemporary groups. That implied that the contemporary group effects were being sampled from different populations. Consequently, both the genetic and residual variation could be allowed to differ between contemporary groups. Bayesian methods were developed to simultaneously compute solutions to mixed model equations and to estimate the necessary contemporary group variances. Often, only the phenotypic variances within contemporary groups were assumed to differ such that the heritability was constant across contemporary groups.

Robust Estimation

Related to heterogeneous variances was the problem of outliers, records that were extreme values in the distribution of phenotypes. Sometimes the extremes were deliberately created by producers who thought they knew how to manipulate the data so that their good animals would receive high EBVs. Other times, the extremes were due to errors in recording, and occasionally were naturally extreme. Regardless of the reason for their existence, the result was a bias to EBVs of one animal or an entire group of animals. Robust estimation methods were introduced to pull the extreme records back toward the mean. Either the record itself or the estimated residual effect of the record could be modified toward the mean if it was beyond two and half standard deviations from the mean. Robust methods usually reduce the biases caused by extreme outliers, but the method requires nonlinear estimation.

Count Data

In swine and sheep breeding, interest is in litter size, which is not normally distributed and also not categorical, but is known as *count* data. Another trait is number of services to attain conception, or number of ovulations.

Behavioral traits can also be count data, such as the number of times an animal takes water during the day. The appropriate distribution for count data is a Poisson distribution, and Bayesian methods for handling and modeling this distribution have been developed.

Hierarchical Models

Another Bayesian development was that of hierarchical models where a trait is modeled in the usual way, but then a component of that model has its own separate model. Thus, two models are solved in steps, until the solutions satisfy both models.

Model Comparisons

Methods have also been developed to compare different models, but even further to select from among a group of models for the better fitting model. Model comparisons are usually conducted using an "estimation" data set (with which to estimate variances and solutions to mixed model equations), and a second, sometimes smaller, "validation" data set to judge how well observations and rankings of animals can be predicted.

International Comparisons

Some species of livestock are traded among countries, depending on health restrictions, and this necessitates comparing the genetic abilities of animals between countries. Usually the genetic evaluation of animals within a country has enough complexities to it such that the models used in country A are very different and incompatible with the models in country B. Thus, EBVs from the different countries are collated and a multiple trait model (where each country is a different trait) is applied to the EBVs of male animals. Genetic correlations among countries are less than unity and rankings of animals may differ between countries. The Interbull organization in Uppsala, Sweden routinely computes evaluations for dairy bulls from 23 or more countries every year.

Future Directions

Molecular genetics will dominate animal breeding research in the next 20 years. The discovery of millions of single nucleotide polymorphisms as genetic markers has forced animal breeders to restudy their basic quantitative genetics notes. The DNA genomes of animal species are being completely mapped, and someday soon all of the genes and their locations in the genome will be known. This is an exciting time for animal breeders and the opportunities for research will be huge.

Already DNA markers are being used to determine the relatedness of individuals to each other, to measure the amount of genetic diversity in a species, to identify genes with major effects on production traits, and to select future breeding animals more accurately than previously possible and as soon as an animal is born. Models for genetic evaluation of animals have been modified to include genetic marker information and are thus becoming more complicated, involving many thousands of markers such that computing issues are more demanding than for multiple trait models. The result is that animals are being selected more intensely at an early age, and the samples of progeny are no longer a random group of all possible progeny of a particular mating, but are highly selected. This lack of randomness of sampling progeny may cause biases in current genetic evaluation models. The possibility also exists that rates of inbreeding could be increased. Modeling of production systems and strategies for making continued genetic change will be utilized.

Bayesian methods will likely be utilized more heavily than the methods of Henderson [2] in order to deal with DNA modifications to statistical models. Structural equation models [39] will try to determine the cause and effects of multiple traits. Statistical models, linear and nonlinear, continue to be required background training for animal breeders. The complexity and usefulness of models will grow even with the emergence of genomics.

Bibliography

1. Lush JL (1945) Animal breeding plans. Iowa State University Press, Ames
2. Henderson CR (1984) Applications of linear models in animal breeding. University of Guelph, Ontario
3. Gianola D, Fernando RL (1986) Bayesian methods in animal breeding. J Anim Sci 63:217–244
4. Sorensen D, Gianola D (2002) *Likelihood,* Bayesian and MCMC methods in quantitative genetics. Springer, New York
5. Johannsen WJ (1909) Elemente der Exakten Erblichkeitslehre. Gustav Fischer, Jena
6. Wright SE (1933) Evolution and the genetics of populations, vol I. University of Chicago Press, Chicago

7. Henderson CR (1976) A simple method for computing the inverse of a numerator relationship matrix used for prediction of breeding values. Biometrics 32:69–79
8. Meuwissen THE, Luo Z (1992) Computing inbreeding coefficients in large populations. Genet Sel Evol 24:305–313
9. Quaas RL (1988) Additive genetic model with groups and relationships. J Dairy Sci 71:1338–1345
10. Robinson GK (1986) Group effects and computing strategies for models for estimating breeding values. J Dairy Sci 69:3106–3111
11. Patterson HD, Thompson R (1971) Recovery of interblock information when block sizes are unequal. Biometrika 58:545–554
12. Graser H-U, Smith SP, Tier B (1987) A derivative free approach for estimating variance components in animal models by restricted maximum likelihood. J Anim Sci 64:1362–1370
13. Meyer K, Smith SP (1996) Restricted maximum likelihood estimation for animal models using derivatives of the likelihood. Genet Sel Evol 28:23–49
14. Wang CS, Rutledge JJ, Gianola D (1993) Marginal inferences about variance components in a mixed linear model using Gibbs sampling. Genet Sel Evol 25:41–62
15. Bijma P (2006) Estimating maternal genetic effects in livestock. J Anim Sci 84:800–806
16. Thompson R (1976) The estimation of maternal genetic variances. Biometrics 32:903–918
17. Schaeffer LR, Kennedy BW (1989) Effects of embryo transfer in beef cattle on genetic evaluation methodology. J Anim Sci 67:2536–2543
18. Heydarpour M, Schaeffer LR, Yazdi MH (2008) Influence of population structure on estimates of direct and maternal parameters. J Anim Breed Genet 125:89–99
19. Meyer K (2005) Random regression analyses using B-splines to model growth of Australian Angus cattle. Genet Sel Evol 37:473–500
20. Schaeffer LR, Dekkers JCM (1994) Random regressions in animal models for test-day production in dairy cattle. In: Proceedings of the 5th world congress of genetics applied to livestock production, Guelph, 7–12 Aug 1994, vol 18 p 443
21. Schaeffer LR (2004) Application of random regression models in animal breeding. Livest Prod Sci 86:35–45
22. Misztal I (2006) Properties of random regression models using linear splines. J Anim Breed Genet 123:74–80
23. Henderson CR, Quaas RL (1976) Multiple trait evaluation using relatives' records. J Anim Sci 43:1188–1197
24. Pollak EJ, Quaas RL (1981) Monte Carlo study of within-herd multiple trait evaluation of beef cattle growth traits. J Anim Sci 52:248–256
25. Schaeffer LR (1984) Sire and cow evaluation under multiple trait models. J Dairy Sci 67:1567–1580
26. Pollak EJ, van der Werf J, Quaas RL (1984) Selection bias and multiple trait evaluation. J Dairy Sci 67:1590–1596
27. Gianola D (1982) Theory and analysis of threshold characters. J Anim Sci 54:1079–1096
28. Harville DA, Mee RW (1984) A mixed model procedure for analyzing ordered categorical data. Biometrics 40:393–408
29. Foulley JL, Gianola D (1984) Estimation of genetic merit from bivariate all or none responses. Genet Sel Evol 16:285–306
30. Gianola D, Foulley JL (1983) Sire evaluation for ordered categorical data with a threshold model. Genet Sel Evol 15:201–223
31. Meijering A, Gianola D (1985) Linear versus nonlinear methods of sire evaluation for categorical traits: a simulation study. Genet Sel Evol 17:115–132
32. Snell EJ (1964) A scaling procedure for ordered categorical data. Biometrics 20:592–607
33. Foulley JL, Gianola D (1986) Sire evaluation for multiple binary responses when information is missing on some traits. J Dairy Sci 69:2681–2695
34. Simianer H, Schaeffer LR (1989) Estimation of covariance components between one continuous and one binary trait. Genet Sel Evol 21:303–315
35. Veerkamp RF, Brotherstone S, Engel B, Meuwissen THE (2001) Analysis of censored survival data using random regression models. Anim Sci 72:1–10
36. Meuwissen THE, Veerkamp RF, Engel B, Brotherstone S (2002) Single and multitrait estimates of breeding values for survival using sire and animal models. Anim Sci 75:15–24
37. Jamrozik J, Fatehi J, Schaeffer LR (2008) Comparison of models for genetic evaluation of survival traits in dairy cattle: a simulation study. J Anim Breed Genet 125:75–83
38. Ducrocq VP, Solkner J (1994) "The survival kit", a FORTRAN package for the analysis of survival data. In: Proceedings of the fifth world congress on genetics applied to livestock production, Guelph, 7–12 Aug 1994, vol 22, pp 51–52
39. Wu XL, Heringstad B, Gianola D (2010) Bayesian structural equation models for inferring relationships between phenotypes: a review of methodology, identifiability, and applications. J Anim Breed Genet 127:3–15

Animal Genetic in Environment Interaction

Erling Strandberg
Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala, Sweden

Article Outline

Glossary

Genotype by environment interaction It exists when the difference between the phenotypic values of two genotypes is not the same in two environments.

Plasticity The ability of an individual to respond to changes in the environment.

Reaction norms The phenotypic expression of a genotype as a function of the environment.

Definition of the Subject

The existence of genotype by environment interaction ($G\times E$) makes animal breeding more complicated. It means that the same genotype is not the best in all environments, and it implies that separate breeding programs might be needed to cater for these different environments. However, separate (and therefore, smaller) breeding programs might be less efficient than one large program. Small breeding programs might also encounter problems with inbreeding depression, but on the other hand, several populations with different breeding programs and breeding goals might increase the overall genetic diversity. Therefore, $G\times E$ is an important factor to consider when creating breeding programs for animals, especially in a global setting.

Introduction

The ability to respond to changes in the environment is a vital characteristic of all organisms. This ability is called *phenotypic plasticity* or sometimes, *environmental sensitivity*. Genetic variation in plasticity will lead to *genotype by environment interaction*. This paper starts by describing the phenomenon of plasticity, a term which is not well known in animal breeding. To describe it, several situations will be illustrated, with and without plasticity, by use of reaction norms. This will be followed by a description of various statistical models that can be used to study $G\times E$, including a brief description of genetic heterogeneity of residual variance. Finally, some consequences of $G\times E$ for breeding programs will be discussed.

Plasticity, Environmental Sensitivity, Reaction Norms, and Genotype by Environment Interaction

A *reaction norm* describes the phenotypic expression of a genotype as a function of the environment. One can say that the reaction norm translates the environmental values into phenotypic values. In Fig. 1, the reaction norm for a genotype is shown. The horizontal x-axis describes the environment, for simplicity let's assume a continuous scale, e.g., ambient temperature for a certain organism. The vertical y-axis gives the phenotypic value for this genotype for each environmental value. In this example, the reaction norm is linear, but it could have any form.

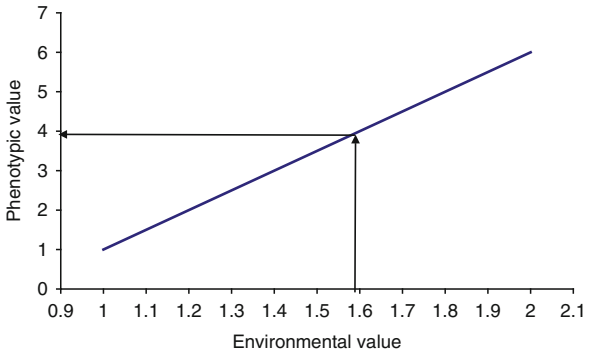
In Fig. 2a, genotypes that show no plasticity are depicted. Note that there is still variation in the level among genotypes, so there is genetic variation in the trait. In Fig. 2b, genotypes showing plasticity are presented. However, all genotypes react in the same way to an environmental change, i.e., there is no variation in plasticity.

In Fig. 2c, the genotypes are plastic, but some are more plastic than others. In other words, there is also variation in plasticity. Now, the genotype with the highest phenotypic value in the low environment also has the highest value in the highest environment. If high phenotypic value is desirable, which genotype to choose would be indisputable.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

In Fig. 2d, there is also variation in plasticity; however, here the reaction norms also cross. Which genotype is the best, now depends on in which environment this genotype should be used.

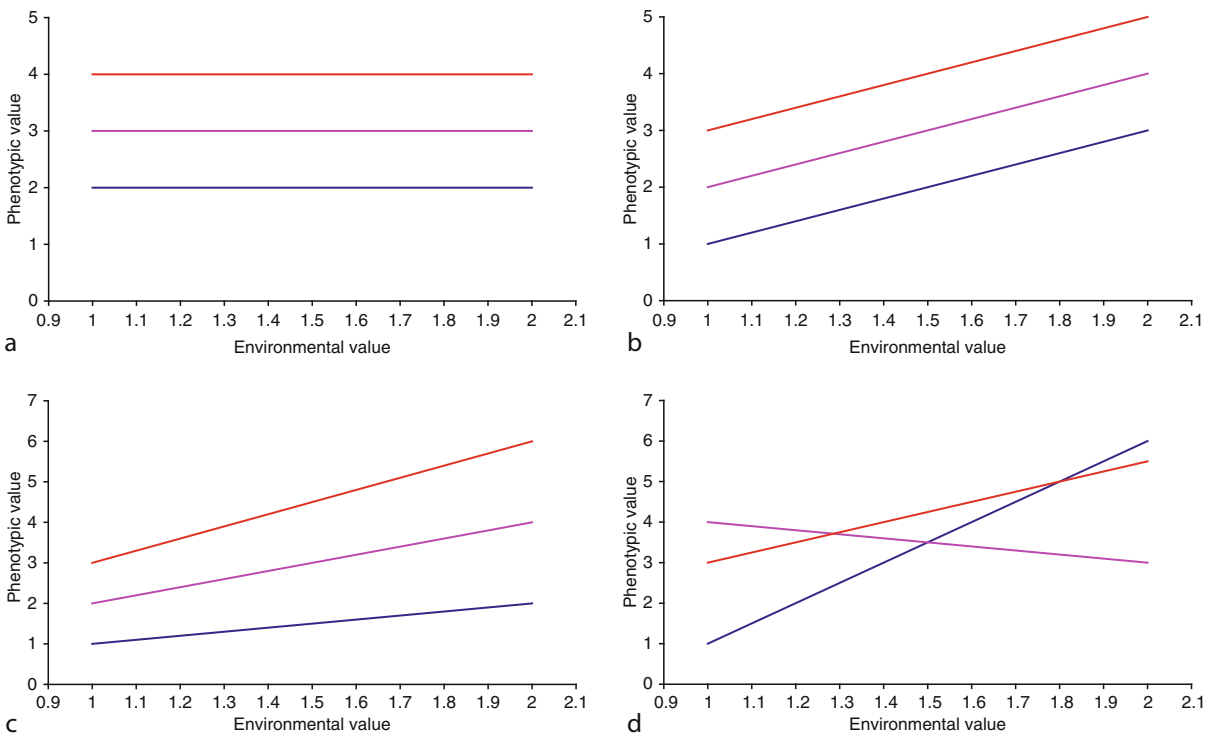


Animal Genetic in Environment Interaction. Figure 1
Schematic description of a reaction norm for a genotype

Thus, differences in phenotypic plasticity between genotypes result in *genotype by environment interaction*. Strictly speaking, $G \times E$ means that the difference between the phenotypic values of two genotypes is not the same in two environments. If the difference changes sign between environments, there is re-ranking of genotypes (Fig. 2d). If the difference changes in size only, there is a scaling effect (Fig. 2c). Note, however, that if the environmental scale had been drawn further to the left in Fig. 2c, there would have been re-ranking also here.

Comparison with the Usual Genetic Model

The most common quantitative genetic model is $P = G + E$ [1]. In this description, G is defined as the *genotypic value* and E as the *environmental deviation*. "We may think of the genotype conferring a certain value on the individual and the environment causing



Animal Genetic in Environment Interaction. Figure 2
Description of the reaction norms for three genotypes. (**a**) No plasticity but genetic variation in level. (**b**) Plasticity but no variation in plasticity. (**c**) Variation in plasticity but no re-ranking of genotypes. (**d**) Variation in plasticity and re-ranking of genotypes

a deviation from this, in one direction or the other" [1]. This might already from the beginning give the impression that G is the central factor, whereas E is just a nuisance, the contribution of which should be decreased as much as possible.

This standpoint actually makes sense from a traditional animal production point of view. The intention is to give all animals as good and standardized treatment as possible in order for them to produce in the best possible way. Therefore, as little as possible should be out of human control (E). Then it can be considered that all animals have the same macroenvironment (same production system, same kind of feed, etc.) and are only affected by microenvironmental effects. To the microenvironmental effects, one might count the effect of the individual farmer (within the rather standardized system), season of production or parturition, etc. These factors can be adjusted for, if known. Thus the environmental or residual variation (σ^2_E) can be decreased. However, there are always some unknown microenvironmental effects (e.g., that the cow is standing close to a door with a cold draft during winter). This will end up in the residual. Therefore, given a certain phenotypic variance, the more that can be adjusted away, the higher the heritability of the trait, and the higher the expected selection response.

Expressed in a graphic way, this traditional model ($P = G + E$) is described in Fig. 2a, the genotypic value only gives a shift in the level of the curve. There may be environmental effects affecting the phenotypic value (as in Fig. 2b), but they affect all individuals in the same way. After adjusting for them (e.g., by a regression), Fig. 2a still applies.

If there are sufficiently different environments (e.g., Northern hemisphere vs. tropical environments), one would still be able to use the traditional model, or rather, two of the traditional models. One would assume that the trait expressed in the tropics is actually a different trait genetically from that in, say, Europe. However, within each environment i, the model $P_i = G_i + E_i$ would still apply.

The difference between this viewpoint and that embodied in the reaction norm and phenotypic plasticity approach is quite substantial, certainly in a conceptual way. In the reaction norm approach, *the environment and the genotype are on equal standing* – there is no way a phenotype can appear without an environment. Philosophically, this makes a lot of sense; a genotype without its environment is nothing, or at least not a phenotype. Certainly, the environment is not something to be "adjusted away" [2, 3].

Staying on the rather philosophical level, it might even be somewhat hard to define what "the environment" is. Obviously, the external environment will qualify (temperature, climate, amount and quality of food etc.). However, the internal environment within an organism is also an environment for the genome. If Richard Dawkins were asked, the whole organism (except the genome) could be considered as the environment (for the genes) [4]. In animal breeding, these fine distinctions are usually disregarded; it is difficult enough to define the external environment!

As stated earlier, the $P = G + E$ model can be said to make sense from an animal (and plant) breeding perspective, at least given certain conditions. It is clear why this model was developed within this setting. The same applies to the reaction norm model and the developments related to phenotypic plasticity. These models were developed mainly within evolutionary biology and genetics, where the focus is on organisms living under natural conditions. Here, there is no way to standardize the environment – the environment is what it is and it is up to the organism to adapt (or die). Populations may adapt (to a changed or variable environment) by changing genetically, i.e., individuals with higher fitness give rise to more offspring that in turn survive better and so on. After some generations, the population has a different genetic constitution (gene frequencies have changed), which is better suited to this environment, and fitness is higher than before [2].

However, changing genetically is not an option for a given individual. So, what an organism really needs is plasticity! If it can change its phenotype – at least somewhat – it will have a better chance of surviving. Now, it is not necessarily so, that even though plasticity would be good for the individual, that it would occur: there has been a lot of discussing in evolutionary genetics literature about when plasticity is expected to develop in natural populations, but that will not be covered here [2, 5]. Students of natural populations and evolutionary genetics from very early on discovered that *there is phenotypic plasticity* in all organisms studied, at least for some traits. Therefore, it is a reality, and it should be modeled somehow.

Canalization, Homeostasis, and Plasticity

There are some terms that have been used in the evolutionary literature that need some explanation because they are sometimes referred to as the opposite of plasticity [2, 5].

Canalization means that a genotype can *buffer against small changes in the environment and in its own genotype* (mutations). The buffering means that even if small changes occur, the resulting phenotype is more or less the same. These terms are most commonly used for developmental traits, e.g., even if the environment is somewhat variable developmental plan is still followed and the resulting body, say, is still the same. It is, for instance, canalization that makes sure that cows have four legs whether they are born in Sweden or in Africa. In developmental genetics, if the environment has an effect on the phenotype, it is usually an either-or effect, i.e., one type or another, and not a gradual effect.

Homeostasis is the outcome of canalization. Homeostasis measures the degree of (in)variance in the phenotype when the individual is perturbed by changes either in the environment or in the genome (by mutation). A more canalized genotype has higher homeostasis (is changed less) and shows lower variance. In his definition of canalization, Waddington [6] defined it for *minor* variations in conditions, what might be called microenvironmental variation. As an example of canalization, he used the environmentally triggered metamorphosis of axolotls (salamanders)! They produce one of two distinct phenotypes; which one is defined by a large change in environment. Once the developmental pathway is chosen, however, small variations in environment do not affect the outcome, and within each phenotypic outcome, there is canalization.

Models to Describe Genotype by Environment Interaction

There are basically three different models to describe the extent of $G\times E$. For all methods, observations on the same or related individuals in two or more different environments are needed to study $G\times E$. In some organism (e.g., some plants), it is possible to use clones (i.e., numerous copies of the same genotype) and put them in different environments. With animals, that is generally not possible. However, the common use of artificial insemination in, e.g., dairy cattle makes it possible to compare the performance of daughters of the same sires in different environments [7].

In the following, the three methods will be described, not with the intent of understanding how to estimate $G\times E$ using these models (which is relatively straightforward), but more to understand the interpretation of these models, and the type of $G\times E$ that is detected.

Interaction Term Model

The traditional genetic model (e.g., as in [1]) is usually written as: $P = G + E$, where the phenotype P is made up of a genotypic value and an environmental deviation (a residual term) (The mean is either assumed to be included in G or that P is expressed as a deviation from the overall mean). This model corresponds to Fig. 2a, where the effect of the genotype is just to shift the level by a certain amount, regardless of the environment. In this terminology, the genotype by interaction is often simply written as $P = G + E + G\times E$. This terminology is incorrect and confusing: it doesn't make sense to have an interaction with the residual.

The model can be rewritten slightly such that the phenotypic value of an individual is described as the sum of the genotypic value, an environmental value, and a residual:

$$P = G + E + e \tag{1}$$

The environmental value E could, e.g., be classification into herds, herd production classes, production systems or countries. When interaction between genotype and environment exists an interaction component, $G\times E$, is added to the equation:

$$P = G + E + G \times E + e \tag{2}$$

The phenotypic variance (σ^2_P) of the observed phenotypes (P) can be derived from Eq. 2 as:

$$\sigma^2_P = \sigma^2_G + \sigma^2_E + \sigma^2_{GE} + \sigma^2_e \tag{3}$$

assuming all covariances being zero, and that all main effects are random (E might normally be considered as fixed, having only few levels).

If this model is compared to the graphs in Fig. 2, it is seen that in neither Fig. 2a nor 2b would this model detect any $G\times E$ (correctly so). The phenotype in Fig. 2b can be exactly described by the sum of the

genotypic values and an environmental value, which is the same for all genotypes. However, in both Fig. 2c and 2d, a nonzero $G\times E$ term would show up, simply as a deviation from main effects model. Whether the $G\times E$ would give rise to re-ranking or not is not directly seen from the size of the $G\times E$ term (σ^2_{GE}), however. One would have to add $G + E + G\times E$ for each genotype and check whether there is much re-ranking for the environments chosen.

From this model, one could define the amount of $G\times E$ as a "heritability" of plasticity as σ^2_{GE}/σ^2_P. This measure is similar to a heritability in that it is a variance ratio; however, it is not useful to predict response to selection for plasticity in the same way as the "additive," narrow sense heritability [2, 8–10].

There is no specific limitation to the number of environments that can be defined in the term E in Eq. 2, e.g., this type of model has been used to estimate sire by herd interactions.

Multiple-Trait Model

The second method used to describe $G\times E$ is based on phenotypic values in different environments and genetic correlations (r_g) between these. The phenotypic expression in the two environments is seen as two separate traits and r_g can be studied to see whether $G\times E$ exists. When r_g between the phenotypic values of the same genotype expressed in different environments is high, the phenotypic expression is considered as the same trait in the different environments. In other words, if r_g between the phenotypic expressions of the trait in two different environments is close to 1, there is no $G\times E$. When r_g is low, the phenotypic expressions in the different environments are not the same trait and this is an indication of $G\times E$ [1, 11].

The genetic correlation (r_g) can be estimated using a multiple-trait analysis based on grouping herds with similar production environments to clusters and treating the observations from the different clusters as separate traits.

By just considering the genetic correlation between two environments, the $G\times E$ of interest is that type which gives rise to re-ranking (Fig. 2d). However, one could also use the estimates of genetic variances from the multiple-trait analysis to describe the kind of $G\times E$ that only gives rise to a scaling effect (Fig. 2c). The genetic correlation is not affected by scaling if the scaling is purely multiplicative.

This method can be used even if the environments cannot be ordered according to some meaningful scale. However, if there exists a continuous underlying scale and the environments chosen are just some representations of that scale (e.g., herd production levels: low, medium, and high), this approach can be modified to describe, by a covariance function, an infinite number of separate traits over a continuous gradient. The covariance function model was developed to model, e.g., growth trajectories, morphology, and reaction norms [12, 13]. Briefly, this method is based on applying a function to the estimated (co)variance components from a limited number of traits. Using this function, one can predict the variance for any environment (normally within the range studied in the data) and also covariances between two environments. Further developments make it possible to estimate the covariance function directly without first using a MT approach (review in Gilmour and Thompson [14]).

Reaction Norm Model

When the production environment can be described as a continuous variable, a third method, called the reaction norm model, is possible to use [15]. The definition of the reaction norm (RN) has already been given: the phenotypic expression of a genotype as a function of the environment [16].

In population and evolutionary genetics, this model has often been statistically analyzed using a fixed regression approach as genotypes have been placed in the different environments. However, in animal breeding, predicting breeding values as random effects is a common practice, and therefore it is natural to estimate the parameters of the RNs for each genotype from a random regression approach [17]. A simple model based on ordinary polynomials with a fixed set of regression coefficients (an average RN) and a random set for each individual is:

$$y = \mu + \sum_{f=1}^{nf-1} \beta_f x^f + \sum_{i=0}^{ni-1} b_{im} x^i + e \qquad (4)$$

where x is the environmental value for the phenotypic value y, μ is the overall mean (fixed intercept), β_f are fixed regression coefficients, and b_{im} are random

regression coefficients for animal (or sire), m: b_{0m} is the BV for level, b_{1m} is the BV for slope, etc. $G\times E$ is defined by variation in linear and higher terms.

If the (co)variance matrix for the random coefficients is defined as $\mathbf{G}$ (order $n_i \times n_i$), then the genetic covariance between environments x_j and x_k can be written as $\mathbf{x}'_j\mathbf{G}\mathbf{x}_k$, where $\mathbf{x}_j$ is a column vector with elements $\{x_j^i\}$ for $i = 0\ldots n_i\text{-}1$. For the linear RN, the vector $\mathbf{x}_j = [1\ x_j]'$. The genetic correlation between environments j and k is $\mathbf{x}'_j\mathbf{G}\mathbf{x}_k/\sqrt{\mathbf{x}'_j\mathbf{G}\mathbf{x}_j\ \mathbf{x}'_k\mathbf{G}\mathbf{x}_k}$ and the genetic variance in each environment is $\mathbf{x}'_j\mathbf{G}\mathbf{x}_j$. These can then be used to describe the re-ranking and scaling $G\times E$. The heritability in each environment can be estimated as $h^2 = \mathbf{x}'_j\mathbf{G}\mathbf{x}_j/(\mathbf{x}'_j\mathbf{G}\mathbf{x}_j + \sigma_e^2)$, where σ_e^2 is the residual variance from Eq. 4.

Using phenotypic average as environmental scale. In the ideal situation, the environmental scale is determined by the researcher, e.g., by subjecting the animal to various temperature, light, or nutrient conditions, and the trait measured is some phenotypic character. Although this situation may occur also in animal breeding situations, more often the environmental scale is directly related to the phenotypic values studied. The simplest example is probably the use of herd (or herd-year) average milk yield as the environmental scale when analyzing the phenotype milk yield [18, 19].

The herd average is most likely a combination of many factors, but it may give a good overall description of the type of environment the cow is exposed to, and the herd average is a practically useful description of the environment. However, the same trait occurs as both dependent and independent variable. To avoid a direct relation, the individual's own phenotypic value could be excluded from the herd average used for that individual. But a further problem is that the phenotypic herd average contains also the genetic component of those animals' phenotypes and is not only a measure of the environment. If the genetic material is not used randomly over herds, one would expect some bias to be introduced. One suggestion is to use an iterative procedure, either in a repeated REML [20] or a Gibbs sampling approach [21], in a model where the fixed regression in Eq. 4 is replaced with a herd effect. Simply expressed, this effect is estimated, used as the x-value, estimated again, and so on. It has been shown that this method gives more unbiased estimates of $\mathbf{G}$ than using the phenotypic herd averages. The latter approach results in more uncertainty in the x-values, which gives an underestimation of variance of the slopes of the reaction norms [21].

Group size. The group size used as basis for the calculation of the environmental values is also of importance. Naturally, a large group size is desirable to get as precise estimate of the environment as possible. Imprecise values for the x-values in a regression analysis is expected to lead to lower regression coefficients, which in this situation means lower variance of slopes and $G\times E$ may be underestimated [20]. However, increasing the group size (e.g., using herd average over several years rather than herd-year average) may also mean that the same environment is not measured anymore, leading to the same problem as above. These two factors must be balanced in a pragmatic way.

Improved environmental scales. There has been some work on defining environmental scales that are not directly dependent on the trait analyzed. Climate and weather conditions (temperature, humidity, rainfall), herd-year SD of production, replacement rate, persistency of lactation curves, calving patterns, and other measures of management practices have been studied [22–26]. This work should continue, and factor or principal component analysis might be of help in defining few but distinct environmental scales [e.g., 27]. Improved farm and animal data (e.g., management practices, feeding system, and intensity) would be of great value in this respect.

Heterogeneous residual variance. In model (4), it was assumed that the residual variance was constant over all environments. For many traits and environmental scales, this is not reasonable. For a sire model, it makes even less sense. By including the random regression, it is assumed that ¼ of the genetic variance is dependent on the environment, but the remaining ¾ (included in the residual) are not. A rough description of the residual variance structure can be found by saving the residuals from fitting model (4), dividing them into groups along the environmental scale, and calculate the residual variance within each group. If this indicates heterogeneous variance, it should be accounted for [e.g., 28].

Multiple environmental scales. A given phenotypic trait may be influenced by several environmental scales. One can accommodate this by extending model (4) to:

$$y = \mu + \sum_{i=1}^{ni-1} \beta_i x^i + \sum_{i=0}^{ni-1} b_{im} x^i + \sum_{j=1}^{nj-1} c_{jm} z^i + e \qquad (5)$$

In a linear reaction norm, there is one intercept, and one slope for each environmental scale, x and z. The genetic covariance and correlation between environments can now be depicted as a function of both environmental scales, i.e., as three-dimensional graph [18, 22].

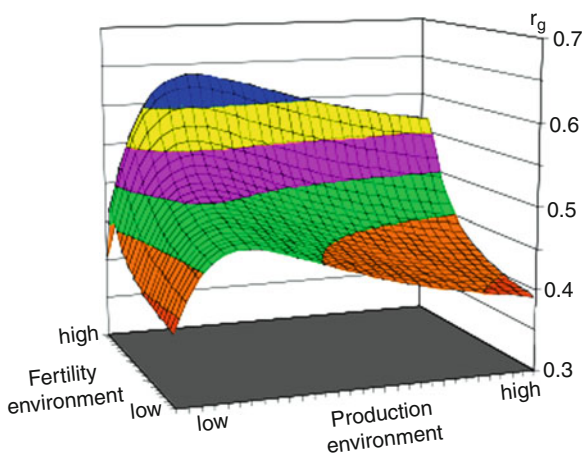
Multiple-trait reaction norms. Reaction norm models, as other models, can be analyzed for more than one trait at a time. The **G**-matrix would then contain off-diagonal sub-matrices pertaining to the covariance between the traits, and the diagonal sub-matrices would have the meaning as before. As an example, in Fig. 3, the genetic correlation between the traits protein yield and fertility (days open) is shown [18]. It can be seen that the genetic correlation changes with the environment. Note that traits could share the same environmental scale but also have specific scales. That a certain trait shows $G\times E$ with respect to a certain environmental scale does not mean that all traits will do so.

Type of reaction norm function. In the example given, ordinary polynomials were used with the origin set to the average environment. It is also recommendable to standardize the environmental scale(s); this makes (co)variance parameters easier to interpret. Another common approach is to use Legendre polynomials. These often make convergence easier than ordinary polynomials. The resulting regression coefficients are only defined within a predetermined interval (usually from minimum to maximum environmental value, redefined from −1 to +1). This means that the level is defined in the middle of this interval, which is not necessarily the average environment. For other purposes, splines have been successfully used. Regardless of the approach used, it is vital to describe exactly what was done, otherwise parameter estimates may be difficult to interpret.

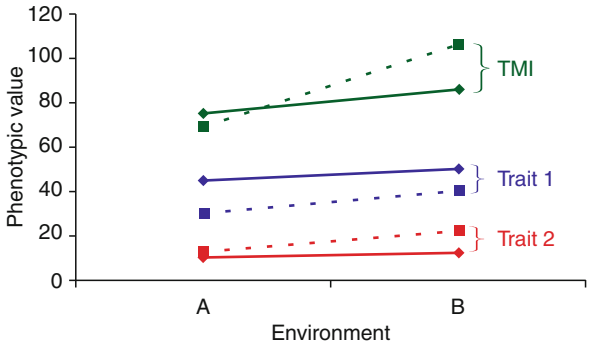
The shape of the reaction norm and the shape of the variance function are strongly connected. For a linear reaction norm, the variance function becomes $\sigma_a^2 + 2\sigma_{a,b1}x + \sigma_{b1}^2 x^2$. Because the term in front of the quadratic term is always positive, the variance curve will always be concave with an intermediate low point. In a certain data range, it may be increasing or decreasing as the minimum may be outside the range. However, it will never have a maximum intermediate point. This is a rather limiting feature of the linear reaction norm approach. With higher polynomial terms, the variance function becomes less constrained. The covariance function or character process approaches in a way work in the other direction and estimate the shape of the covariance function first. The shape of that function could be an indicator of what shape the reaction norms should be allowed to have. In most applications, only linear RNs have been found; however, there are exceptions where also higher-order RNs have been found [29–31].

Scaling can give re-ranking. It can be shown that even if there is only the scaling type of $G\times E$ for the traits, there can be re-ranking in the total merit index. A simple example is given in Fig. 4. Therefore, scaling $G\times E$ should not be considered irrelevant until the total evaluation has been done [32]; however, generally re-ranking $G\times E$ for traits is expected to be much more important.

The indication of $G\times E$ is that there is variation in the coefficients of the reaction norms. For linear reaction norms, this means that there is variation in the slopes. This definition would pick up plasticity of the kind shown in Fig. 2c–d. As for the interaction term



Animal Genetic in Environment Interaction. Figure 3
Genetic correlation between protein production and fertility (days open) in dairy cattle, in relation to environmental conditions (Data from Kolmodin et al. [22])



Animal Genetic in Environment Interaction. Figure 4
Breeding values for two individuals for two traits and total merit index (TMI) in two environments. There is scaling $G\times E$ for both traits, but re-ranking $G\times E$ for the total merit index (TMI = 1 × trait 1 + 3 × trait 2)

model, this variation does not directly tell us how much re-ranking there is: in order to find out, one could calculate the predicted performance in various environments and get the correlation (or rank correlation) between these values.

Genetic Heterogeneity of Residual Variation

The discussion so far has been related to macro-environmental sensitivity, i.e. the reaction of genotypes to large and known changes in the environment. However, as already mentioned, there is always unknown microenvironmental variation, variation that cannot be adjusted away or analyzed with any of the above methods, because it is not associated with any known factor. If there is genetic variation in how animals react to this variation, then there is genetic heterogeneity of residual variation (GHRV).

There are several different models possible to analyze GHRV, well summarized in Mulder [33]. The simplest additive model draws the residual not from a distribution with a constant variance but where it also depends on an additive breeding value, A_v. Thus, each individual has two breeding values, one (usual) breeding value for the mean (A_m) and one for the residual variance (A_v) [34]. One perhaps slight drawback with this model is that A_v is drawn from a normal distribution, and the sum of the average residual variance and A_v could become negative. The exponential model overcomes this problem: here the residual distribution is an exponential function of both the average residual variance and A_v, thus ensuring positive values [35]. Yet another option is to envision the microenvironmental sensitivity to be a special case of the reaction norm model, assuming an unknown environmental *x*-variable.

Selection for decreased heterogeneity is of interest for traits where large variation is undesirable. This might be true for, e.g., carcass traits where uniformity is favorable for the slaughtering process, but in general for traits with an intermediate optimum. When the mean performance is close to the optimum, more of the selection pressure will move toward reducing the residual variance [36]. Selecting for reduced residual variation might also be a way to select for more robust animals, animals that can cope with unknown changes in the environment [33]. It would also be interesting to study the relationship between macro- and microenvironmental sensitivity.

Consequences for Breeding Programs

The first obvious consequence of re-ranking $G\times E$ (Fig. 2d) is of course that if you select individuals to become parents based on information from one environment and plan to use them in another environment, you have partially selected the wrong animals. One example of this could be a nucleus herd with very good environment but where the production animals are used under less optimal commercial conditions. The equally obvious solution is to include information from the production environment into the genetic evaluation. Mulder and Bijma [37] showed that the loss in genetic gain (compared with if there was no $G\times E$) was lowest when progenies of males were tested in the production environment, and that this system was better than testing of sibs in that environment, and both systems were superior to testing only in the selection environment.

Another question is whether two breeding programs should cooperate even if their breeding goals differ to a certain extent. This could apply to two countries or to different production systems, e.g., organic and conventional production. It has been shown that long-term cooperation was beneficial if the correlation between breeding goals were higher than 0.8–0.9. However, initial cooperation was beneficial even when the correlation was as low as 0.4–0.6, but after some generations,

the two populations had diverged so much that selection was practiced only within population. Furthermore, small breeding programs benefited from long-term cooperation at values of genetic correlation also below 0.8 [38]. It was also shown that for dairy cattle breeding programs, the genetic correlation had to be lower than 0.6 before it was beneficial to run two separate breeding programs rather than progeny testing bulls in both environments. If the selection intensity was high, the genetic correlation increased to 0.7–0.8, below which separate programs were optimal. Again, for a small population (e.g., organic or niche production), the genetic correlation had to be even lower before two programs were optimal [39].

So, in summary, it seems that even if there is $G\times E$, it is beneficial to cooperate and use information from other environments, unless the $G\times E$ is extremely large. This is not the same as ignoring the existence of $G\times E$, rather it is to acknowledge its existence as something natural, and then adapting to that reality.

Future Directions

The trend toward globalization of breeding will emphasize the importance of $G\times E$. A breeding company that wants to be successful in a global setting must also take into account what environment the animal is going to encounter. This might be even more problematic with the advent of genomic selection because it requires reference populations with phenotypic observations from the appropriate environments, if $G\times E$ exists. And, in a global setting, there is no doubt that $G\times E$ exists to a large extent, e.g., between the industrialized production systems in Europe or USA and systems in tropical environments in Africa. Because an increase in production efficiency in developing countries is both a key factor in poverty alleviation and to decrease the ecological footprint, it is important that genetic improvement is aimed at the appropriate traits expressed in the appropriate environments.

Bibliography

Primary Literature

1. Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman Group, Essex, UK
2. Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. The Johns Hopkins University Press, Baltimore
3. Falconer DS (1952) The problem of environment and selection. Am Nat 86:293–298
4. Dawkins R (1982) The extended phenotype: the long reach of the gene. Oxford University Press, Oxford
5. Schlichting C, Pigliucci M (1998) Phenotypic evolution: a reaction norm perspective. Sinauer, Sunderland
6. Waddington CH (1942) Canalization of development and the inheritance of acquired characters. Nature 150:563–565
7. de Jong G, Bijma P (2002) Selection and phenotypic plasticity in evolutionary biology and animal breeding. Livest Prod Sci 78:195–214
8. Scheiner S (1993) Genetics and evolution of phenotypic plasticity. Annu Rev Ecol Syst 24:35–68
9. Scheiner SM, Lyman RF (1991) The genetics of phenotypic plasticity II. Response to selection. J Evol Biol 4:23–50
10. Scheiner SM, Lyman RF (1989) The genetics of phenotypic plasticity I. Heritability. J Evol Biol 2:95–107
11. Robertson A (1959) The sampling variance of the genetic correlation coefficient. Biometrics 15:469–485
12. Kirkpatrick M, Heckman N (1989) A quantitative genetic model for growth, shape, reaction norms, and other infinite-dimensional characters. J Math Biol 27:429–450
13. Kirkpatrick M, Lofsvold D (1989) The evolution of growth trajectories and other complex quantitative characters. Genome 31:778–783
14. Gilmour A, Thompson R (2006) Statistical models for multidimensional (longitudinal/spatial) data. In: Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, Belo Horizonte, Brazil
15. de Jong G (1995) Phenotypic plasticity as a product of selection in a variable environment. Am Nat 145:493–512
16. Kolmodin R (2003) Reaction norms for the study of genotype by environment interaction in animal breeding. Swedish University of Agricultural Sciences, Uppsala
17. Strandberg E et al (2000) Genotype by environment interaction in Nordic dairy cattle studied by use of reaction norms. Interbull Bull 25:41–45
18. Kolmodin R et al (2002) Genotype by environment interaction in Nordic dairy cattle studied by use of reaction norms. Acta Agric Scand A Anim 52:11–24
19. Calus M, Groen AF, De Jong G (2002) Genotype × environment interaction for protein yield in Dutch dairy cattle as quantified by different models. J Dairy Sci 85:3115–3123
20. Calus M, Bijma P, Veerkamp RF (2004) Effects of data structure on the estimation of covariance functions to describe genotype by environment interaction in a reaction norm model. Genet Sel Evol 36:489–507
21. Su G et al (2006) Bayesian analysis of the linear reaction norm model with unknown covariates. J Anim Sci 84:1651–1657
22. Kolmodin R et al (2004) Reaction norms for protein yield and days open in Swedish Red and White dairy cattle in relation to various environmental variables. Acta Agric Scand A Anim 54:139–151
23. Fikse WF, Rekaya R, Weigel K (2003) Genotype by environment interaction for milk production in Guernsey cattle. J Dairy Sci 86:1821–1827

24. Zwald N et al (2003) Identification of factors that cause genotype by environment interaction between herds of Holstein cattle in seventeen countries. J Dairy Sci 86:1009–1018
25. Raffrenato E et al (2003) Genotype by environment interaction for yield and somatic cell score with alternative environmental definitions. J Dairy Sci 86:2470–2479
26. Ravagnolo O, Misztal I (2000) Genetic component of heat stress in dairy cattle, parameter estimation. J Dairy Sci 83:2126–2130
27. Windig JJ, Calus M, Veerkamp RF (2006) Influence of herd environment on health and fertility and their relationship with milk production. J Dairy Sci 88:335–347
28. Meyer K (2001) Estimating genetic covariance functions assuming a parametric correlation structure for environmental effects. Genet Sel Evol 33:557–585
29. Calus M, Veerkamp RF (2003) Estimation of environmental sensitivity of genetic merit for milk production traits using a random regression model. J Dairy Sci 86:3756–3764
30. Pollott GE, Greeff JC (2004) Genotype × environment interactions and genetic parameters for fecal egg count and production traits of Merino sheep. J Anim Sci 82:2840–2851
31. Fikse WF, Rekaya R, Weigel K (2003) Assessment of environmental descriptors for studying genotype by environment interaction. Livest Prod Sci 82:223–231
32. Namkoong G (1985) The influence of composite traits on genotype by environment relations. Theor Appl Genet 70:315–317
33. Mulder HA (2007) Methods to optimize livestock breeding programs with genotype by environment interaction and genetic heterogeneity of environmental variance. In: Animal Breeding and Genetics Group, Wageningen University, Wageningen
34. Mulder HA, Bijma P, Hill W (2007) Prediction of breeding values and selection responses with genetic heterogeneity of environmental variance. Genetics 175:1895–1910
35. SanCristobal-Gaudy M et al (1998) Prediction of the response to a selection for canalisation of a continuous trait in animal breeding. Genet Sel Evol 30:423–451
36. Mulder HA, Bijma P, Hill W (2008) Selection for uniformity in livestock by exploiting genetic heterogeneity of residual variance. Genet Sel Evol 40:37–59
37. Mulder HA, Bijma P (2005) Effects of genotype by environment interaction on genetic gain in breeding programs. J Anim Sci 83:49–61
38. Mulder HA, Bijma P (2006) Benefits of cooperation between breeding programs in the presence of genotype by environment interaction. J Dairy Sci 89:1727–1739
39. Mulder HA et al (2006) Optimization of dairy cattle breeding programs for different environments with genotype by environment interaction. J Dairy Sci 89:1740–1752

Books and Reviews

Annicchiarico P (2002) Genotype × environment interaction: challenges and opportunities for plant breeding and cultivar recommendations. FAO Plant Production and Protection Paper (174). Food and Agriculture Organization of the United Nations, Rome

Hoffmann I (2010) Climate change and the characterization, breeding and conservation of animal genetic resources. Anim Genet 41:32–46

Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. The Johns Hopkins University Press, Baltimore

Schlichting C, Pigliucci M (1998) Phenotypic evolution: a reaction norm perspective. Sinauer, Sunderland

Thiruvenkadan AK, Panneerselvam S, Prabakaran R (2010) Layer breeding strategies: an overview. Worlds Poult Sci J 66(3):477–501

Animal Molecular Genetics from Major Genes to Genomics

Asko Mäki-Tanila
Biotechnology and Food Research, MTT Agrifood Research Finland, Jokioinen, Finland

Article Outline

Glossary

Additive genetic variance is usually the largest part of genetic variation in the quantitative trait and is due to average effects of genes. The change in mean caused by selection is proportional to the additive genetic variance.

BLUP is a short name for Best Linear Unbiased Prediction. It is now a norm in estimating breeding values within populations. It uses information on all kind of relatives and corrects the data for differences in production environment.

Breeding value of an individual is the expected value of its progeny relative to the population mean.

Candidate gene is a possible mutation underlying the mapped QTL. A positional candidate is a gene located in the same region as a mapped QTL. A biological candidate for a QTL is a gene which has a function related to the quantitative trait.

Effective population size (N_e) is the number of individuals that with random mating result in the same rate of inbreeding as the population itself.

Genetic marker is a specific detectable sequence of DNA with a know location in the genome.

Heterosis Is the extent to which the performance of crossbred animals is better than the mean of two parental populations.

Infinitesimal model assumes the genetic variation of a quantitative trait is due to infinitely many unlinked genes each with an infinitesimally small additive effect, so that selection produces negligible changes in gene frequency and variance at each locus.

Linkage disequilibrium is a non-random association of alleles across loci. Recombination between loci will gradually reduce the associations, more slowly the closer the loci are to each other. Adjacent markers with correlated allele frequencies could be used for mapping and selection.

Marker-assisted selection is selection on a quantitative trait where also the information on associated markers is used as a selection criterion. Gene-assisted selection is a special case where the marker is at the major gene causing the variation.

Mixed model equations are providing a method to simultaneously solve the predicted breeding values (random effects) for animals and estimate the fixed effects due to differences in production environment.

Morgan is the unit for a map distance in the genome.

Quantitative trait locus (QTL) A short genomic region with a large effect on a quantitative trait.

Single nucleotide polymorphism (SNP) is variation caused by a mutation at a single nucleotide.

Definition of the Subject

Animal breeding is a major contributor to the vast improvements in animal production over decades. The main tool in breeding operations is selection; now, more and more attention is also paid to the amount and nature of genetic variation. It is on these topics that the chapter is built on. It starts from the fundamental methods in determining the genetic value of animals. The normal distribution and linear methods stemming from the concept of large number of loci with tiny effects causing variation are the base line. For quite some time, there have been observations on major loci causing deviation in linear prediction of genetic values. There is a part introducing methods to get around such cases and turning them advantageous

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

by directly utilizing the variation mediated by major loci. The longest section is on introducing molecular genetic tools to find areas in the genome harboring such major loci and to further characterize the actual underlying genes. The most recent development in DNA technology is the availability of high throughput analysis of individual genomes with tens of thousands of signposts or markers. Without knowing the causal factors, the extensive marker panels can dissect the variation for named markers and recompose the piecewise information for prediction and selection for individuals with respective marker information. Thus, with a sufficiently large number of genotyped and phenotyped animals, selection decisions can be made immediately after birth and very speedy genetic progress is achieved. The same marker panels could be used to have a detailed picture on the state of genetic variation in a population and genome areas causing heterosis in crosses between populations. The theory of animal breeding is thoroughly introduced and discussed in many books, for example those by Falconer and Mackay [1] and Lynch and Walsh [22]. The recent developments in the utilization of DNA markers in characterizing the variation is well covered in the textbook by Weller [2].

Introduction: From Infinitesimal Model to Major Genes

Animal breeding has been very successful in improving production efficiency through utilizing genetic variation between animals. Most of the economic traits are measurable and require own type of approach in analyzing the genetic variation, that is, quantitative genetics. Quantitative genetics theory provides a statistical description of the genetic and environmental variation affecting a particular measurement in a random breeding population as it is at the moment and allows some short-term predictions of the response to selection. In essence, the *additive genetic variance* and its related concept, heritability (h^2), give a coherent framework into which observations on individuals and population, such as the effect of selection or the similarity between relatives, can be fitted.

When there is additive gene action across loci and random mating, the contributions from different loci will be independent, so that their sum will become asymptotically normal as the number of loci increases (while each locus has an infinitesimally small effect). This is referred to as an *infinitesimal model*. Also, the joint distribution of parent and offspring – or of any related individuals – will be approximately normal when the number of loci is large. Therefore, linear methods could be used for prediction. Animal breeding data is typically influenced by many nongenetic factors. The Best Linear Unbiased Prediction (BLUP), due to Henderson [3], has been developed to simultaneously handle both fixed (environmental) and random (mainly genetic) effects in a mixed model. In symbols, denoting individual performance record by y, production environment effects by β and individual animal's genetic value by u, there is an equation for the genetic value of the ijth animal (with known sire and dam and n progeny of varying genetic value u_p with known mates)

$$\beta_i + (1 + 2k + {}^1\!/_2 kn)u_{ij} - k(u_{\text{sire}} + u_{\text{dam}}) + \sum_{\text{progeny}} \left({}^1\!/_2 u_{\text{mate}} - u_p\right)k = y_{ij} \qquad (1)$$

where $k = (1 - h^2)/h^2$. The conventional *breeding value* estimation model for a large number of animals would contain the observation vector $\boldsymbol{y}$ explained by $\boldsymbol{u}$ of random additive genetic effects and $\boldsymbol{e}$ of random residual effects simultaneously correcting for the fixed environmental effects $\boldsymbol{\beta}$. The fixed effects and random genetic effects are connected to observations with the incidence matrices $\boldsymbol{X}$ and $\boldsymbol{Z}$, respectively, and $\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Zu} + \boldsymbol{e}$. The genetic values are made of additive effects within and between loci, dominance deviations due to interaction within loci, and epistatic deviations due to interaction between loci. The covariance matrix of additive genetic effects can be expressed with the additive relationship matrix ($\boldsymbol{A}$) as $\boldsymbol{A}\sigma_a^2$. For solving the *mixed model equations*, one needs $\boldsymbol{A}^{-1}$ which is actually easier to form than the matrix itself [4]. From the inverse elements, one can extract equations like (1). Indeed, an approximate BLUP based on the consideration of a chosen set of close relatives gives satisfactory accuracy in comparing different selection schemes [5].

Matings between relatives cause inbreeding (increased probability for homozygosity) and inbreeding depression in loci with segregating recessive alleles with undesirable effects. In order to improve the genetic evaluation, the *mixed model equations* should

also include dominance effects, although the inverse of the joint covariance matrix of additive and dominance effects is very challenging under inbreeding [6]. It is indeed the inbred populations where the influence of dominance is more pronounced, or the crosses of inbred animals recovering from inbreeding depression or showing *heterosis*.

Selection at the Locus Level

All the concepts of quantitative variation can be expressed at the gene level [1], and therefore it is possible to describe how selection is altering allele frequencies of any locus causing variation in the quantitative trait. The genetic variance is made of contribution from individual loci. The change in mean or response to selection is proportional to the *additive genetic variance*. Hence, the contribution of any locus (or several loci) to the genetic progress under selection will be in proportion to its (their) contribution to the overall *additive genetic variance*. If a locus contributes 10% of the genetic variance, it will explain the same 10% of the total response to selection. Modifying the illustration in Alan Robertson's unpublished lecture notes at the University of Edinburgh, assume that in a dairy cattle population, there is an additively acting biallelic locus where the difference in average milk yield between two homozygotes is $2a$ = 400 kg. Further suppose that the two alleles have the same allele frequency of 0.5. If the selection lifts the mean milk yield by 500 kg, how much has the frequency (x) of the allele with increasing effect changed at the locus. Before selection, the locus contributes to the *additive genetic variance* by ½ x (1 − x) $(2a)^2$ = 20,000. The total genetic variance is – say – 250,000, so that the current locus controls 8% of the total variance and will therefore be responsible for a selection change of 40 kg. This kind of change could be caused by a change 0.1 in allele frequency so that after selection the allele frequency in the progeny generation is 0.6.

The relationship between individual loci and selection response could be described yet another way. The population must respond to selection with an increase in the frequency of those alleles which increase the trait value. The population genetic theory says that the change in gene frequency caused by selection is ½ s x (1 − x) where s is the selective advantage of one homozygote over the other. The aim is to find in the quantitative genetic context the selective advantage of the desirable homozygote compared to the other homozygote after imposing a certain selection intensity (i) for a measured trait. Or to know what is the relationship between a genetic effect on the quantitative trait and the consequent selective advantage caused by the selection on the trait. Falconer and Mackay [1] are showing that there is a linear relationship between the two so that $s = i\,2a/\sigma_p$ where σ_p is the phenotypic standard deviation, so the change in the population mean is equal to $2a$ times the change in allele frequency = $2\,i\,a^2x\,(1-x)/\sigma_p$. Since $2\,a^2x\,(1-x)$ is the *additive genetic variance* due to the locus, the response produced by a change in allele frequency at the locus is proportional to the *additive genetic variance* caused by the locus. Summing over all loci to compute the total response yields again the classical formula for the selection response $\Delta G = i\,\sigma_a^2/\sigma_p$.

Regarding the example and writing $\sigma_p = 1000$ (assuming h^2 = 0.25), if the selection response of 500 kg had taken place in just one generation, it would have to be caused by an intensity i = 2. The expected change of frequency at the studied locus then equals $^1/_2\ i\ 2ax\,(1-x)/\sigma_p$= 0.1. The example has been on a simple additive locus. The same exercise could be done for dominance – or even for epistasis – considering the average effect of allele substitution on the mean of individuals within the population and the effect of small changes of allele frequency on the population mean.

Major Genes

Genes with smaller effects may have their gene frequencies altered less by selection. Highly selected populations might therefore be expected to be segregating for loci with smaller effects on a selected trait compared to those with no previous history of selection. For loci with a tiny effect on the characteristic, directional changes of gene frequency due to selection will be low and changes will occur mostly by chance, depending on the number of parents used each generation.

Loci with very large effects on the trait will have their frequencies changed much more by selection. Since their contribution to the genetic variance depends on the allele frequency, the genetic variance

will also change. The immediate effect will depend on the starting frequencies in the population. When the allele effects are large, it is necessary to include also second-order terms of a/σ_p for the allele frequency change (e.g., [7]) and the respective response would have a quadratic terms of phenotypic values (or selection intensity). These effects would be more pronounced when there are dominance effects between the alleles. In case of carrying selection in one sex only, for example, if the variance is due to a locus with complete dominance and $x = 0.9$ and $a/\sigma_p = .5$, the response upward is going to be overestimated with linear prediction by up to 31% and 21%, when the proportion selected is 10% and 20%, respectively [8]. Also the *mixed model equations* would work less efficiently when the variation is caused by a small number of loci [9].

Over the decades, there have been findings on major genes segregating in farm animal populations: the halothane gene mutation causing stress syndrome and leanness in pigs [10], dwarf affecting body size in chicken [11], double muscling in beef cattle [12], booroola lifting prolificacy in sheep [13]. There is clear evidence that the contributions from the loci affecting the quantitative variation are vastly unequal, there being a small number of major loci and a larger number of minor loci. Such an understanding has generated research on developing tools to utilize major genes and to detect them with new available mapping tools made available by molecular genetics.

Improvement Schemes Utilizing Major Genes

The previous discussion shows that segregating major genes may bias the linear prediction of response in selection. On the other hand, a direct selection on a gene with large effect should give higher overall gains in a selection scheme. If one is able to type the different genotypes at the major locus, it is possible to carry out gene-assisted selection. Until recently, this has not been possible and selection has been based on marker loci close to the actual gene, therefore the term "*marker-assisted selection*" (MAS). Earlier the markers were typed by utilizing protein variants, now the markers are solely based on DNA polymorphism.

While conventional selection is favoring desirable alleles in an indirect way, it should be beneficial to have a tool which can influence the allele frequencies in a much more direct way. The ordinary selection with intensity i would change the mean by $i\,h\,\sigma_a$. Denoting the variance due to the major locus with σ^2_M and its size relative to the total *additive genetic variance* as R, it is possible to assess the advantages of exploiting the marker information in selection.

Lande and Thompson [14] used the selection index methodology in investigating the influence of gene or marker-assisted selection. The selection can be based both on phenotype (P) and marker genotype (M). First step is to compute the selection weights b_1 and b_2 in the index

$$b_1 \text{ phenotype} + b_2 \text{ marker genotype} = b_1 P + b_2 M$$

This could be used to generate equations for the covariance of the index with P and M (the genotypic value of the trait is denoted with G).

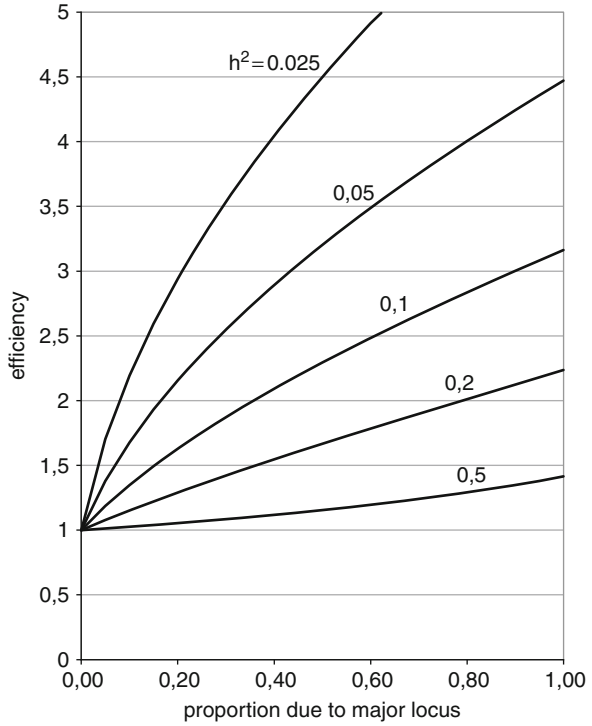
$$b_1 \operatorname{cov}(P,P) + b_2 \operatorname{cov}(M,P) = \operatorname{cov}(G,P)$$

$$b_1 \operatorname{cov}(P,M) + b_2 \operatorname{cov}(M,M) = \operatorname{cov}(G,M)$$

where $\operatorname{cov}(P,P) = \sigma^2_p, \operatorname{cov}(G,P) = \sigma^2_a$, $\operatorname{cov}(P,M) = \operatorname{cov}(G,M) = \operatorname{cov}(M,M) = \sigma^2_M$. The weights in the selection index are $b_1 = h^2(1-R)/(1-Rh^2)$ and $b_2 = (1-h^2)/(1-Rh^2)$. The correlation between the index and genotypic value is approximately $h\sqrt{1+R/h^2}$. In conclusion, the marker information would be useful, when R is high or when a major part of the variation is due the QTL and when the heritability of the trait is low (Fig. 1). Therefore, the selection of low heritability traits such as longevity traits (fertility or disease resistance) would benefit if useful genes or linked markers could be detected.

There are traits which are expressed only in one sex. The use of markers would be useful for such traits. Dairy cattle breeding is a good example: markers would allow pre-selection of bulls on milk traits which would mean savings in progeny testing. Selection could be carried out as early as the embryo stage, resulting even higher reductions in generation interval.

Genes or markers are also beneficial for traits that are difficult to improve under traditional selection. Good examples on such are carcass traits that require slaughtering before they can be measured. Meat pH, tenderness, and color are typical carcass traits. Traits that are available late in life, like longevity and lifetime



Animal Molecular Genetics from Major Genes to Genomics. Figure 1
Efficiency of marker-assisted selection relative to conventional phenotypic selection with the same selection intensity in large population with respect to the proportion of variance explained by a major gene, for different heritability values of the trait

fecundity, would benefit from MAS. The measurement and data collection of some traits, for example, disease resistance, is very expensive and therefore there is much interest in finding major genes for them.

In general, MAS has advantage over conventional selection for alleles that are initially rare and for alleles that are recessive. It is customary to think that the variation in a quantitative trait is caused by a large number of nongenetic factors. However, the marker genotype would be accurately known without environmental noise.

The consideration of gene or *marker-assisted selection* could therefore include the following points: difficulties of collecting performance records, heritability of the trait, proportion of variance explained by the major gene, and the availability of performance in the life cycle. Methods and applications of MAS are comprehensively reviewed by Dekkers [15].

The *marker-assisted selection* has also drawbacks. The direct selection on a gene would be efficient only for a short time. The desirable allele would quickly become common and new beneficial alleles should be found or new potential markers should be searched for useful segregation. If the selection is not directly on the gene, it is possible that the recombination between the used marker and QTL would gradually weaken the association. The use of markers would even be harmful if the association is not properly validated and efficiency of selection reduced compared to conventional selection.

There are also consequences for the overall genetic variation. The larger the effect of the QTL is, the faster it is fixed by selection and the more it will also reduce the variation in the surrounding genome area (e.g., [16]). An accelerated increase in the marker allele frequency may be accompanied by very unbalanced use of family lines. This is increasing the risks for the reduction of variance and enrichment of harmful recessives.

Introgression from Other Populations

Many local breeds carry interesting genetic variants that are considered beneficial to be introduced to a commercial main stream breed. The former is termed as a donor breed and the latter one as a recipient breed. The operation is usually done by an introgression program. It consists of forming an initial cross between the breeds followed by repeated backcrosses to the recipient one to recover the economically important genome. The target gene is maintained in the backcross generation through selection of donor gene carriers. After some generation of backcrossing the program will finish by a generation of intercrossing to make the population homozygous for the desired allele.

Genetic markers could be useful in introgression programs in two ways [17]. First, markers can be used to select individuals at each backcross generation which are heterozygous for the desired allele or homozygous in the last generation of intercross (foreground selection). Secondly, markers can be used to enhance the recovery of the recipient genome (background selection).

The introgression strategy is a popular genetic improvement method in plants, while there are still very few applications in domestic animals. An example is the naked neck gene in chicken which reduces plumage in chicken and makes animals more tolerant to heat. It was introgressed from low body weight landrace chickens into a commercial meat-type Cornish chicken [18].

Most of the economic traits in farm animals are complex ones with polygenic variation. One of the most promising cases of introgressing exotic germplasm to commercial production populations has been the impact made on western pig production by the Taihu breeds of the Shanghai area (China). The background for the interest and the results have been reviewed by Knap and Neeteson [19]. Some of these pig breeds (Meishan and Jiaxing) were imported into France, the UK, and the USA in the 1980s. The interest is in reproductive traits: low age at puberty, high ovulation rate and embryo survival resulting in high litter size, and high teat numbers. The disadvantage is in high body fat levels and slow growth rates.

Several commercial breeding organizations in France, UK and Canada have invested in the introgression of Jiaxing and particularly Meishan pigs into their dam lines. This was accompanied by studies into the trade-off between improved fertility and reduced leanness (e.g., [20]). These suggested that the feasible way to commercially exploit these genotypes would be to have commercial sows with 12.5% Taihu genes [21].

While the fertility is improved, as a compromise, leanness is reduced in Taihu-based genotypes, which is a serious obstacle toward large-scale commercial exploitation, although persistent genetic improvement in the latter trait obviously would solve the problem. A complicating factor here is that a few years after the Taihu imports, large data bodies and improved statistical methods allowed for a faster genetic change of reproductive traits in pigs, while genetic improvement of leanness continued at the same rate as before.

Western commercialization of the Taihu breeds has been successful so that almost 100,000 Taihu-based parent gilts are currently sold per year in Europe and North America. It represents less than 1.5% of the total market volume. A good working example on successful introgression of exotic genotypes into advanced animal breeding program is still missing for a polygenic trait.

QTL Mapping

Markers and Linkage Maps

Better understanding and utilization of major genes would benefit from the location of genes or genome areas responsible for the variation in quantitative traits. The prerequisite for this is to have a reasonable skeleton of the genome sites to survey and locate the findings. The genome is made of chromosomes. There are two homologues of each chromosome in a typically diploid animal genome. One homologue is originating from sire (paternal chromosome) and the other one from dam (maternal chromosome). The alleles at the loci on the paternal (maternal) chromosome tend to be inherited together or they are said to be linked. The genome is full of variable sites which could be used as markers to construct a detailed map of locations for further work. The closer the markers are to each other, the more likely they are inherited together or the tighter is the linkage between them or the rarer are the recombinants between them. The distant markers are on the other hand showing independent segregation. Physically, recombination is seen as a crossing over of chromosome strands. The chromosomes could be termed as linkage groups.

The linkage is measured as recombination frequency (c) or the proportion of recombinant ones among all the gametes. If markers are very far from each other, they may be several crossing-overs between them. When the loci are freely combining, the number of recombinant and nonrecombinant gametes is equal and $c = 0.5$. The map distance is expressed in *Morgan*s (M) with its hundredth being cM (centimorgan). A low recombination frequency 1% corresponds to 1 cM. For longer distances, the possibility of several crossing-overs should be considered, as, for example, two crossing-overs between distant markers would often result in nonrecombinants of the marker pair itself.

The detection of linkage or computing map distances would require variable sites and use of heterozygotes. Ideal markers for mapping are the (codominant) loci with alleles not showing dominance (or recessivity). Linkage could be demonstrated with different mating options. For example, there can be

double heterozygote $M_1\,N_1/M_2\,N_2$ mated with a double homozygote $M_1\,N_1/M_1N_1$, in which case the offspring numbers in different genotype classes would give a straightforward measure for linkage. Inbred lines are homozygous for all loci and the first generation cross F_1 heterozygous, respectively. When F_1 animals are crossed with one of the parental lines, the resulting backcross animals would give direct estimates on recombination frequencies. Using inbred line terminology, the mating is called double backcross.

Although there are often heterozygotes available, only a limited number of cases are informative on possible recombinants and determining the amount of linkage. There are five different cases with respective information content.

$\frac{M_1\ N_1}{M_1\ N_1}$	$\otimes$	$\frac{M_1\ N_1}{M_1\ N_1}$	Not informative
$\frac{M_1\ N_1}{M_2\ N_1}$	$\otimes$	$\frac{M_1\ N_1}{M_1\ N_1}$	Not informative
$\frac{M_1\ N_1}{M_2\ N_2}$	$\otimes$	$\frac{M_1\ N_1}{M_1\ N_1}$	Informative (double backcross)
$\frac{M_1\ N_1}{M_2\ N_2}$	$\otimes$	$\frac{M_1\ N_1}{M_2\ N_1}$	Informative (single intercross)
$\frac{M_1\ N_1}{M_2\ N_2}$	$\otimes$	$\frac{M_1\ N_1}{M_2\ N_2}$	Informative (double intercross)

From the last mating type, there are nine different progeny classes with respective expected numbers according to the recombination frequency.

Before a marker is used, its suitability is assessed studying the allele frequencies (x_i). So far it is obvious that homozygous parents are of no use and among the progeny of heterozygous parents only the homozygotes (or for multiple alleles, the heterozygotes M_1/no M_2 or M_2/no M_1) would signal which allele of the parent has been transmitted to the progeny. So the quality of the marker is based on polymorphism information content (PIC), which is the probability of identifying which homologue of a given parent was transmitted to a given offspring, the other parent being genotyped as well, or in other words, probability that the parent is heterozygous $\times$ probability that the offspring is informative. Summing over alleles, the PIC for a multiallelic marker would be $1 - \sum x_i^2 - 2\sum\sum_{i<j} x_i^2 x_j^2$.

Mapping Function

In order to fill the linkage map with markers and genes, there is a need to have a good measure for the map distance. As there can be several crossing-overs between distant markers, recombination frequency as such is not an appropriate measure. If there are markers *M*, *N*, and *O* (with respective recombination frequencies c_{MN}, c_{MO} and c_{NO}) and crossing-overs in the adjacent genome regions would be independent, then the recombination frequency $c_{MO} = c_{MN} + c_{NO} - 2\,c_{MN}c_{NO}$. In other words, the recombination frequencies are additive only if they are small enough so that the product term could be ignored. A map distance is required that would give the total number of crossovers between even very distant markers. The measure should be additive so that the number of crossovers between *M* and *O* is the number of crossovers in the interval *M*–*N* plus those in the interval *N*–*O*.

The probability of no crossovers is $e^{-\lambda}$ (from the Poisson distribution) and the probability of at least one recombinant gamete is $c = ½(1 - e^{-\lambda})$. The latter is true, because for each crossover event only one-half of the gametes will be recombinant types there being only two of four strands involved in a crossover. The map distance is then $-½\ \ln(1 - 2c)$ in *Morgan*s for an observed recombination rate *c*. For short chromosome segments ($c < 10\%$), the map distance equals the recombination rate, while 50 cM corresponds to recombination rate 32%. The approach is called Haldane mapping function and is valid when the crossing-overs across the genome occur independently. If some degree of interference needs consideration, Kosambi mapping function should be applied. For more details, see Lynch and Walsh [22].

The number of chromosomes varies quite much over the animal species:

Cattle	Chicken	Pig	Sheep	Carp	Human	Mouse
30	39	17	27	52	23	32

When there are so many chromosomes in livestock species, the average recombination across the genome is very high. In comparison, the well-known lab organism Drosophila has only four chromosomes and no recombination in males.

The genome in mammals is about 3×10^9 b (base) and 3 M. The chicken genome is only about 1×10^9 b, while the linkage map is still around 3 M. The well-known lab organism Drosophila has only 275 cM and 0.13×10^9 b.

DNA Markers

Ideal markers for mapping purpose should be widely distributed over the genome. At the same time, it is important that there are several markers available within short genomic regions allowing high-resolution mapping. Good markers should have many alleles, and with frequencies as equal as possible. The alleles should be codominant to allow easy detection of heterozygotes. The allele typing should be straightforward with satisfactory repeatability across analyses. Alleles should also be stable from generation to generation with a low mutation rate. The cost of genotyping could be reduced if the genotyping could be done simultaneously for many loci and the analysis could be easily automatized to provide high throughput.

The most common markers are either microsatellites (based on loci which have a variable number of repeats of a same short sequence) or single nucleotide polymorphism (SNP, which is a nucleotide site in the genome sequence, predominantly biallelic, but possibly having four alleles with each of the four nucleotide bases appearing in the same location). Both these marker types are widely distributed and codominant. Microsatellites are now less popular because their repeatability is lower and there is a limited number of them for a high-resolution mapping in a chosen short region. SNPs have gained popularity and it is nowadays possible to have very large SNP panels typed moderately cheaply in a single analysis with a small microarray chip.

Microsatellites are still used when sufficient information is delivered by only few markers (e.g., as required for parentage testing), and in some applications, this will offset the relative disadvantage of throughput in comparison to SNP markers.

From Segregation Analysis to Use of Markers

Since the genes affecting quantitative genetic variation are behaving like genes with classical discrete genotypes, there have been attempts to characterize them. If in the population there is segregation at a major locus mediating quantitative trait variation, alleles with very large effect and extreme frequency could be causing a skewed phenotypic distribution. Even if allele classes are equally common, the distribution may have several peaks. The consequences due to major gene can be studied with segregation analysis (e.g., [23]). Segregation analysis is full of factors which are hard to be itemized and tested as the effect of several major genes, extreme allele frequencies and dominance gene action produce similar distribution patterns and are therefore hard to separate. Segregation analysis is historically important and would still be used for providing preliminary understanding about the inheritance pattern, especially for human familial diseases.

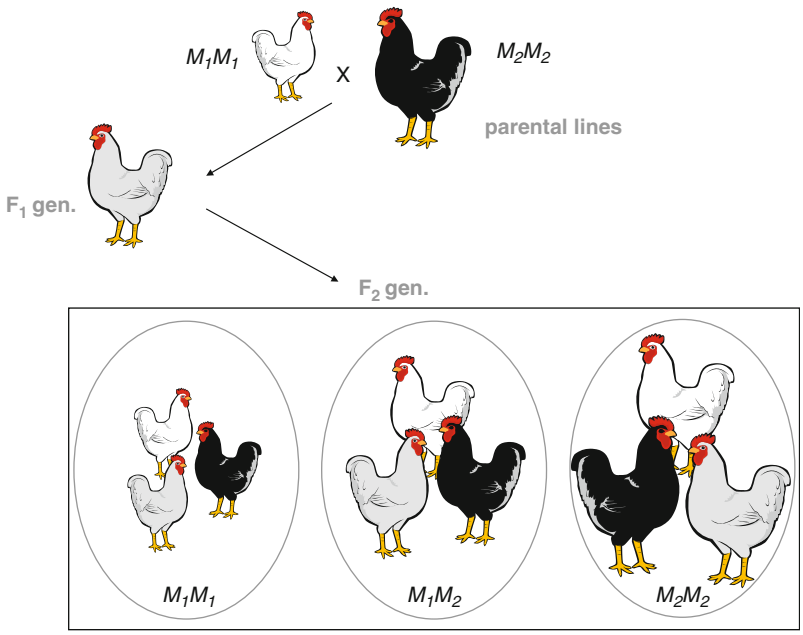
If one wants to demonstrate the existence of a major gene, it is better to anchor the analysis of distribution features to a known locus with visible genotype classes, and hope that the locus is a useful marker to demonstrate the existence of a linked major gene. Thereby, the analysis could be narrowed down to investigating one gene (or marker) at the time (Fig. 2). The statistical model could include the location of the gene, the effect size, dominance, and frequency of the alleles. Only the availability of marker loci supporting the localization was for a long time restricting the possibility for such a work.

It was half a decade ago when the blood group alleles became immunologically identifiable and possible signposts for analyzing quantitative genetic variation in farm animals. These markers were followed by electrophoretically (molecule size and electric charge) detected protein. The number of markers was still very low and even their rough location was not known. Enthusiastic researchers were facing frustrating results in hunting major genes, although the possibilities and optimum designs for analyzing quantitative traits were clearly envisaged by some groups (e.g., [24]).

The research chances improved a lot when molecular genetic techniques opened completely new kind of possibilities for mapping. With a lot of markers available all around the genome, it was soon rather rewarding to detect regions responsible for the variation. Also a new term, QTL or *quantitative trait locus*, for such genomic regions was coined.

QTL Mapping from Crosses of Inbred Lines

Inbred lines would be ideal resource populations for QTL mapping. They are homozygous for most of the genome, including, hopefully, the used marker locus and putative QTL, in which case the crossbred F_1 population would be heterozygous at both the loci. In other



Animal Molecular Genetics from Major Genes to Genomics. Figure 2

Two chicken lines differing in body weight and plumage color are fixed for a marker allele (M_1 or M_2). The lines are crossed to follow the possible co-segregation of the traits and also of the marker locus in the F_2 animals where all possible combinations of phenotypic classes and marker alleles are appearing. The animals are grouped by the genotypes M_1M_1, M_1M_2, and M_2M_2. The M_1M_1 animals are the heaviest, while the M_2M_2 ones are the lightest with the M_1M_2 ones being intermediate. This would suggest that the marker is close to a QTL related to body weight. However, the plumage color which is also showing a simple pattern of inheritance, is segregating independently as the color types occur with equal frequencies in the different weight classes

words in the F_1 generation, there is a very high correlation of allele frequencies across the loci, or using population genetic terms, the *linkage disequilibrium* between the marker and possible QTL is maximized. *Linkage disequilibrium* is reduced over generations as recombination is gradually breaking the associations. However, very little of *linkage disequilibrium* will be lost within short map distances in producing backcross and F_2 generation. Starting from inbred lines, it is easy to follow the variation in the marker loci and the allele phases across marker and QTL would remain the same over further generations. While the use of line crosses is enhancing marker variation, it is also beneficial to choose lines that are phenotypically very divergent. Such a starting point would increase the chances of finding QTL.

Assume there are parent lines (P_1 and P_2) homozygous for marker locus (two alleles M_1 and M_2) and QTL (B and b). The crossed generations are mixtures of distributions with respect to the marker genotypes. The line differences could be associated with the marker classes using alternative crossing designs. Then the expected distribution and contrasts for the marker genotypes between the lines are

	Expected distribution among marker genotype classes			
	M_1M_1	M_1M_2	M_2M_2	Contrasted classes
Backcross with P_1	½	½		M_1M_1–M_1M_2
Backcross with P_2		½	½	M_1M_2–M_2M_2
F_2	¼	½	¼	M_1M_1–M_2M_2 M_1M_1–M_1M_2 M_1M_2–M_2M_2

The efficiency of associating the computed phenotypic differences with the marker variation depends on how closely the analyzed single marker is to a gene affecting the variation. The F_2 generation (produced by intercrossing F_1 animals) is better than the backcrosses (crossing F_1 with one of the parent lines) as it is possible to estimate also the dominance effect via comparing the heterozygote class with the homozygote ones.

Random Breeding Populations

Typically, farm animals are forming a random breeding population. Such a population has many limitations for successful QTL mapping. The available phenotypes are within a relatively narrow range of variation and would generate much smaller class differences than in crosses from diverse inbred lines. The markers would also show less systematic polymorphism than crossbred populations, since in a large random breeding population the *linkage disequilibrium* is expected to be very low over moderate map distances. In that situation, the only chance to find QTL is to have a detectable marker at the gene itself or its immediate neighborhood. Still, some families or parent individuals may be useful for mapping studies as by chance they have marker and QTL variation phased in a useful manner. Although there is no *linkage disequilibrium* at the population level, there may be partial disequilibrium within families depending on the recombination rate. Such a *linkage disequilibrium* could be exploited in QTL screening. Assume sires that are heterozygous for the marker (M_1M_2) and have large progeny group of half-sibs with phenotype and marker genotype data. The data could be modeled for an analysis of variance around the average level μ as performance record = μ + marker genotype + residual.

Using the notation above, at the QTL locus the separation of two homozygotes is $2a$, the frequency of B allele in the population is x and the recombination rate between the marker and QTL is c. The analysis could be extended to include dominance by allowing the heterozygotes to deviate from the mean of the two homozygotes by da ($d = 1$ dominant, $d = 0$ additive, $d = -1$ recessive gene action with the intermediate values indicating partial dominance or recessivity).

Therefore the frequency of different progeny types would be across heterozygous sires with different QTL allele configuration as follows:

	QTL genotype in progeny		
	BB	*Bb*	*bb*
	0	$(1 + d)a$	$2a$
Sire genotype	M_1 allele from sire		
M_1B/M_2B	x	$1 - x$	0
M_1b/M_2b	0	x	$1 - x$
M_1B/M_2b	$(1 - c)x$	$(1 - c)(1 - x) + cx$	$c(1 - x)$
M_1b/M_2B	cx	$c(1 - x) + (1 - c)x$	$(1 - c)(1 - x)$
	M_2 allele from sire		
M_1B/M_2B	x	$1 - x$	0
M_1b/M_2b	0	x	$1 - x$
M_1B/M_2b	cx	$c(1 - x) + (1 - c)x$	$(1 - c)(1 - x)$
M_1b/M_2B	$(1 - c)x$	$(1 - c)(1 - x) + cx$	$c(1 - x)$

From these frequencies, one can compute for different progeny groups across the population:

Sire genotype	The expected phenotypic difference between M_1 and M_2 progeny
M_1B/M_2B	0
M_1b/M_2b	0
M_1B/M_2b	$(1 - 2c)[1 + (1 - 2x)d]a$
M_1b/M_2B	$-(1 - 2c)[1 + (1 - 2x)d]a$

Hence, the difference between subgroups disappears when recombination between the marker and QTL is 0.5 (or when the marker is very far from QTL). In general, it is not possible to separate the effect and location, for example, QTL which is 25 cM away from the marker would have an estimate like with the marker with half of the effect locating exactly at the QTL position. On the other hand, the frequency and dominance action will also affect the estimation and obviously the detection of rare recessives is very hard.

When at the population level there is no linkage disequilibrium, the occurrence of informative haplotypes in progeny depends on the allele frequencies at the marker and QTL. The heterozygous sires M_1M_2 are

investigated for the difference in performance between M_1/no-M_2 and M_2/no-M_1 progeny classes. In principle, the analysis should pool the results across sires to improve the power of the analysis. However, there are sires (like M_1 B/M_2 B, M_1 b/M_2 b) where the marker genotype contrast is zero. In some cases, the difference is positive (M_1 B/M_2 b) and in other cases negative (M_1 b/M_2 B). Therefore, the analysis is carried out within sires. Thereby, it is possible to include all the possible existing gametes (and alleles) with different combinations of marker and QTL alleles, and at the same time eliminate the spurious association caused by between sire variation [24, 25].

Interval Mapping

When the QTL screening is done marker by marker over the genome, the available information is utilized in a suboptimal way, and, for example, the effect and location of QTL cannot be separated. On the other hand, when there are many markers, some of them may by chance give a statistically significant result at the chosen risk level. If the significance threshold is 5%, then five out of hundred analyzed map positions may give a "significant" result. This could be taken into account by having a more stringent rejection criterion and have for testing each individual marker a statistical significance level of 1/(no. markers) times what it would be if only one hypothesis were tested (so called Bonferroni correction). When there are several (linked) markers available on the same chromosome, one should consider utilizing all the markers jointly and screen the whole chromosome interval by interval for existing of QTL.

Assume that the QTL is at the marker locus or in its immediate neighborhood. Therefore, it is possible to ignore how the recombination may reduce the estimate on the effect. When several linked markers are used, the localization part could be sharpened by the adjacent markers and subsequently, the estimation of QTL effect would receive better attention. Such an approach is called interval mapping. It was first developed for analyzing inbred lines [26].

Suppose there are now two markers M and N to test a case where B locus affecting the quantitative trait variation is assumed to be in the interval between them. Further, the alleles at B recombine with the alleles of M at the rate c_1 and with N with c_2. If the parental lines P_1 and P_2 are homozygous M_1 B N_1/M_1 B N_1 and M_2 b N_2/M_2 b N_2, respectively, then in the backcross to P_2 the expected progeny mean computed with respect to inheritance probabilities at the B locus is

$$\begin{aligned}&\text{prob}(B) \times \text{effect of } B \text{ allele} + \text{prob}(b)\\ &\times \text{effect of } b \text{ allele} = \text{effect of } b \text{ allele} + \text{prob}(B)\\ &\times (\text{effect of } B \text{ allele} - \text{effect of } b \text{ allele})\end{aligned}$$

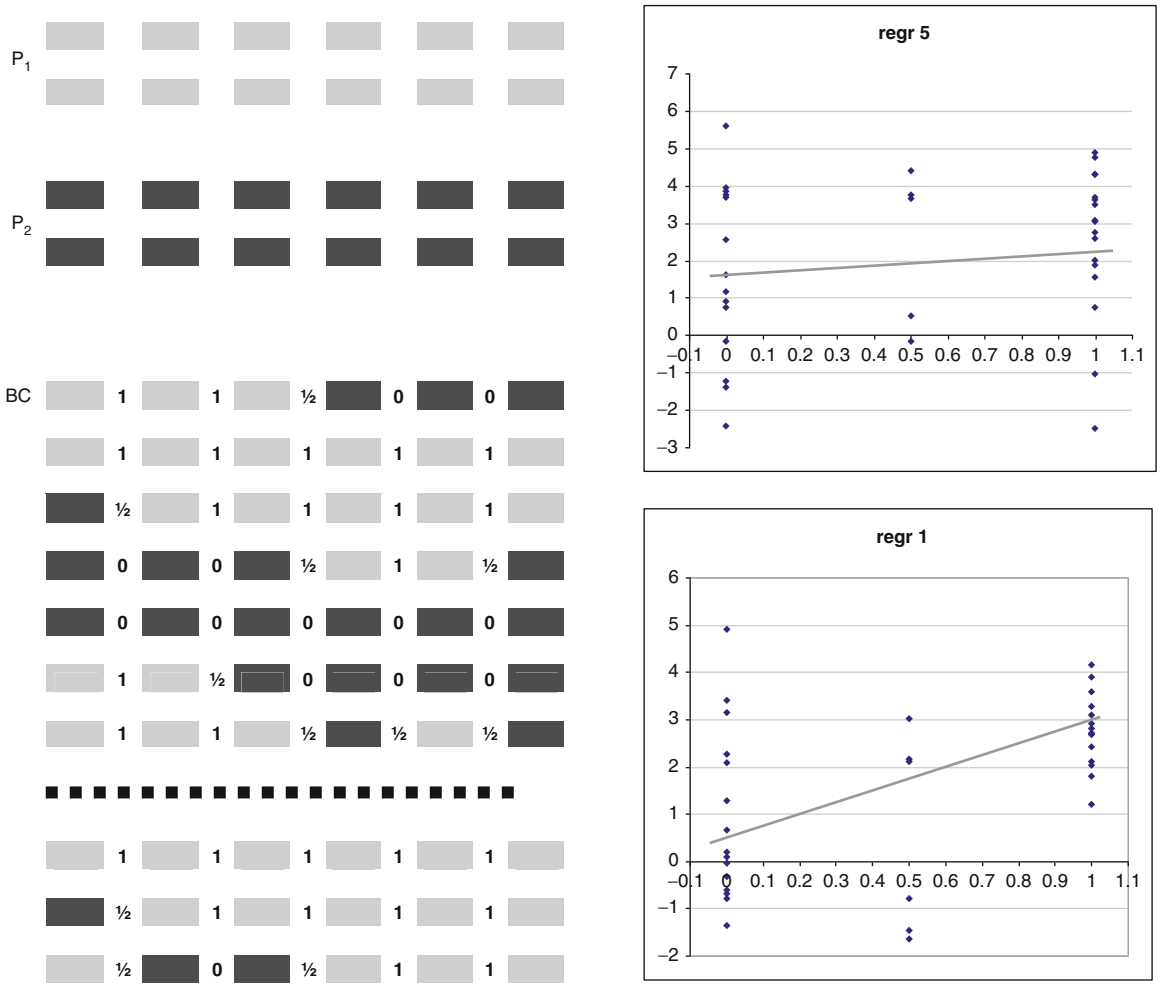
In terms of different combinations of M and N alleles in the backcross progeny, the probability for them having B allele from F_1 parent is (recalling that the recombination rate c between M and N can be expressed as $c_1 + c_2 - 2\,c_1\,c_2$)

$$\begin{aligned}&M_1N_1 \quad 1 - c_1c_2/(1-c)\\ &M_1N_2 \quad (1-c_1)c_2/c\\ &M_2N_1 \quad 1 - (1-c_1)c_2/c\\ &M_2N_2 \quad c_1c_2/(1-c)\end{aligned} \tag{2}$$

The prob (B) varies across the different positions between M and N. Different map positions could be converted to recombination rates in finding the map positions and allele effects that best explain the observations. The estimation could be done with computing the regression [27] at several positions between M and N with the model: performance record = intercept + regr coeff × prob (B) + residual. The regression coefficient is an estimate of the average effect of allele substitution at (biallelic) QTL. This is illustrated in Fig. 3. Like for any regression analysis, fitting the regression could be assessed with F test at different positions of the interval. If there are more markers along the studied chromosome, the analysis could be repeated over all the possible intervals.

When there is a reasonable coverage of the genome with interval mapping, the analysis is screening the whole genome for QTL. The same type of QTL analysis could be carried out for F_2 cross animals. In that case also the dominance deviations could be estimated for QTL.

In farm animals, the closest to an inbred line is a breed. The first major genome scan utilizing breed differences was reported in the mid-1990s in pigs from a cross between the Large White breed and the wild boar [29]. Large QTL effects were found influencing



Animal Molecular Genetics from Major Genes to Genomics. Figure 3
QTL interval mapping via regression for backcross setting. The backcross (BC) population is produced by crossing phenotypically diverse "light" and "dark" parental lines (P_1 and P_2). The light line animals of P_1 have a higher phenotype. The BC population is genotyped with a marker panel covering the shown genomic region at regular intervals of 20 cM. Markers are represented by shaded bars. The BC population is formed by crossing F_1 animals with the P_2 animals. When all the transmitted P_2 markers are black, the BC animals are illustrated with the chromosomes from F_1 parents, with possible recombinants. Marker intervals are considered one by one for the presence of QTL. The probability that individual has received the "light" QTL allele from F_1 parent is computed (values in the brackets between the markers) and used as an explanatory variable to compute the regression of phenotypic value on it. The regression coefficient provides an estimate of the allele substitution effect. The QTL is in the first bracket and leads to higher regression coefficient than in the last (5th) bracket, far away from the QTL, both examples shown on the right. The illustration is modified from Georges [28]

both growth and fatness. After that study, several genome scans have been developed involving the commercial and exotic breeds.

In random bred populations, there is less regularity in many respects and the interval analysis should be preceded by the examination of allele phases across adjacent markers in heterozygous (say $M_1/M_2N_1/N_2$) sires. The phase determination would require a large genotyped half-sib family from the sire. The non-informative heterozygous progeny M_1/M_2 cannot be

automatically used for the analysis but the considered interval should be extended until an informative locus in the progeny is found. If the sire chromosome phases over the markers are M_1N_1/M_2N_2 and there is a progeny that has inherited the combination M_1N_1 (and assuming that the QTL allele B is on the same chromosome with M_1 and N_1), the progeny has B allele with probability $1 - c_1\, c_2/(1 - c)$. The other probabilities could be derived and would resemble the ones listed for the backcross case above at (2).

The interval analysis is again performed within a family. The power of the analysis could be improved (or residual variation reduced) by analyzing several families and pooling the results at the end. The statistical model for progeny mean is therefore average level + sire_i + $\text{regr coeff}_i \times \text{prob}\ (B)$ + residual. The sum of squares due to the regression is then obtained by pooling the sum of squares due to regression across sires as $\sum \text{SS-regr}_i$/number of sires. The degrees of freedom in the F test for the residual variation are the total number of observations minus twice the number of sires. The analysis is repeated at several positions, say at cM intervals, between M and N and further across other intervals. The highest value for the test parameter is indicating the likely position for QTL with the regression coefficient itself giving the substitution effect. The first genome-wide screening for QTL was done in the outbred half-sib design for dairy cattle by Georges et al. [30].

Design of QTL Mapping Studies

It is important to understand what would be an optimal design and number of observations (n) for QTL mapping studies. First consider the regression with a single marker for a crossbred population (or single family). The explanatory variable (*prob*) is made of probabilities for the inherited QTL allele. For simplicity, there are so many observations (>50) that one can formulate the test for the regression coefficient as regr. coeff./its standard error = regr coeff/$\left(\sigma_e^2/SS_{\text{prob}}\right)^{1/2}$ = $\sqrt{n}\ \sigma_{\text{prob}}$ regr. coeff./σ_e In other words, the test parameter depends on the number of observations, variation range of explanatory variable, size of QTL effect and residual variation(σ_e).

Considering the type I error rate α and test power $1 - \beta$, then in terms of the respective standardized variates (z) of normal distribution, the number of observations required to detect a QTL with an effect a/σ can be deduced from $(z_{1-\alpha} + z_{1-\beta})^2/(\sigma_{\text{prob}}\ a/\sigma)^2$. For the 5% risk and power 90% with the effect sizes

0.1	0.4	0.7	1.0

the required number of animals (with observation and marker genotype) in a backcross is

4,202	262	85	42

Compared to backcross, the explanatory variable would double its variation in F_2 and therefore the F_2 cross would have twice the efficiency given the same number of genotyped individuals. So far it is assumed that in the single marker analysis, the marker is at QTL itself. When this is not so and the recombination between marker and QTL is c, then with the same criteria the number of genotyped individuals should be $1/(1 - 2\ c)^2$ times higher (for more details see [31]).

In conclusion, a cross between divergent lines is very powerful to detect QTL because the statistical power is high and a reasonable number (200–300) of animals is sufficient to detect a major gene. Experimental cross is also a useful approach for QTL screening as the production could be controlled in a small scale study and uncommon traits (disease resistance and quality traits) can be monitored. However, in farm animals, uniform lines are not common and rarely inbred enough to fully exploit the crossbreeding advantages. It is also very costly to maintain such lines or find facilities for generating and maintaining experimental crosses.

The random bred populations should be analyzed pooling within family results across families. When there are several groups in the analysis, the testing should follow the framework for the analysis of variance (F ratio). The test is improved with a large number of progeny. When there are s sires, the family size requirement could be computed from $F^2\sigma_e^2\ s/\Sigma\ SS_{prob}$. On the other hand, the more sires there are, the higher are the chances of finding a family with a segregating QTL.

With the half-sib design, the number of genotyped animals could be reduced by moving from daughter design to granddaughter design [32]. Instead of genotyping daughters for markers, the progeny tested

sons of the sires are now typed and their evaluation results are used in the analysis. Thereby the number of genotypings can be kept reasonable and the accurate genetic value of sons reduces the residual variation and thereby increases the power of analysis. The sons are genotyped for markers that are heterozygous in their sire. The analysis for a single marker is carried out within sires with the model: progeny test result value of son j from sire $i = \mu + \text{sire}_i + \text{son's genotype}_{ij} +$ residual.

With a given number of genotypings the test would be more powerful than the daughter design. When there are 200 daughters per sire versus 200 sons per sire and 100 daughters per son, one can compare the test power (risk 5%) with different effect sizes for daughter/granddaughter design.

Number of sires/paternal grandsires	No. genotypings	a/σ =.1	a/σ =.3
5	1,000	3/48	50/97
10	2,000	5/73	76/99
20	4,000	7/95	95/99

In order to reach the power of granddaughter design, the daughter design would need large progeny groups which – in terms of collecting DNA samples and carrying out genotypings – are workwise almost impossible.

For the interval mapping, both the variance of the explanatory variable and residual would be reduced while in general the aim should be to push down the residual variation as low as possible while keeping a wide range of alternatives for the explanatory variable. If only animals with extreme phenotypes are genotyped, the latter could be increased and the efficiency enhanced. Ultimately, genotyping only the very highest and lowest phenotypes, the variance of the explanatory variable is increased, and in the best situation the analysis would have only the extreme homozygotes for QTL. It is important to notice that the selective genotyping should be done within families to make sure that the selection is targeted on the QTL instead of the general genetic background. Selective genotyping has its drawbacks, as it would focus on a single trait at a time.

Genes Underlying QTL

In farm animals, the QTL mapping has been very popular since the microsatellites and later SNPs became available as mapping markers. Depending on the species, two designs have been used: crosses mainly in pigs and chicken and half-sib families in dairy cattle. The QTL investigations have been carried out with marker sets of 100–300 microsatellites covering the whole genome. This would correspond to a marker density of some 10–30 cM. QTL mapping in farm animals has been very prolific as it has been possible to have sufficiently large experiments or population samples to yield an adequate statistical power for QTL detection. Until today, thousands of QTL for numerous traits have been reported (see http://www.animalgenome.org/QTLdb/).

Year	Number of QTL (number of traits in brackets)		
	Cattle	Pigs	Chicken
2006	630 (89)	1,287 (246)	657 (112)
2011	4,682 (376)	6,344 (593)	2,451 (248)

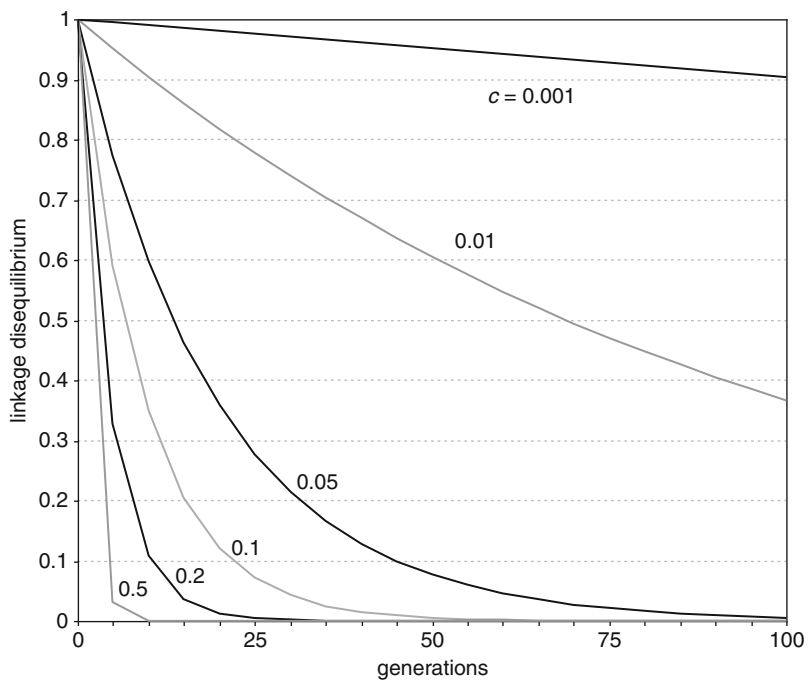
Fine Mapping

A successful *marker-assisted selection* requires that the applied marker is at the QTL itself or very close to it. Otherwise there is a need to resort to linked markers which may differ across families, depending on how they are showing *linkage disequilibrium* with the targeted QTL. There are other reasons for the interest to find the underlying genes, as the general aim is to understand more about their function and utilize them in further studies and applications. It is hard to resist the temptation of trying to find the genes, the allelic variants within them, the allelic effects and frequencies, co-effects with the alleles of other loci, and effects on other traits. Moreover, the never-ending curiosity drives research to distinguish coding areas and regulatory elements (e.g., [33]). In a typical QTL study, it is possible to locate a QTL only within a fairly wide region of a chromosome. A region of 10–30 cM may still contain 100–300 genes which is making difficult to identify and characterize the responsible mutations [28].

There are several factors that affect fine mapping. An obvious criterion is high marker density. The design consideration showed how the sample size of animals contributes substantially to the mapping resolution. As high as 5,000 individuals are required to increase the resolution down to 5 cM in crossbreeding designs. Therefore, it is necessary to think about ways to produce new recombinants. It is easier with experimental laboratory strains serving as model organisms where with a short generation turnover it is possible to have intercross populations F_3, F_4, F_5, . . . In that context, by generation t the confidence interval for the QTL location is reduced to $1/t$ of the F_2 one given the same number of animals per generation [31]. In farm animals, such operations would be money and time consuming and practically unfeasible.

It is easy to produce a large number of markers for an interesting QTL area and find the markers with the best association with QTL alleles. Usually, combining individual marker variation over the area and assessing the association between haplotypes and QTL alleles may be more fruitful than analyzing single markers one by one [34]. In farm animals, the density of observable recombinants could be increased by exploiting historical recombination events in comparing the same genomic region within population or even across breeds (Fig. 4). Many breeds are originating from a small number of founders or have experienced bottlenecks alternating with periods of population growth. These events affect sampling of haplotypes and thereby cause *linkage disequilibrium* which can be used in fine mapping QTL regions. *Linkage disequilibrium* extends over longer genome stretches in young populations which hampers localizing causal mutations. In modern dairy breeds, *linkage disequilibrium* is seen as long haplotype blocks [35]. The older haplotypes shared by breeds can be exploited in high-resolution mapping.

In a QTL analysis one is following the transmittance of the variants over a chromosome section and computing probabilities for assumed QTL in the marker bracket. The more detailed variation within the chromosome interval is ignored and further it is assumed that the variants of the interval are unrelated. The intervals could be filled with more markers, especially



Animal Molecular Genetics from Major Genes to Genomics. Figure 4
The decay of linkage disequilibrium over generations for different values of recombination rate (*c*) between loci. It is noticeable that with a recombination rate 1% it takes some 80 generations to halve very high linkage disequilibrium

around the found QTL, to classify the relationships between the interval variants given the linkage disequilibrium. When there is evidence for a QTL from linkage analysis and from *linkage disequilibrium* around the putative QTL position, the linkage and *linkage disequilibrium* analysis could be combined [36]. The analysis would eliminate QTL effects from distant regions of the chromosome and other chromosomes because they do not appear as linked QTL. Therefore, the combined analysis would produce a clearer signal for the QTL position compared to the two separate analyses [37]. Such a combined linkage and *linkage disequilibrium* approach has been successfully exploited in cattle to map QTL within less than 1 cM [38].

The discussion above shows how important it is to eliminate the spurious associations due to linked genes or other genetic background. One approach is to use transmission disequilibrium test or TDT [39]. This compares the effect of the two alternative types of gametes from a common parent. For example, a sire heterozygous for a studied marker (or a putative gene) may have a large progeny group allowing a test to find how regular the difference is between progeny inheriting the alternative alleles. Such an analysis is extended over sires by treating them fixed.

Few Examples on Causal Mutations

The characterization of mutations underlying QTL is very hard in any species, and it is not too surprising that there are only few validated examples on such mutations in domestic animals. The work has usually started from a genome-wide screening for QTL and then proceeded to find genetic and functional support for possible causative mutations.

Many independent QTL mapping studies in dairy cattle have shown that there is QTL for milk traits on chromosome 14. After lots of effort and international collaboration it has been shown that the associations could be explained by the *DGAT1* gene that is coding acyl-coenzyme A: diacylglycerol acyltransferase. The enzyme is known to catalyze the last step in the triglyceride synthesis and a missense mutation (K232A) influences milk fat content in cattle (e.g., [40]). The fine mapping and gene identification took some 7 years.

The second example is from pigs. A single-nucleotide substitution of IGF2 (insulin-like growth factor 2) is a causal gene behind a major QTL for growth [41]. This is a regulatory mutation. It has been present in four different breeds selected for lean growth and a major reason for the successful detection is the finding of an ancestral haplotype that differs only by one nucleotide substitution from the mutant haplotype [42].

Another example is a single nucleotide substitution in the myostatin gene (*MSTN*) causing increased muscle mass in Texel sheep [43]. This mutation creates a new target site for two microRNAs expressed in muscle which leads to down regulation of MSTN transcripts.

The findings have a clear message: farm animal populations have a high haplotype diversity allowing successful genetics research to locate genes underlying QTL irrespective of the mode of action. The examples show that the molecular nature of a QTL can be as simple as a missense mutation in a coding sequence or a single nucleotide substitution in noncoding DNA. It is also impressive how in farm animals the genes behind the found QTL are regulatory mutations. The regulatory factors are much more challenging in showing the effect on gene function than mutation changing the protein sequence and structure.

There are others reported but in many cases more work is needed to demonstrate their effect in independent samples. The number of actual genes in the QTL seems very low so far but is comparable with the modest findings in man and mice where the work volume is more substantial. One obvious reason for a small number is the poor resolution in QTL mapping experiments. The confidence interval for a QTL is at best very wide and may contain hundreds of genes. Another reason is that many QTL will not have such a simple explanation as the examples described above. Some QTL will be due to several mutations present in one or many genes. Such QTL may break up into several linked loci for the fine mapping. Mutations in noncoding DNA make their detection and functional characterization also very difficult (see [28, 42] for more discussion).

Candidate Gene Approach

In the characterization of genetic factors, there are basically two types of approaches. The linkage studies

are aiming to locate genomic regions or QTL that are important sources of variation, or biological knowledge is used to deduce the genes most likely responsible for the variation. The examples above are indicating that studies to identify positional *candidate gene*s have a rather limited success. It is not therefore surprising that many researchers have restricted their studies to investigate the role of biologically deduced *candidate gene*s in explaining the variation in the available data. This approach is much more economical, on the other hand there is very little new knowledge to be discovered as the analysis only spotlights the chosen gene or genes. Sometimes, the studies have switched to candidate approach after the linkage approach has failed or included *candidate gene*s as a marginal part of wider analysis not originally designed to explore individual genes. The research angle is naturally highly subjective in choosing candidates from numerous promising ones.

The report on functional candidates should provide full details why the particular genes were chosen, where the genotyped group of animals is originating, what kind of marker set was used to eliminate the other associated effects in the genome, etc. For any gene, the combined data on its vicinity should be used to convince others about hitting the true gene in the face of too much interfering information from dozen other genes. A good design in such studies should be aiming at balanced genotype frequencies. If a gene is picked up because of ease of genotyping, the only hope is to have much luck to hold marker variants that are in *linkage disequilibrium* with the alleles causing a trait deviation. There is also the trick of having a "significant" result surface from the data by permuting several genes and traits (see [44, 45]).

It is a common problem that *candidate gene*s are not providing consistent evidence when exposed to validation in subsequent generations of the same population or more importantly, in other populations. The most beautiful investigations are finalized by functional mutation studies (knock-out or in) or complementation tests in a modeled mouse experiment, practically impossible in livestock species. One way to improve the analysis is to define a trait more explicitly. For example, calving interval could be itemized to several sub-traits, which in turn may prove to be more rewarding objects for QTL analysis. Likewise with the markers, instead of associating gene variants, it is now possibly to go a level deeper and excavate the respective gene expression patterns. The difficulties are not over when it comes to deciding which tissues and which developmental stage would provide appropriate samples for expression studies and how to plan a comparable and uniform production environment for such studies. The expression profiles are obviously an outcome influenced by several factors and their interactions are ever so hard to interpret (for more discussion, see [28]).

The complex traits in medical studies have experienced similar disappointing conclusion, and there are hardly any major genes manifesting the familial multifactor diseases. Researchers have voiced this by talking about missing explanations for the observed genetic variation or heritability [46]. In quantitative genetics, it is more and more obvious that the variation is simply caused by lots of genes with tiny effects and variants that are not appearing among the prior candidates [47]. With the rapid growth of high throughput marker typing and re-sequencing, there is now a possibility with very large population samples and concerted actions to detect reliable associations even for the small effects [48]. Different techniques, such as detailed mapping and functional studies, could be combined [49]. What remains is the requirement for large bodies of data, as has always been in analyzing genetic variation for animal breeding purposes.

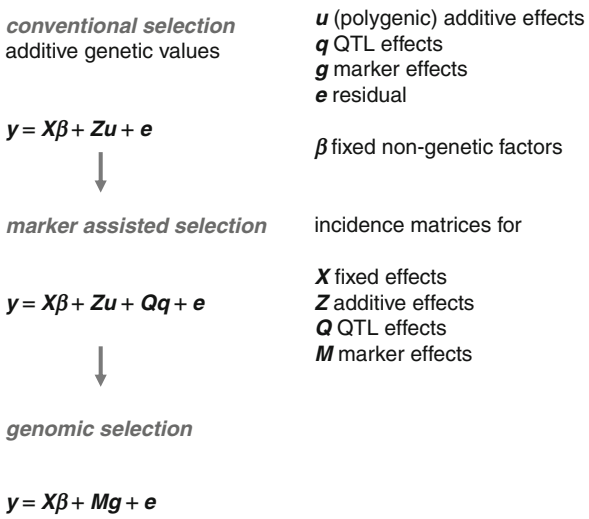
Genomic Revolution

The very fast development of molecular genetic techniques has resulted in exploitation of the existing nucleotide diversity. The genome sequence is available for chicken [50] and cattle [51]. The sequences of the whole genome in pig and sheep are still on the way. The sequence information is indicating that a typical animal genome has almost endless amount of potential variable sites. Several descriptive measures are used to summarize polymorphisms of DNA sequences. Under a neutral model, the expected level of diversity can be deduced from the generation of new alleles by mutations and from the elimination of alleles by drift (which is inversely proportional to effective population size), that is, $4\ N_e \times$ mutation rate. For comparison, in a human population two randomly chosen individuals differ at ~1 in 1,000 nucleotides (1 SNP per kilo base).

The genetic diversity of mankind is low compared to other (older) species. In cattle and sheep the mean nucleotide diversity is 2–2.5 SNPs per kilo base [52], whilst for chicken the estimate is 4–5.5 [53]. With modern DNA chip technology up to hundreds of thousands of loci across the genome could be used to genotype animals with a reasonable cost. These possibilities have opened up completely new kind of possibilities to understand and utilize the genetic variation.

MAS with More Markers

When there are several markers available, the *mixed model equations* for *breeding value* estimation could be extended to accommodate several markers with a vector of QTL effects. Using the previously introduced notation (Fig. 5), the use of several markers is with matrix presentation $\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Za} + \boldsymbol{Qq} + \boldsymbol{e}$, where $\boldsymbol{Q}$ is the incidence matrix for $\boldsymbol{q}$ [54]. Var $(\boldsymbol{q}) = \boldsymbol{G}\,\sigma_q^2$ where σ_q^2 is the variance due to QTL and $\boldsymbol{G}$ is the matrix of probabilities for QTL alleles being identical by descent. These probabilities can be computed from the pedigree, marker, and linkage map information. So far QTL mapping has produced fewer useful genes or markers for MAS than was anticipated when the QTL screening work started. The found QTL are contributing much less to the variation than would be satisfactory for a successful use of MAS, as its efficiency is affected by the proportion of variation due to a major locus [55]. Much caution should be also taken in MAS, as most of the found QTL have an overestimated effect (so-called Beavis effect). The availability of vast amounts of SNP markers should improve both these aspects: a dense marker panel covering the whole genome would be able to wrap all the genetic variation and avoid the problems of few overemphasized markers.



Animal Molecular Genetics from Major Genes to Genomics. Figure 5
The mixed model methodology is a flexible tool to accommodate the models ranging from simple breeding value prediction with polygenic model to QTL effects associated with known markers. The effects of genome-wide marker sets are computed through a straightforward summation over the genome. ***X***, ***Z***, ***Q***, and ***M*** are the incidence matrices linking the observations to respective effects

From MAS to Genomic Genetic Values

For the future practical application in estimating the genetic values, the most promising approach seems to be simultaneous utilization of a vast number of markers over the entire genome [56]. The map density is so high that the recombination between markers and QTL can be ignored. With a genome-wide set of markers, the estimate of individual's genetic value or genomic estimated *breeding value* is obtained by estimating effects associated with the markers from animals with both the phenotype and marker genotype information and summing the marker effects for recently genotyped animals available for selection. With genome-wide marker panel one can hopefully catch most of the genetic variation and produce a more attractive way to exploit markers.

The process of predicting the *breeding value*s utilizing genomic information could be described in three steps:

1. Use a genome-wide marker panel to genotype animals. It is now a norm to use densely mapped SNPs as markers. The SNP variation is seen as two alleles (potentially with four). When the marker panel is very dense and genome-wide and each marker is wrapping the genetic variation for a measured trait in its neighborhood, the whole panel is covering all the genetic variation. With additive gene action the biallelic marker is assumed to have a substitution

effect. As the whole genome is covered with the dense map, the effects of the n SNP loci would sum the genetic value of the animal.

2. Estimate the effects of each marker locus. The model for the phenotype of animal i is $y_i = \sum_{j=1}^{n} m_{ij} g_j + e_i$ where m_{ij} (2, 1 and 0) is representing the marker genotypes in the ith animal, g_j is half of the substitution effect at the marker locus for the quantitative trait and e is the residual. The phenotype and genotype data could therefore be used to estimate the marker effects (ignoring all the nongenetic factors) with the model in matrix notation $\boldsymbol{y} = \boldsymbol{M}\,\boldsymbol{g} + \boldsymbol{e}$. The new method could be seen as part of the development in mixed model methodology (Fig. 5). The first two steps are carried out in a sufficiently large reference population with information on both marker genotypes and trait phenotypes.
3. The genomic *breeding value* could be computed for genotyped selection candidates by summing the estimated effects over the markers.

There are several options how the equations could be solved. Treating the marker effects as random, it is customary to assume that their variance is constant over the genome or that there are few loci with large effects with the majority having small effects. In addition, there are options for the distribution of the effects. The case of normally distributed effects with constant variance is interesting because the *breeding value* predictions would be equivalent with *BLUP* where the pedigree-based relationship matrix A is replaced by a relationship matrix estimated from the marker information [57]. The latter is also called genomic relationship matrix and is technically *MM'*. The genomic information has also been successfully used to incorporate the realized relationships into the A matrix used in *BLUP* [58].

It is the Bayesian methods that are able to cope with situations where an allowance for graduated effects of markers is made. This is beneficial as the QTL screening works is clearly showing that there are genomic regions having relatively large effects. Many distributions have been tried: a case where most markers have a small effect and very few a large effect, alternatively assuming 0 for several markers and nonzero for few markers. These were studied in the original paper [56] and the former approach is known as BayesA and the latter one as BayesB. Meuwissen and Goddard [36] also presented an alternative where the variances of marker effects are sampled from a mixture of two distributions allowing the variation in the effect size across markers. Simple methods are possibly favored so far, as pure Bayesian approaches would require more computing time. Related to this, the rapidly increasing number of markers – hundreds of thousands – and availability of sequence data and larger number of genotyped animals would also challenge the computing capacities.

The accuracy of genomic *breeding value*s depends of course on the number of records and proportion of genetic variation or heritability h^2. Small *effective population size* is generating linkage disequilibrium. Related to linkage disequilibrium, the genome contains regions of reduced haplotype diversity, termed haplotype blocks [59], separated by blocks of higher diversity. Within such blocks the frequencies of marker alleles may be highly correlated across loci. The genome blocking sets limits to the estimation even if the marker density is very high. The number of independent segments for a chromosome of length L (in *Morgan*s) is $2\,N_e\,L/\ln(4N_e\,L)$ [57]. So, a chromosome of 1M has some 33 independent segments and the whole genome of 30 such chromosomes would have 1,000 independent segments. If the *effective population size* is small, increasing more markers would not improve the accuracy or resolution in explaining the genetic variation and the best one can do in that situation, is increasing the number of animals in the analysis [57, 60]. In conclusion, the accuracy of genomic *breeding value*s would depend on the number of animals in the reference population, the heritability of the trait, the marker density, the number of independent genome segments (or effective population size) and the total length of the genome.

With best linear unbiased prediction and Bayesian method, the accuracy of predicting genetic values for offspring of the recorded animals has been shown to be of the order 0.7–0.8 [56], comparable to that of the progeny test. The genomic selection method was presented in 2001 and did not receive much attention, as at that time the required coverage of the whole genome with markers was not feasible, at least in terms of costs. When the high throughput genotyping

of SNPs with microarrays became available and Schaeffer [61] pointed out the remarkable returns and savings that the genomic selection could produce in dairy cattle breeding, the breeding companies rushed out to exploit the tool with unparalleled enthusiasm. He compared genomic selection with conventional progeny testing and assumed that there were marker effects available with accuracy 0.75 to select bulls and bull dams. As much less bulls would be kept after genomic "pre-selection," the cost of the marker-based improvement would be only 10% of the traditional one. Another radical feature of genomic selection, is the sufficiently reliable evaluation of young animals which would reduce the generation interval drastically, for example, in the bull–bull path from 6 years down to less than 2 years. When it takes 5–7 years to have the progeny test result for a dairy bull, the genomic genetic value is available at birth. Putting the slightly lower accuracy and shortened generation interval together, the annual genetic change is predicted to be doubled.

Much of the work on assessing the advantages of genomic selection has been done by simulation. The reliabilities of genomic *breeding value*s could be assessed by dividing the real data into two parts, usually over birth date and compare how the predictions based on older animals compare with the estimates obtained for the more recent ones with all the data. VanRaden et al. [62] concluded that the reliability is proportional to the size of the reference population while the increments in marker density had a smaller impact. They were implying that a reference population of few thousand progeny tested bulls is required to arrive at a satisfactory accuracy of genomic *breeding value*s. Only the Holstein breed has such numbers and even there the breeding companies would need collaborative efforts across countries for reaching a sufficient number of accurately known sires.

Breeds with smaller populations (and progeny tested bulls) could in principle resort to the marker effects estimated in a large population. If the marker density is very high, genomic *breeding value*s estimated in one breed may support the estimation in the other breed [63]. They may be some common *linkage disequilibrium* within a short distance across the breeds otherwise recombination has broken associations since the divergence. Selection and drift have also changed the genotype frequencies over time.

Management of Variation

Very intensively selected populations may suffer from losing variation or, in a more extreme case, from inbreeding depression and appearance of recessives defects. This is due to the reduced number of selected parents to generate the breeding bulls. The risk for inbreeding and drift is expressed with *effective population size* or rate of inbreeding ($\Delta F = 1/(2N_e)$). The safe areas for these would be over 50 and less than 1%, respectively (e.g., [64]). In dairy cattle, the awareness on rate of inbreeding was triggered by the breeding schemes utilizing multiple ovulation and embryo transfer (MOET). It was shown that the schemes could be optimized with respect to genetic progress and costs, by concentrating the MOET operation on cows in a nucleus of few hundred cows only. The apparent small number of parents and larger full-sib families accompanied by early selection would easily mean risks for higher rates of inbreeding. This kind of worries generated lots of research and now the better understanding about the related risks in selection programs has resulted in new tools. The most important aspect is to maximize the variation in selected groups of males and females while the formation of mating pairs is less relevant. Meuwissen [65] has developed so-called optimum contribution method which yields the guidance in choosing parents and their progeny numbers to maximize the genetic gain while minimizing the coancestry. The constraining on the coancestry is based on the relationship matrix and therefore the optimum contribution method could be easily integrated with the information needed for *BLUP* evaluation.

Genomic *breeding value*s are based on information on the individuals themselves and are therefore better than *BLUP breeding value*s which may more often lead to co-selection of sibs due to family information [66]. So the genomic selection has short-term advantages for the management of genetic variation. In the long-term an efficient genomic selection is automatically reducing variation. The genomic information is on the other hand giving an accurate estimate on the relationship between individuals. While the pedigree based matrix gives the expected relationships, the marker-based matrix yields the realized relationships. The earlier work (e.g., [67]) showed that tens or even hundreds

of markers (e.g., microsatellites), is of limited value in estimating the relationships and in controlling the rate of inbreeding. The use of genome-wide SNP panels has changed the situation and molecular similarities could be calculated very accurately and used like the genealogical information. Just like the relationship matrix in the *BLUP* context is improved by the genomic information, in the same way the genomic relationship matrix could enhance the management of variation. There are only preliminary results [68] which are indicating that high benefits could be anticipated from the use of genomic relationship matrix in promoting the sustainable use of genetic variation.

Genetic Correlation

It is a common observation that there is a negative genetic correlation between production traits and longevity, made of such traits as disease resistance and fertility. It is widely accepted that such a correlation is caused by pleiotropy so that the same genes would have opposite effects on the two sets of traits. This could be understood as a consequence of allocating limited metabolic resources to competing physiological compartments within an animal. If a genetic correlation between two traits is positive, it can be thought that the effects on the two traits are on average influencing to the same direction. On the other hand, if the majority of effects are of opposite "sign," a negative correlation would be seen. However, there should be some variation over the pleiotropic genes in the genome, there being also some with positive effect on both the traits. Therefore, selection could be differentiated over the genome to avoid undesirable effects in longevity traits while improving the production efficiency. This is something one is going to learn when multiple trait analysis are thoroughly investigated with the dense genome-wide marker information.

Extensions to a Pair of Populations

The introgression of multifactor traits from one population to another should enjoy the powerful genomic tools in making the procedure more precise and diminishing the compromises of receiving undesirable sections of the donor genome. Ødegård et al. [69] showed that genomic selection results in an efficient introgression of desirable QTL alleles from a donor line, as the genome of the donor apart from the QTL could be selected against. Lots of data is obviously needed for separating the beneficial and unattractive genome parts in a candidate exotic breed.

Heterosis or hybrid vigor in crosses has been long observed. Predicting which lines will give good crosses is a hit and miss affair. Simple dominance is sufficient to give crossbreds better performance than either parent provided that both parents are fixed for the dominant allele at some locus at which the other parent is fixed for the recessive one – that is with one parent being Bc/Bc and the other bC/bC, where B and C are the dominant alleles.

Several breeding procedures have been suggested and used in order to make best use of the *heterosis*, among them the reciprocal recurrent selection, proposed by Comstock et al. [70] makes the most use of quantitative genetic principles. The benefits from crossbreeding are highest with widely deviating allele frequencies between the breeds. There is a need for efficient methods with immediate returns to evaluate the most promising breed crosses jointly with the most potential mating pairs.

Starting from the results by Smith and Mäki-Tanila [6], theory and methods to compute genotypic means and covariances in a two-breed population under dominance inheritance have been presented by Lo et al. [71]. They showed that the genotypic mean is a linear function of 5 location parameters and that the genotypic covariance between relatives is a linear function of 25 dispersion parameters. These would include the additive (and the corresponding heritability) and dominance variance in both the purebred populations and the variation of contributions from the breeds to the F_1 individuals. Clearly simpler and more parsimonious methods are needed.

Genomic research has proven to be a powerful approach in quantifying the genetic distances between populations, in revealing history of animal populations, number and sites for domestication, population expansions and contractions, selection, origin, and mixing of maternal and paternal lineages (see [72]). Because *heterosis* is proportional to the differences in gene frequencies in the parental lines, it is possible to make marker-based prediction of hybrid performance based on genetic distances, despite having

only indirect estimates of allele frequencies for the interesting traits *via* the anonymous markers.

Xu [73] stimulated by the elegance of genomic selection has extended a Bayesian analysis to the F_2 population of inbred lines and has successfully also estimated the dominance deviations. However, the cases starting from random breeding populations – in subdivided population or in crossbred animals, typical in animal breeding – need research. Toro and Varona [74] have investigated the use of genomic information in predicting also dominance effects and the advantages of designing matings to have a full advantage of the extra component in the evaluation. First of all, the genomic approach was possible compared to the overcomplicated polygenic parameterization. Secondly, the prediction of additive effects had a higher accuracy. Finally, the immediate response was higher when dominance effects were estimated, although the subsequent progress stayed very much the same. If the cost of genotyping is reasonable, the estimation of dominance effects could be seen as a new tool in utilizing *heterosis* in chicken and pig production where crosses of different lines are a norm.

Research on Beneficial and Harmful Genes

Along the use of genome-wide marker sets in large reference populations, it is possible to map medium to large size QTL. This would yield genomic predictions that are stable across families and generations. If the genes underlying QTL are found, they may be further studied for the pleiotropic effects on other traits, dominance effects, interactions with other QTL (epistasis), or response to environmental changes. Information derived from such studies will lead to better models and better predictions and even management of phenotypes, which can be used for selection and production planning.

There are several Mendelian defects identified in farm animals. Recessive disorders are a problem in efficient improvement schemes, because healthy carriers can spread the disease allele quickly to a large number of progeny. A good example is BLAD (bovine leukocyte adhesion deficiency) causing a severe immunodeficiency. The mutation got widely distributed in the Holstein cattle due to a famous bull few decades ago. The missense mutation is now identified [75] and the eradication program has been successful. Charlier et al. [76] reported fine-scale mapping of five recessive disorders in cattle using large SNP panel. Between 25,000 and 50,000 SNPs were used in the discovery populations. Homozygosity mapping is used to detect mutations that cause disease when both copies are present (recessivity). Three disorders were mapped with a sufficient resolution so that the molecular basis could be characterized and effective eradication tools were established and used in mating planning. A prerequisite for finding recessive disorders is a well-organized recording of defects and diseases in the population. In general, molecular genetic techniques could be used to develop diagnostic tests. On the other hand the disorders are providing animal models to study human diseases.

Future Directions

The conventional prediction methods based on *infinitesimal model* have produced very impressive results in quantitative traits of animal production. There is, however, plenty of evidence on the existence of major genes mediation the variation in such measured traits. For some time now, it has been possible to map such genes in the genome using molecular genetic markers. The accurate localization is needed for estimating the gene effects and integrating their direct selection into methods predicting the polygenic effects. There has been extensive genome screenings for QTL carried out all the farm animal species, either with crossbreeding or half-sib design. The outcome is thousands of QTL while the high-resolution localization has yielded only very few causal mutations underlying the found QTL. Also the variation contributed by these QTL is far too low for efficient marker- or gene-assisted selection. The molecular genetic technology has in the mean time taken further leaps and now there are available microarray chips containing thousands or even hundreds of thousands of SNPs to allow genomic selection. Genomic selection is in principle genome-wide *marker-assisted selection*. As it covers all the genetic variation in the genome, the estimated of marker effects would give sufficiently reliable predictions of genetic values for young animals. Hence, the genetic improvements programs could be accelerated and high savings could be done in testing schemes. Genomic information could be further used for management of genetic

variation, mating planning utilizing non-additive variation, understanding genetic correlation architecture, etc. The animal breeding industry has quickly adopted the new technology and is now widely exploiting the latest SNP panels in the selection schemes. As a side product, the systematic genome-wide screening is picking up harmful and beneficial mutations for further research and applications. It is very tempting and certainly very challenging to try to model the variation via complicated pathways and interactions due to individual genes and regulatory factors. No doubt many would try. Luckily animal breeding research has a long and flourishing history of sophisticated mathematics and statistics. Hence, much research will be devoted to the area. The main test for a new method in animal breeding would consist of questions like: how it is going to help us in understanding variation, how would it help to improve prediction of breeding value, what gains are made in efficiency of selection programs, what kind of savings could be made in testing, etc. Finally, to have firm ground in analyses and decision making, there is a continuous need to have lots of information, both on the genomes and the measurable traits.

Acknowledgment

I am grateful to colleagues, especially Pekka Uimari, at MTT, for useful comments on the draft of the text, and to Ignacy Misztal for lots of encouragement.

Bibliography

Primary Literature

1. Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman, Harlow, 464 p
2. Weller JI (2009) Quantitative trait loci analysis in animals, 2nd edn. CABI, Wallingford
3. Henderson CR (1984) Applications of linear models in animal breeding. University of Guelph, Guelph
4. Henderson CR (1976) A simple method for computing the inverse of a numerator relationship matrix used for prediction of breeding values. Biometrics 32:69–79
5. Wray NR, Hill WG (1989) Asymptotic rates of response from index selection. Anim Prod 49:217–227
6. Smith SP, Mäki-Tanila A (1990) Genotypic covariance matrices and their inverses for models allowing dominance and inbreeding. Genet Sel Evol 122:65–91
7. Silvela L (1980) Genetic changes with generations of artificial selection. Genetics 95:769–782
8. Mäki-Tanila A (1982) The validity of the heritability concept. Ph.D. thesis, University of Edinburgh, 120 p
9. Mäki-Tanila A, Kennedy BW (1986) Mixed model methodology under genetic models with a small number of additive and non-additive loci. In: Proceedings of 3rd world congress on genetics applied to livestock production 12, Lincoln, pp 443–448
10. Webb AJ, Carden AE, Smith C, Imlah P (1982) Porcine stress syndrome in pig breeding. In: Proceedings of 2nd world congress on genetics applied to livestock production 5, pp 588–608
11. Merat P, Ricard FH (1974) Etude d'un Gene de nanisme lié au sexe chez la poule: importance de l'état d'engraissement et gain de poids chez l'adulte. Ann Genet Sel Anim 6:211–217
12. Rollins WC, Tanaka M, Nott CFG, Theissen RB (1972) On the mode of inheritance of double muscled conformation in bovines. Hilgardia 41:433–456
13. Piper LR, Bindon BM (1982) The genetic segregation for fecundity in Booroola Merino sheep. In: Barton RA, Smith WC (eds) Proceedings of world congress on sheep and beef cattle breeding. Dunmore, Palmerston North, pp 315–331
14. Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743–756
15. Dekkers JCM (2004) Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. J Anim Sci 82:E313–E328
16. Pong-Wong R, Woolliams JA (1998) Response to mass selection when an identified major gene is segregating. Genet Sel Evol 30:313–337
17. Dekkers JCM, Hospital F (2002) The use of molecular genetics in the improvement of agricultural populations. Nat Rev Genet 3:22–32
18. Yancovich A, Levin I, Cahaner A, Hillel J (1996) Introgression of the avian naked neck gene assisted by DNA fingerprint. Anim Genet 27:149–155
19. Knap PW, Neeteson-Van Nieuwenhoven AMA (2005) Private and public roles in conservation. In: Proceedings of options and strategies for conservation of FAnGR., Montpellier, pp 62–67
20. Bidanel JP (1989) Etude de stratégies de valorisation en croisement de la race Meishan. 3 – evaluation comparée de différents systèmes de croisement. J Rech Porcine en France 21:361–366
21. Legault C (1998) Génétique et prolificité chez la truie: la voie hyperprolifique et la voie sino-européenne. INRA Prod Anim 11:214–218
22. Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer, Sunderland
23. Elston RC, Stewart J (1973) The analysis of quantitative traits for simple genetic models from parental, F1 and backcross data. Genetics 73:695–711
24. Neimann-Sorensen A, Robertson A (1961) The association between blood groups and several production characteristics in three Danish cattle breeds. Acta Agric Scand 11:163–196

25. Soller M, Genizi A (1978) The efficiency of experimental designs for the detection of linkage between a marker locus and a locus affecting a quantitative trait in segregating populations. Biometrics 34:47–55
26. Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199
27. Haley CS, Knott SA (1992) A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. Heredity 69:315–324
28. Georges M (2007) Mapping, fine mapping, and molecular dissection of quantitative trait loci in domestic animals. Annu Rev Genomics Hum Genet 8:131–162
29. Andersson L, Haley CS, Ellegren H, Knott SA, Johansson M, Andersson K, Andersson-Eklund L, Edfors-Lilja I, Fredholm M, Hansson I et al (1994) Genetic mapping of quantitative trait loci for growth and fatness in pigs. Science 263:1771–1774
30. Georges M, Nielsen D, Mackinnon M, Mishra A, Okimoto R, Pasquino AT, Sargeant LS, Sorensen A, Steele MR, Zhao X, Womack JE, Hoeschele I (1995) Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. Genetics 139:907–920
31. Darvasi A (1998) Experimental strategies for the genetic dissection of complex traits in animal models. Nat Genet 18:19–24
32. Weller J, Kashi Y, Soller M (1990) Power of daughter and granddaughter designs for determining linkage between marker loci and quantitative trait loci in dairy cattle. J Dairy Sci 73:2525–2537
33. Mackay TF (2009) The genetic architecture of complex behaviors: lessons from *Drosophila*. Genetica 136:295–302
34. Calus MP, Meuwissen TH, Windig JJ, Knol EF, Schrooten C, Vereijken AL, Veerkamp RF (2009) Effects of the number of markers per haplotype and clustering of haplotypes on the accuracy of QTL mapping and prediction of genomic breeding values. Genet Sel Evol 41:11
35. Farnir F, Coppieters W, Arranz JJ, Berzi P, Cambisano N, Grisart B, Karim L, Marcq F, Moreau L, Mni M, Nezer C, Simon P, Vanmanshoven P, Wagenaar D, Georges M (2000) Extensive genome-wide linkage disequilibrium in cattle. Genome Res 10:220–227
36. Meuwissen THE, Goddard ME (2004) Mapping multiple QTL using linkage disequilibrium and linkage analysis information and multitrait data. Genet Sel Evol 36:261–279
37. Goddard ME, Meuwissen TH (2005) The use of linkage disequilibrium to map quantitative trait loci. Aust J Exp Agric 45:837–845
38. Meuwissen THE, Karlsen A, Lien S, Olsaker I, Goddard ME (2002) Fine mapping of a quantitative trait locus for twinning rate using combined linkage and linkage disequilibrium mapping. Genetics 161:373–379
39. Spielman RS, McGinnis RE, Ewens WJ (1993) Transmission test for linkage disequilibrium: the sinsulin gene region and insulin dependent diabetes mellitus. Am J Hum Genet 52:506–516
40. Grisart B, Farnir F, Karim L, Cambisano N, Kim JJ, Kvasz A, Mni M, Simon P, Frère JM, Coppieters W, Georges M (2004) Genetic and functional confirmation of the causality of the DGAT1 K232A quantitative trait nucleotide in affecting milk yield and composition. Proc Natl Acad Sci USA 101:2398–2403
41. Van Laere AS, Nguyen M, Braunschweig M, Nezer C, Collette C, Moreau L, Archibald AL, Haley CS, Buys N, Tally M, Andersson G, Georges M, Andersson L (2003) A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature 425:832–836
42. Andersson L (2009) Genome-wide association analysis in domestic animals: a powerful approach for genetic dissection of trait loci. Genetica 136:341–349
43. Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibé B, Bouix J, Caiment F, Elsen JM, Eychenne F, Larzul C, Laville E, Meish F, Milenkovic D, Tobin J, Charlier C, Georges M (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet 38:813–818
44. Tabor HK, Risch NJ, Myers RM (2002) Candidate-gene approaches for studying complex genetic traits: practical considerations. Nat Rev Genet 3:391–397
45. Zhu M, Zhao S (2007) Candidate gene identification approach: progress and challenges. Int J Biol Sci 3:420–427
46. Maher B (2008) Personal genomes: the case of the missing heritability. Nature 456:18–21
47. Hill WG (2010) Understanding and using quantitative genetic variation. Phil Trans R Soc B 365:73–85
48. Yang J, Benyamin B, McEvoy BP, Gordon S, Henders AK, Nyholt DR, Madden PA, Heath AC, Martin N, Montgomery GW, Goddard ME, Visscher PM (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42:565–569
49. Karim L, Takeda H, Lin L, Druet T, Arias JA, Baurain D, Cambisano N, Davis SR, Farnir F, Grisart B, Harris BL, Keehan MD, Littlejohn MD, Spelman RJ, Georges M, Coppieters W (2011) Variants modulating the expression of a chromosome domain encompassing PLAG1 influence bovine stature. Nat Genet 43:405–413
50. Wallis JW, Aerts J, Groenen MAM, Crooijmans RPMA, Layman D, Graves TA, Scheer DE, Kremitzki C, Fedele MJ, Mudd NK, Cardenas M, Higginbotham J, Carter J, McGrane R, Gaige T, Mead K, Walker J, Albracht D, Davito J, Yang S-P, Leong S, Chinwalla A, Sekhon M, Wylie K, Dodgson J, Romanov MN, Cheng H, de Jong PJ, Osoegawa K, Nefedov M, Zhang H, McPherson JD, Krzywinski M, Schein J, Hillier L, Mardis ER, Wilson RK, Warren WC (2004) A physical map of the chicken genome. Nature 432:761–764
51. Bovine Genome Sequencing and Analysis Consortium (2009) The genome sequence of taurine cattle: a window to ruminant biology and evolution. Science 324:522–528
52. Meadows JRS, Hawken RJ, Kijas JW (2004) Nucleotide diversity on the ovine Y chromosome. Anim Genet 35:379–385
53. Hillier LW, Miller W, Birney E et al (2004) Sequence and comparative analysis of the chicken genome provide unique

perspectives on vertebrate evolution. Nature 432:695–716 (International Chicken Genome Sequencing Consortium)
54. Fernando RL, Grossman M (1989) Marker assisted selection using best linear unbiased prediction. Genet Sel Evol 21: 467–477
55. Meuwissen THE, Goddard ME (1996) The use of marker haplotypes in animal breeding schemes. Genet Sel Evol 28:161–176
56. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
57. Goddard ME (2009) Genomic selection: prediction of accuracy and maximisation of long term response. Genetica 136:245–257
58. Legarra A, Aguilar I, Misztal I (2009) A relationship matrix including full pedigree and genomic information. J Dairy Sci 92:4656–4663
59. Wall JD, Pritchard JK (2003) Haplotype blocks and linkage disequilibrium in the human genome. Nat Rev Genet 4:587–597
60. Daetwyler HD, Villanueva B, Woolliams JA (2008) Accuracy of predicting the genetic risk of disease using a genome-wide approach. PLoS One 3:e3395
61. Schaeffer LR (2006) Strategy for applying genome-wide selection in dairy cattle. J Anim Breed Genet 123:218–223
62. VanRaden PM, VanTassell CP, Wiggans GR, Sonstegard TS, Schnabel RD, Taylor JF, Schenkel FS (2009) Invited review: reliability of genomic predictions for North American Holstein bulls. J Dairy Sci 74:2737–2746
63. De Roos APW, Schrooten C, Mullaart E, van der Beek S, de Jong G, Voskamp W (2009) Genomic selection at CRV. Interbull Bull 39:47–50
64. Woolliams J, Berg P, Mäki-Tanila A, Meuwissen T, Fimland E (2005) Sustainable management of animal genetic resources. NGH Nordisk Genbank Husdyr, Norway, 95 p
65. Meuwissen THE (1997) Maximizing the response of selection with a predefined rate of inbreeding. J Anim Sci 75:934–940
66. Daetwyler HD, Villanueva B, Bijma P, Woolliams JA (2007) Inbreeding in genome-wide selection. J Anim Breed Genet 124:369–376
67. Fernandez J, Villaneuva B, Pong-Wong R, Toro MA (2005) Efficiency of the use of pedigree and molecular marker information in conservation programmes. Genetics 170:1313–1321
68. Sonesson AK, Woolliams JA, Meuwissen THE (2010) Maximising genetic gain whilst controlling rates of genomic inbreeding using genomicoptimum contribution selection. In: Ninth world congress on genetics applied to livestock production, Leipzig
69. Ødegård J, Yazdi MH, Sonesson AK, Meuwissen TH (2009) Incorporating desirable genetic characteristics from an inferior into a superior population using genomic selection. Genetics 181:737–745
70. Comstock RE, Robinson HF, Harvey PH (1949) A breeding procedure designed to make maximum use of both general and specific combining ability. J Am Soc Agron 41:360–367
71. Lo LL, Fernando RL, Cantet RJC, Grossman M (1995) Theory for modelling means and covariances in a two-breed population with dominance inheritance. Theor Appl Genet 90:49–62
72. Toro MA, Mäki-Tanila A (2007) Genomics reveals domestication history and facilitates breed development. In: Oldenbroek K (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic, The Netherlands, pp 75–102, 232 p
73. Xu S (2003) Estimating polygenic effects using markers of the entire genome. Genetics 163:789–801
74. Toro MA, Varona L (2010) A note on mate allocation for dominance handling in genomic selection. Genet Sel Evol 42:33. http://www.gsejournal.org/content/42/1/33
75. Shuster DE, Kehrli ME, Ackermann MR, Gilbert RO (1992) Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc Natl Acad Sci USA 89:9225–9229
76. Charlier C, Coppieters W, Rollin F, Desmecht D, Agerholm JS, Cambisano N, Carta E, Dardano S, Dive M, Fasquelle C, Frennet JC, Hanset R, Hubin X, Jorgensen C, Karim L, Kent M, Harvey K, Pearce BR, Simon P, Tama N, Nie H, Vandeputte S, Lien S, Longeri M, Fredholm M, Harvey RJ, Georges M (2008) Highly effective SNP-based association mapping and management of recessive defects in livestock. Nat Genet 40:449–454

Aquaculture and Renewable Energy Systems, Integration of

Bela H. Buck[1,2,3], Gesche Krause[4]
[1]Biological Sciences – Marine Aquaculture, Alfred Wegener Institute for Polar and Marine Research (AWI), Bremerhaven, Germany
[2]Marine Aquaculture for Sustainable Fisheries, Institute for Marine Resources (IMARE), Bremerhaven, Germany
[3]Maritime Technologies – Applied Marine Biology, University for Applied Sciences, Bremerhaven, Germany
[4]Leibniz Center for Tropical Marine Ecology (ZMT), Bremen, Germany

Article Outline

Glossary

Aquaculture Following the definition of the FAO [1, 2], *aquaculture* is the farming of aquatic organisms, including fish, molluscs, crustaceans, and aquatic plants with some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators. Specifically, *marine aquaculture*, also called *mariculture*, concentrates on aquatic organisms cultivated in brackish or marine environments.

Integrated Coastal Zone Management (ICZM) A process for the management of the coast using an integrated approach, regarding all aspects of the coastal zone, including geographical and political boundaries, in an attempt to achieve sustainability. The EU Commission [3] defines ICZM as a dynamic, multidisciplinary, and iterative process to promote sustainable management of coastal zones. It covers the full cycle of information collection, planning (in its broadest sense), decision making, management, and monitoring of implementation. ICZM uses the informed participation and cooperation of all stakeholders to assess the societal goals in a given coastal area, and to take actions towards meeting these objectives. ICZM seeks, over the long term, to balance environmental, economic, social, cultural, and recreational objectives, all within the limits set by the natural dynamics.

Mariculture See "aquaculture".

Offshore aquaculture A culture operation in a frequently hostile open ocean environment exposed to all kinds of sea states as well as being placed far off the coast.

Offshore co-management A dynamic partnership using the capacities and interests of different stakeholder groups for managing cross-sectoral activities in cooperation with governmental authorities in the open sea.

Offshore wind farms A group of wind turbines in the same confined area used for production of electric power in the open ocean. Moving off the coast to the offshore, wind turbines are less obtrusive than turbines on land, as their apparent size and noise is mitigated by distance. Since water has less surface roughness than land (especially in deeper waters), the average wind speed is usually considerably higher over the open water. Therefore, the capacity factors are considerably higher than for onshore and nearshore locations [4].

Open ocean aquaculture See "Offshore aquaculture".

Definition of the Subject

"Fisheries have rarely been sustainable." This statement by Pauly et al. [5] was based on the recognition that this lack of sustainability was induced by a serial depletion of wild stocks worldwide. Causative for this trend is due to the improved fishing technology, geographical expansion, and exploitation of previously spurned species lower in the food web. In exchange, aquaculture was often either regarded to bridge the gap between supply and demand or, in contrast, even to exacerbate this scenario.

Since the 1970s, aquaculture production has grown quite rapidly and is by now one of the fastest growing

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

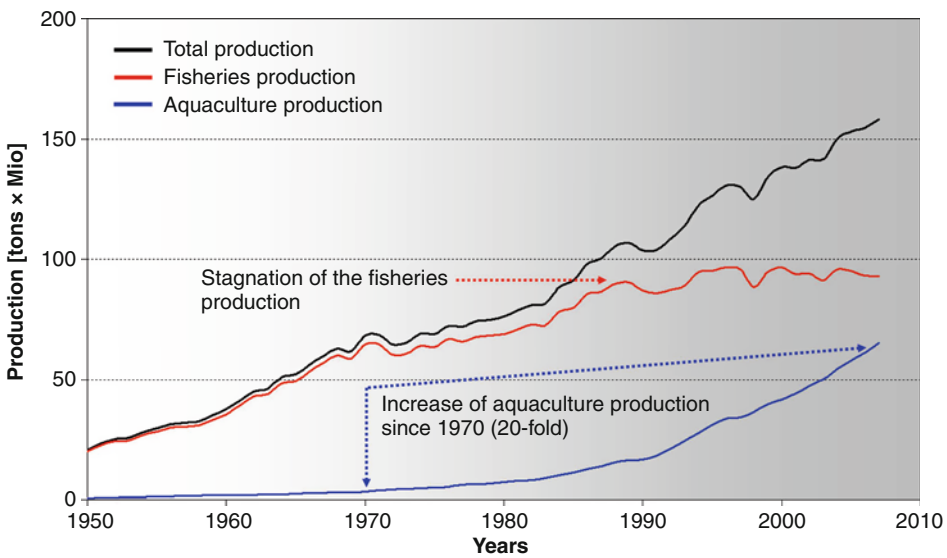
Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

aquatic food production sectors in the world [6]. Besides the rapid development of this sector, the wide-ranging decline in fisheries yields has been enhanced by an increase in public demand for aquatic products. With an annual share of more than 15% of total animal protein supplies, the production of captured fisheries and aquaculture plays a significant role in the global food security [6]. In 2007, approximately 160 million tons of aquatic organisms were produced worldwide (Fig. 1). From that, the share of global aquaculture production amounts to almost 47%, totaling about 60 million tons annually of aquatic organisms [7, 10]. A wide range of aquatic species is raised in various systems, onshore as well as in the ocean. According to the FAO [6], approximately 300 different species, ranging from fish to shellfish, crustaceans and algae are produced in aquaculture systems. Most of these aquaculture enterprises are concentrated in well-protected and therefore favorable inshore water areas [11].

Even probably though over-reporting its aquaculture production [12], the People's Republic of China has contributed approximately 70% to the world's aquaculture production in 2004. It is nevertheless debatable, whether this production can compensate for the global deficiency in aquatic food. In addition, the intensive traditional aquaculture of carnivorous species does not automatically relieve pressure on ocean fisheries [13]. Salmon farming, e.g., requires large inputs of wild fish as fish oil and fish meal for the production of fish feed for aquaculture. Hence, the farming of non-carnivorous species that is not dependent on fishmeal-based feeds is considered a sustainable way of producing food. However, the global increase in production originates from herbivorous species. Further, the balance between carnivorous and non-carnivorous species in aquaculture production is heavily skewed towards herbivorous species [14].

On top of this issue, an increasing limitation of favorable coastal sites for the development of modern aquaculture is evident in various countries, such as Germany, the Netherlands, Belgium, as well as others [15]. This spatial limitation is mainly caused by the lack of protected nearshore areas and by the fact that regulatory frameworks that assign specific areas for aquaculture operations are diverse and still emerging.

Further, the utilizations of coastal marine waters are manifold and quite competitive, such as shipping (trade or private), recreational activities, extraction or



Aquaculture and Renewable Energy Systems, Integration of. Figure 1
Global production of aquatic organisms originating from fisheries and aquaculture within the last 55 years (Data source [7], modified after [8, 9])

disposal of gravel, marine missions, fisheries, mariculture, offshore wind farms, cable and pipelines, establishment of nature reserves, and other marine and coastal protected areas. In addition, overlapping use of coastal habitats adds to the increasing pollution of coastal waters in various situations and gives rise to spatial conflicts, thus leaving little room for the expansion of modern coastal aquaculture systems.

This situation in most industrialized countries is often in contrast to the production progress in developing countries. Here, the installation of aquaculture systems benefits from the often weak enforcement of integrated coastal management schemes, which regulate equal access to the coastal resources [16, 17]. Thus, the rise of aquaculture production has specifically taken place in developing countries, especially in Asia, which holds approximately 91.4% of the global production share [10, 18, 19]. In contrast, the number of competing users within offshore regions is relatively low, thus favoring the offshore environment for further commercial development, such as offshore wind farming and open ocean aquaculture. So far, spatial regulations offshore are scarce and clean water can be expected [20]. Thus, there is an enormous economic potential for *extensive marine aquaculture* in offshore areas.

Introduction

Aquaculture has been increasing dramatically in most parts of the world and now accounts for more than 47% of the total global seafood supply [7]. Many people generally assess aquaculture positively as a potential alternative to global fishery resources, which are globally under stress as a result of overfishing. However, it also raises concerns over pollution, disease transmission, and other socio-economic impacts. Almost all efforts to develop marine aquaculture have focused on state jurisdictional waters of the coastal sea, which are generally situated within 3 nautical miles off the shore [21, 22]. With the convergence of environmental and aesthetic concerns, aquaculture, which is already competing for space with other more established and accepted uses, is having an increasingly difficult time expanding in nearshore waters [23]. Therefore, alternative approaches are needed in order to allow the expansion of the marine aquaculture sector to make a meaningful and sustainable contribution to the world's seafood supply.

The political recognition – on a national as well as on EU level – that the implementation of integrated coastal zone management (ICZM) is still fragmentary, acted as incentive to investigate in more detail how this could be overcome [e.g., 24]. This lack was recognized and led to the operation of a EU-Demonstration Programme on Integrated Coastal Zone Management from 1996 to 1999. This Programme was designed around a series of 35 demonstration projects and six thematic studies. In 2002, based on the experiences and outputs of the Demonstration Programme, the EU-Commission adopted a recommendation concerning the implementation of Integrated Coastal Zone Management in Europe (Recommendation of the European Parliament and of the Council, 2002/413/EC). In Germany, this generated a call of the Federal Ministry of Education and Research to the various federal states to develop projects that address ICZM on a regional level. In 2004, the program *Coastal Futures* [25], which tied up various administrative and scientific bodies and the public along the west coast of the State of Schleswig-Holstein, was granted funding. This program focused primarily on two issues: [1] to develop the future of the coast as a living, working, and recreational space for the local population, and [2] to consider the potential contribution of coastal resources to the sustainable development on the national and EU/global level, i.e., by providing regenerative energy by wind power. In order to sustain sufficient open space for future development, the idea of combining offshore wind power generation with other uses, such as aquaculture operations, emerged [26]. Marine aquaculture is a growing enterprise in Germany as well as in the whole of Europe, strongly motivated by the decline of fisheries production and the search for alternative income options for rural peripheral coastal regions.

In order to stimulate multifunctional use of marine space, it was decided to develop a project on a showcase basis, which deals not only with different scientific fields but also with private–public partnerships and the relevant institutional bodies. In the following, an overview on the current state of research undertaken within this focus is provided. Offshore

wind farms will hereby act as a case example for renewable energy systems in the open ocean.

Offshore Aquaculture – A New Addition to Marine Resource Use

Farming in the open ocean has been identified as one potential option for increasing seafood production and has been a focus of international attention for more than a decade. *Offshore aquaculture* or *open ocean aquaculture* are operations in a marine environment fully exposed to all kinds of oceanographic conditions [27] as well as located at least 8 nautical miles off the coast [15] to avoid the many stakeholder conflicts in nearer coastal areas [28]. The procedures and applied techniques for the cultivation of organisms mainly depend on the species; their life cycle determines the phases of cultivation and the location for the grow-out, where market size will be reached. First trials of cultivation were based on *extensive marine aquaculture*, which – in contrast to intensive aquaculture – is a line of production with little impact on the marine environment. These aquaculture operations are characterized by (1) a low degree of control (i.e., environmental control, nutrition, predators, competitors, and disease agents), (2) low initial costs, (3) low level technology, (4) low-production efficiency, and (5) high dependence on local climate and water quality (natural water bodies, such as bays, ponds, embayments) [29]. Mostly, they are regarded as a sustainable line of production.

Moving to the open ocean has been considered as a means for moving away from negative environmental impacts and negative public perception issues in the coastal zone. Favorable features for the transfer to open ocean waters include ample space for expansion and thus reduced conflicts with other user groups, lower exposure to human sources of pollution, the potential to reduce some of the negative environmental impacts of coastal fish farming, and optimal environmental conditions for various marine species through the larger carrying and assimilative capacities. However, this move should not be seen as an "out of sight, out of mind" attitude, as open ocean development will also come under scrutiny by the institutional bodies as well as by a more and more educated public. It is expected that, because of economies of scale, the open ocean farms of tomorrow will be larger than the present nearshore farms. Therefore, higher levels of waste can be generated. Even if greater residual effects occur, deeper waters and lower nutrient baselines are expected to reduce impacts from open ocean operations through wider dispersion plumes of nutrients, as compared to similarly sized nearshore operations. However, there will be a point when open ocean ecosystems will eventually reach their assimilative carrying capacities [30].

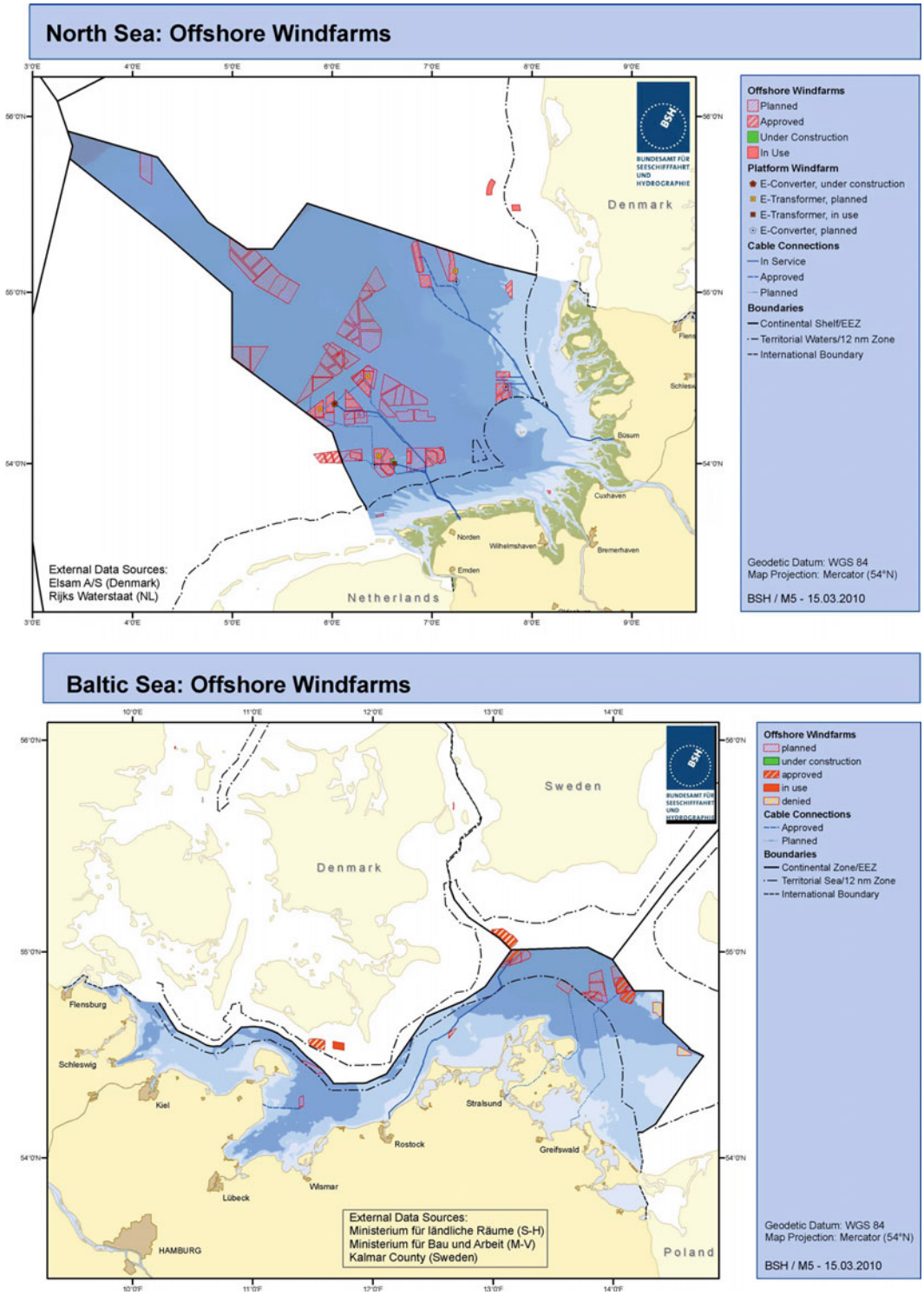
Offshore Wind Farms as a Case Example for Renewable Energy Systems

Wind energy continues to be the world's most dynamically growing energy source [31]. Drawing on the example of Germany, the first initiative toward an economy based on renewable energy resources was set by the governmental decision in the year 2000 to gradually reduce the use of nuclear energy and to respond to the gradually diminishing fossil- and nuclear-energy reserves. Simultaneously, the output of CO_2 to the atmosphere would be reduced in accordance with the Kyoto protocol as well as the dependence on conventional fossil-energy resources is lowered.

As high and reasonably steady wind speeds are characteristic in Northern offshore areas, these areas are prime candidates for renewable energy production by wind-energy farms. For instance in the North Sea, a major political incentive exists currently to install large offshore wind farms [32, 33]. Thus, the emerging branch of offshore wind farms appears as a new stakeholder on the list of users [34, 35].

So far, this development has been successful to such an extent that around 7.2% of the total energy consumption in Germany is covered by this technology. At the end of 2007, Germany had an installed capacity of 22,247 MW, generated by 19,460 mainly land-based operating wind turbines [36]. Within Europe, as the leading market for wind energy with over 57 GW, Germany thus accounted for 39% in terms of the total installed capacity and still remains the world's leader. However, with the North American market currently experiencing a strong growth, it is expected that the US market will soon overtake Germany [37].

At present, 60 project applications for wind farms in the Exclusive Economic Zone (EEZ) of the German



Aquaculture and Renewable Energy Systems, Integration of. Figure 2
Maps indicating all application sites for wind farm projects in Germany. At the top, the North Sea, below the Baltic Sea areas (Modified after [38])

North Sea and in the Baltic Sea are in the planning process stage with the total number of wind turbines per farm ranging between 80 and 500 [26] (Fig. 2). In November 2001, the Federal Maritime and Hydrographic Agency (BSH) granted the first approval for the installation of a pilot offshore wind farm. Since then, a total of 23 wind farm development projects have been approved in German waters, most of them planned seaward of the 12 nautical miles zone [38]. Currently, a larger test farm of about 12 wind turbines (5 MW class) at the "Borkum West" site are in operation (Fig. 3) [39]. Experience gained in this project should give developers practical knowledge in the construction and operation of offshore wind farms at depths (down to 50 m) and at distances from the shore (up to 50 nautical miles and more) that are beyond comparison to those anywhere in the world [31, 33].

In contrast to neighboring European states, the prospect of moving wind energy developments offshore stagnated in Germany for years mainly due to a very complex licensing procedure and the high environmental constraints [33, 40]. A further obstacle roots in the spatial competition of offshore wind farms with other utilization of the marine waters in the German Bight [41, 42]. However, despite the number of competing users within offshore regions being lower compared to coastal areas [43], the quest for spatial efficiency remains to be a key incentive also for offshore developments in the future.

Moving Offshore: The Multiple-Use Concept

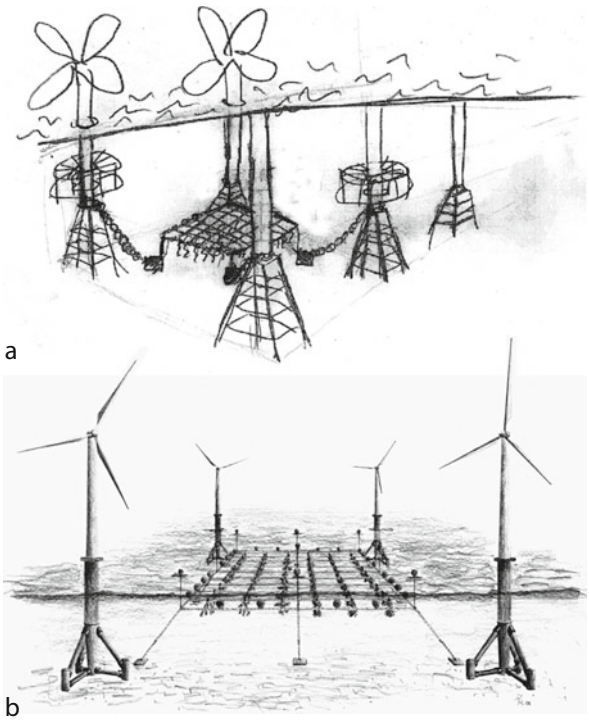
The plans for the massive expansion of wind farms in offshore areas of the North Sea triggered the idea of a combination of wind turbines with installations for extensive shellfish and macroalgae aquaculture [15, 26]. Offshore wind farms provide an appropriately sized area free of shipping traffic as most offshore

Aquaculture and Renewable Energy Systems, Integration of. Figure 3
Offshore wind farm Alpha Ventus. (**a**) Shows the transfer of the windmill tripods to the harbor of Wilhelmshaven and (**b**) displays the setup of an offshore windmill (REpower MI 068 [39])

wind farms are designed as restricted-access areas due to hazard mitigation concerns. Concurrently, the infrastructure for regular service support is readily available, and hence such sites provide an ideal opportunity for devising and implementing a multiple-use concept [42, 44]. However, in contrast to coastal inshore areas where beaches and their adjacent nearshore zones act as buffers to absorb wave energy, offshore regions are high-energy environments, fully exposed to waves, weather, and currents. Numerous studies have demonstrated that waves can reach remarkable heights (up to 10 m) in the offshore areas of the North Sea [e.g., 45, 46]. In this context, the solid foundation structure of wind turbines provides support for anchoring cultivation devices that can withstand the harsh oceanic conditions [47]. Furthermore, offshore structures are well known for their artificial reef function, thus supporting biodiversity in the ecosystem. The offshore water quality, which is a major issue in all kinds of aquaculture operations, is regarded to be excellent in comparison to inshore areas [48, 49]. Finally, the multifunctional use of offshore areas reduces conflicts between stakeholders if activities are concentrated and conjointly managed within so-called multiple-use marine areas. This, in turn, increases the amount of open ocean territory free of utilization by man. All of the above issues are considered as key incentives to move offshore with aquaculture operations.

In view of the many interests for the offshore move, different suggestions for technical structures for open ocean aquaculture were proposed (see proceedings of various OOA-Conferences [e.g. 50, 51]), which could cope with the harsh environmental conditions that place an enormous stress on the employed materials. It would be advantageous for the global offshore aquaculture development to plan for a combination of uses. While windmills use the wind above the surface to produce energy, their fixed pylons, commonly concrete fundaments (gravity foundation), metal jackets, tripods, or triples offer a possibility to connect systems used in aquaculture (Fig. 4). The combination of the respective two industries has to cope with the forces generated by the high-energy environment.

Since 2000, when the co-use of wind farms for off-bottom offshore cultivation [26] in the German Bight was proposed, several studies have been conducted to elucidate the potential as well as constraints of this offshore alternative for extensive aquaculture. Two



Aquaculture and Renewable Energy Systems, Integration of. Figure 4
Potential multifunctional use of fixed underwater structures of wind turbines for the operation of aquaculture facilities: 12 years ago and today (2010). (**a**) First drawing ever for the multi-use concept, including alternative solutions of oyster cages and mussel collectors attached to longlines in the inner section of the wind farm or offshore-rings (collar systems) attached directly to the pylon. The latter system can be submersed in case of wind-turbine maintenance. (**b**) Presents a design of a single mussel plot within a group of four wind turbines (not to scale) (Modified after [52], Buck personal drawing)

pioneer studies, the project *Roter Sand* and *Offshore Aquaculture* were conducted between 2002 and 2004 by the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany. These two projects followed a complex approach to obtain data about suitable indigenous candidates for the offshore cultivation [15], the technical requirements of longline systems for the cultivation of mussels or oysters [8, 9] and algal cultivation systems [53]. Insights into the feasibility of offshore seed and mussel production concerning larval, nutrient, and phytoplankton concentrations [8, 9, 54] were provided, and the existing legislation and regulations concerning marine aquaculture in Germany were listed [21]. In addition, all stakeholders potentially involved in a multifunctional use of offshore wind farms for aquaculture were identified [42]. This successful multifaceted approach helped to disperse many concerns and doubts on the offshore idea and initiated a sequence of and relations between various following projects, which are displayed in Fig. 5.

Candidates and Techniques for the Multi-Use Concept

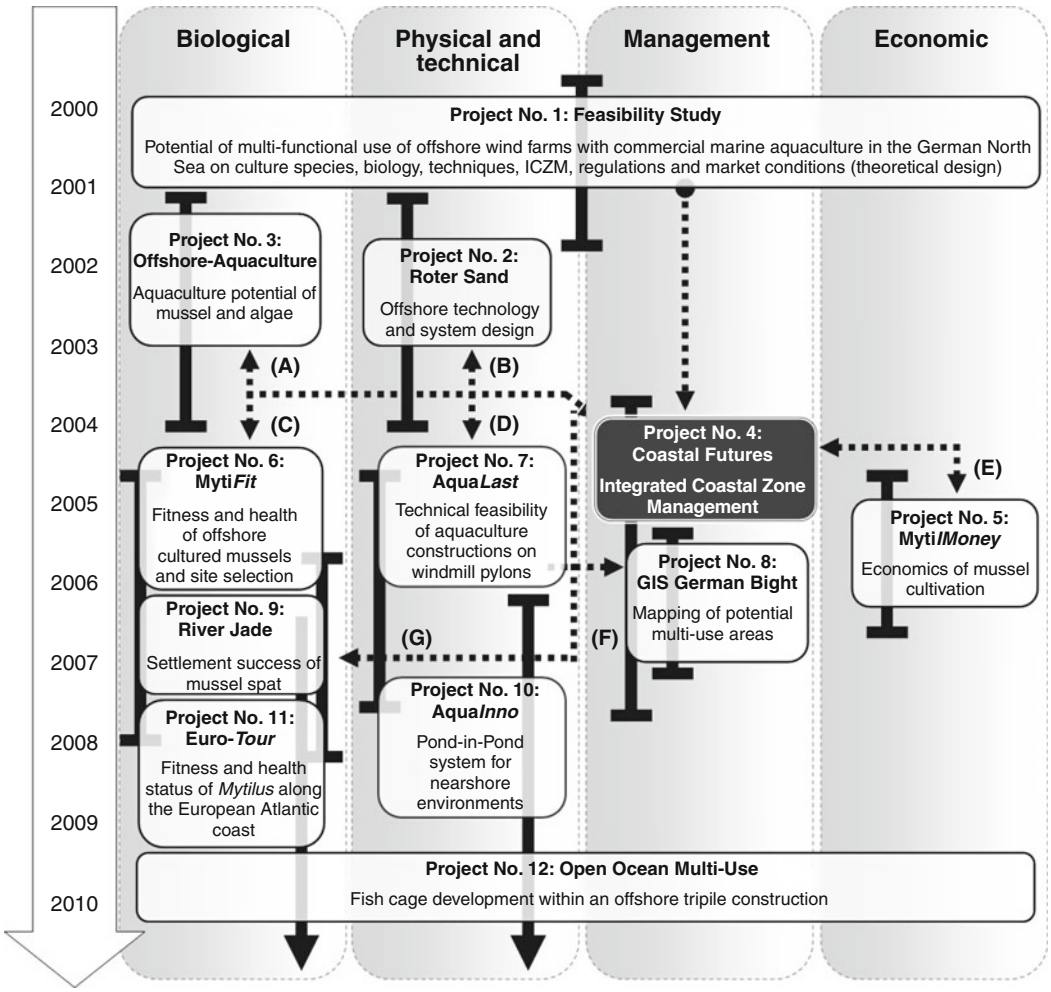
In general, the cultivation process should consider only indigenous species for marine aquaculture operations to avoid the disruption with the local marine flora and fauna. This limits the economic opportunities of marine aquaculture enterprises since in certain sites only a few indigenous candidates are regarded as high-value species. Following a feasibility study by Buck [26], in Germany only culture species with modest service needs can be considered as favorable candidates for offshore aquaculture. In the offshore test trials in Germany, most suitable candidates suggested and tested were the sugar kelp (*Laminaria saccharina*), oarweed (*L. digitata*), dulse (*Palmaria palmata*), the blue mussel (*Mytilus edulis*), and two oyster species, the Pacific oyster (*Crassostrea gigas*) and the European flat oyster (*Ostrea edulis*). Mussels and seaweeds, for example, are cultured mainly in extensive systems throughout the world [8, 56, 57]; the latter occurs for historical and traditional reasons mostly in Asian countries.

According to Tseng [58], the cultivation procedure of brown seaweeds can be divided into two separate steps: In step (1), the seedling phase, spores are artificially released from mature sporophytes and seeded on a given substrate (ropes wrapped around plastic frames), where germination of gametophytes, the sexual maturation of male and female gametophytes, and finally, the development of zygotes into juvenile sporophytes takes place. In step (2), the grow-out phase, culture ropes with juvenile sporophytes are transferred to the open sea. In the grow-out phase, the macroalgal sporophytes grow on ropes for one season to a frond length of approximately 2 m.

When natural reproduction of mussels occurs, gametes are released into the water column where fertilization takes place [59]. The larvae undergo all trochophore and veliger stages when settling on a given substrate to start metamorphosis. According to Pulfrich [60] and Walter and Liebezeit [61], this process normally takes place at spring time (larval peak in May) in the German Bight. The cultivation of blue mussels can be divided into two steps: in step (1) the naturally occurring spat collection is achieved by deploying artificial substrates [62]. Usually, spat collectors are made out of unraveled polypropylene lines or sisal ropes, to offer the mussel's post larvae substrate for settlement [56]. After several months (step 2), collectors are retrieved and mussels thinned out and reseeded on ropes to provide space to improve growth and allow fattening [63, 64].

To operate culture phase (2) of both species, macroalgae and bivalves, an appropriate system design, such as suspended longlines or floating ring-structures, have to be deployed and securely moored in order to resist the stress forces of incoming waves and tidal currents, as well as swell. In addition, it was necessary to assess what kind of technical structure supports best the growth of the organisms (e.g., prevention from loss or mortality) while also assessing whether such systems provide reasonable production returns. Finally, potential combinations with offshore wind turbines had to be assessed.

However, currently even candidates requiring a semi-intensive as well as intensive cultivation process



Aquaculture and Renewable Energy Systems, Integration of. Figure 5
Chronological order of conducted and ongoing research projects dealing with the combination of offshore wind farming and open ocean aquaculture. Project No. 1, the feasibility study, constituted the basis for all subsequent research. The *Coastal Futures Project* acts as a key node project to which the other projects either have contributed or by which they have been stimulated because of its transdisciplinary approach. It is visible that: (**a**) calls the wind farm developers' attention to offshore aquaculture; (**b**) and (**c**) include authorities and fishermen into the planning process for site-selection criteria of appropriate aquaculture sites; (**d**) involves offshore engineers and wind farm developers/operators into the technical part of an offshore aquaculture enterprise; (**e**) introduces (mussel) fishermen to the co-management idea and appraises the economics of mussel cultivation; (**f**) supplies authorities with maps and tools to limit regional stakeholder conflicts, (**g**) establishing an inshore reference station to support the data collected offshore, and (**h**) testing the first fish cage mounted within a tripile construction (Modified after [55])

are in the testing phase. Salmon (*Salmo salar*), seabass (*Dicentrarchus labrax*), seabream (*Sparus aurata*), or some flatfish species are discussed for aquaculture in fish cages below windmill platforms at different offshore sites worldwide. Fish will firstly be reared in land-based facilities and will then be transferred as fingerlings to the offshore site and released into the submergible fish cages. After reaching market size, the fish will be harvested and removed to the land and will undergo normal processing procedures.

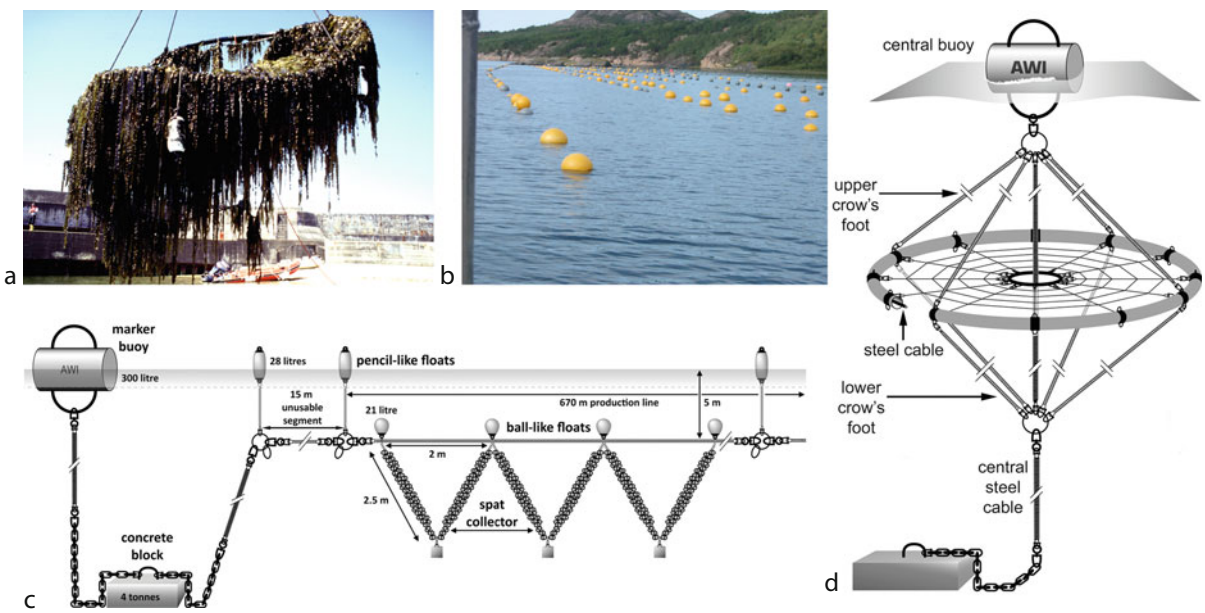
Relocating cultivation systems offshore into high-energy environments requires the development of suitable culture techniques able to withstand the harsh conditions and minimize risk of economic loss [65].

Several techniques exist to cultivate mussels and seaweed either in co-culture or in single culture. Basically, both organisms are cultured in a suspended manner in the water column, floating or submerged. The use of rafts, longlines, and ring methods dominate. The latter two were the main cultivation techniques used in test trials offshore wind farm areas [8, 53, 56] (Fig. 6).

Major difficulties in the development of suitable techniques for open ocean aquaculture are – as mentioned above – the harsh environmental conditions which place an enormous stress on materials. Depending on the acting hydrodynamic forces, different technical setups can be distinguished. One of the interesting possible linkages of aquaculture is the combination with offshore wind farms as these would provide stable fixing structures for the cultivation systems. This is especially relevant from an economic point of view as so far the costly infrastructure for offshore aquaculture systems is one of the major drawbacks in the development.

Status Quo of Offshore Aquaculture Research Activities in Wind Farms

Only a few scientific studies dealing with the prospects of offshore aquaculture were available before 2000, and little was known about the biotechnological requirements, economic potential, or the socio-economic influence on the general feasibility of offshore aquaculture. Very few long-term experiments under harsh hydrodynamic conditions exist, e.g., Langan and Horton for offshore mussel cultivation [66]; Neushul and Harger [67]; Neushul et al. [68] for offshore seaweed cultivation. However, data on system and species performance are urgently needed to derive methodologies for the assessment of its environmental and economic viability. Therefore, the assessment of the potentials and constraints for sustainable aquaculture development in all marine habitats requires input from various scientific disciplines in order to direct this development towards



Aquaculture and Renewable Energy Systems, Integration of. Figure 6
Aquaculture constructions suitable for the cultivation in high-energy environments. (**a**) Offshore ring design for the cultivation of macroalgae (here: harvesting after grow-out in the harbor of Helgoland), (**b**) example of a nearshore, submerged longline design for mussels and oysters, (**c**) schematic drawing of a submerged longline suitable for exposed sites, and (**d**) a technical illustration of the ring design and its mooring system (Modified after [8, 9, 53])

a successful aquaculture undertaking. In particular, this holds true for offshore aquaculture, where little practical experience is available to date, although research in this area is evolving rapidly (e.g., Buck [15], Turner [69]; Pérez et al. [70]; Bridger and Costa-Pearce [51]; Dalton [71]; Naylor and Burke [72]).

The offshore wind farm and aquaculture investigations initiated an integrated assessment of theoretical and practical challenges of aquaculture operations in the North Sea. Several studies were carried out, all of which contributed to specific aspects of such a combined utilization of offshore space. These were:

(a) *Biological studies*, in which the focus was placed on cultivation and subsequent performance characteristics of indigenous bivalve, seaweed, and fish species exposed to extensive offshore aquaculture farming conditions. Further, the health status and infestation rates with parasites, bacteria, and viruses of candidates were determined to gain reliable predictions on where the highest growth rates and best product quality for consumers can be achieved. In nearshore intertidal areas, mussels and oysters are particularly exposed to high concentrations of pollutants, pesticides, near surface agents, estuarine run-offs, etc. that can pose a threat to consumer health. Buck [8, 9, 15] reported high growth rates for mussels cultivated in the German Bight. The scope of growth, i.e., the energy available for growth, is usually directly and positively correlated to a good overall health condition of the respective organism [73]. But organisms with high growth rates and a healthy appearance are no guarantee of a healthy food for human consumers. For instance, in coastal waters, eutrophicated by urban sewage, mussels show good growth performance. The microbial status of these mussels, however, mostly excludes them from consumption since they might carry various human pathogens. Even in developed countries with strict legislation for the treatment of wastewater, mussels can function as carriers of vector diseases. Whether this is also true for offshore cultivated mussels, where the environment is cleaner due to dilution of contaminants, remains open. Data for offshore-produced mussels, generated according to the analysis protocols of controlling authorities, are not readily available for all cultivation sites. However, new regulations are in the implementation process in all of the EU states and will fulfill the prerequisites for an official sampling design and assessment (i.e., sanitary survey).

To evaluate the significance and comparability of the employed parameters in a broader geographical context, the area of investigation was extended along the Atlantic coast from southern Portugal to northern Denmark. Further on, the closely related Mediterranean mussel *Mytilus galloprovincialis* was included in the analysis to test the effectiveness of all the parameters in different species.

Investigations on fish species for submerged cage-systems included aspects on growth, welfare, stress in exposed environments, and health.

(b) *Physical and technical studies* investigated the effects of the prevailing hydrodynamics on candidates and culture constructions at specific offshore sites. At the same time, the necessary technical requirements for farming structures in high-energy environments and their possible combination with offshore wind farms were assessed. New system designs for offshore farming were developed and prototypes (e.g., offshore ring, offshore collector) were tested. Technical details about the microstructure of artificial substrates were addressed to increase production per meter longline under offshore conditions. In addition to offshore seaweed and mussel cultivation, new technologies for submerged fish cages were investigated.

(c) *Management and institutional studies* focussed on the analysis of potential management approaches to implement a multi-use concept of offshore areas. Hereby, the various stakeholders and their respective views and knowledge systems were integrated. Against the background of the social and institutional dimensions, particular emphasis was given to the interrelationship between scientific findings on the one hand and effective implementation on the other. Key aspects included the social acceptance of combined use, as well as the possible management strategies that would govern it. This endorsed the examination of the prevailing case laws and regulative and management framework conditions, as well as a suggestion of decisive offshore co-management strategies to support such activities. In this process, the continuous inclusion of the stakeholders in a participatory manner was

a prerequisite. To address the respective technical, economic, social, and political challenges of mariculture and offshore wind farms, specific co-management strategies were elaborated that are either more results-oriented (e.g., for integrating technical knowledge of the two sectors) or more process-oriented (e.g., for establishing new linkages between different groups). Thus, in cooperation with governmental authorities, co-management in the offshore makes use of the capacities and interests of the respective stakeholder groups and employs these in managing cross-sectoral activities.

(d) *Economic studies* conducted an economic evaluation of such multi-use concepts in offshore locations that take into consideration market conditions as well as investment and operating costs.

All the above listed conceptual approaches relied on results of a theoretical feasibility study (Fig. 5) [26], which was carried out prior to practical research in the field. All of the results contribute to the *Coastal Futures* Program and support the quest to find innovative new approaches for sustainable use and alternative livelihoods of coastal populations.

Overview of Biological and Technical Investigations

Over the last decades, substantial insights have been gained on the terms and conditions active in the offshore environment. However, these data are only partly useful for the selection of offshore aquaculture sites because they have been gathered primarily for other user needs and thus lack the essential specificity to address the biological and cultivable potential of these sites. Prior to a multifunctional development comprising mariculture activities, it is therefore necessary to determine the appropriate biological, technological, and management requirements, as well as the performance characteristics that would allow the employment of favorable and cost-effective methodologies. To meet this end, special focus was placed on the combination of extensive offshore shellfish, seaweed, and fish farming at exposed sites within the proposed offshore wind farm boundaries.

Due to the wide spectrum of open questions, the outcomes are quite manifold. In the following, first results according to their contributions towards the main research topics involved are presented.

Biological Studies The theoretical *Feasibility Study* [13, 24] was aimed to ascertain the biological, technical, and economic feasibility of an offshore marine aquaculture structure with respect to the cultivation of marine organisms within wind farm sites in the German North Sea. One result was that to date, in terms of commercial marine aquaculture, Germany had little knowledge and background in offshore aquaculture compared to many other coastal countries throughout the world. Nevertheless, a synthesis of a selection of parameters (e.g., geophysical and biological parameters) allowed the identification of suitable candidates for commercial offshore aquaculture. These candidates include blue mussels (*Mytilus edulis*) and oysters (*Ostrea edulis*, *Crassostrea gigas*), which could be maintained extensively in the offshore region. Moreover, labor requirement for these candidates as well as for seaweeds, such as the sugar kelp (*Laminaria saccharina*) and dulse (*Palmaria palmata*), is supposed to be low.

Further, the biological feasibility of cultivating mussels, oysters, and kelp within offshore wind farm sites was assessed. The growth of these species is excellent in the rather eutrophicated offshore environments of the North Sea, but can differ depending on exposure sites, system designs, installation modes, and season.

For instance, settlement of young mussels on artificial collector substrates decreases with increasing distance from the shore [74]. However, this does not limit the economic potential if the thinning procedure will be omitted, following a "One-Step-Cultivation" concept [15]. In general it was found that mussels are free of parasites at offshore locations due to dilution effects and the interrupted reproduction cycles of some macroparasites [75]. Special focus was placed on the overall health status of mussels cultured under different conditions, and the impact on economic aspects was investigated [76]. Specific aims of the projects were the development of suitable offshore spat collecting techniques, detailed knowledge about parasites (macro and micro), bacteria and virus infestations at different sites, implementation of biodiagnostic techniques for the health analysis of cultured mussels, and collection of all relevant data (e.g., shell stability and attachment strength of mussels), for the further processing of mussels as a product for human consumption.

Hydrodynamic forces support length increase of seaweed blades when transferring young sporophytes to sea. These algae will adapt to the occurring loads and develop strong holdfasts, preventing detachment of the entire plant [77].

Modified and improved techniques for offshore farming withstand high-energy environments, but will certainly cause higher investment costs. Therefore, site-selecting criteria for a culture area should be clearly identified to assess economic risks. Important for the cultivation success is the water quality. The analysis of the cultured organisms with biodiagnostic tools provides detailed insights into the water conditions the animals live in. By this approach, reliable predictions are possible as to which locations grant highest growth rates and best product quality for consumers. Preliminary results attest offshore areas satisfying settlement success and excellent growth rates [78], and low infestations with macroparasites [79], microparasites, bacteria, and toxins [76]. The results on consumption suitability show that water quality regarding the concentrations of pollutants in offshore areas of the German Bight is quite good. Lysosomal membrane stability is mostly relatively low at all tested nearshore and offshore sites. Interestingly, growth rates of the hanging cultivated mussels are not affected by this low fitness parameter [58].

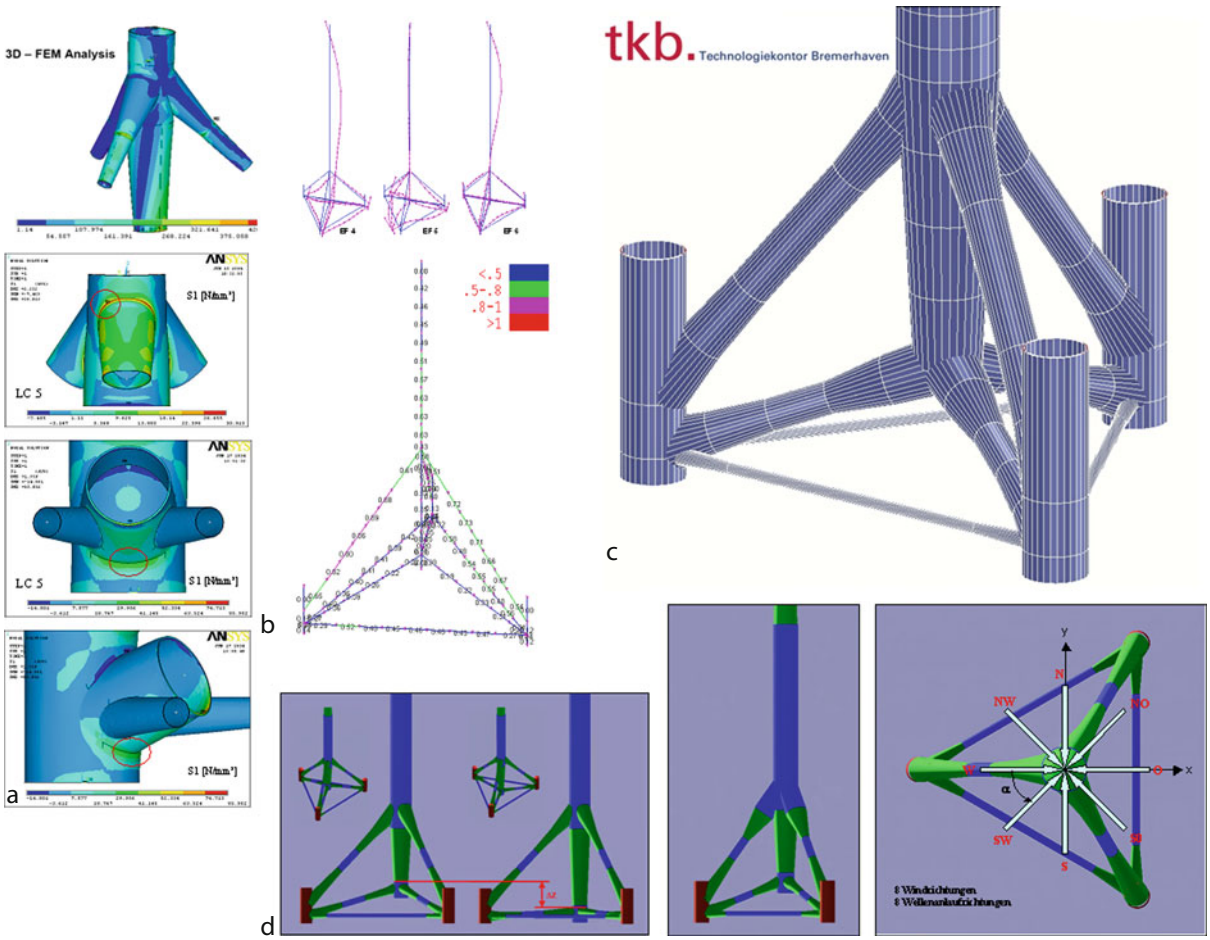
First results on investigations along the Atlantic Coast show that mussels originating from offshore habitats have a better health status regarding the infestation with macroparasites and microparasites (Buck and Brenner, unpublished data). While macroparasites are still infesting mussels in nearshore areas in the Wadden Sea (the Netherlands, Germany, Denmark), microparasites are absent.

Physical and Technical Studies The results above allowed the identification of two offshore aquaculture systems that were best suited for offshore operations from a biological point of view. Depending on the acting hydrodynamic properties, different technical setups are regarded as favorable. The first one is a floating and submergible ring system for the cultivation of seaweed. It withstands rough weather conditions and allows easy handling [53]. The second system is a submerged longline design for blue-mussel culture [8]. The longline should ideally be installed 5 m below the water surface and should be connected to foundations of offshore windmills (Fig. 7) [47]. For the longline, polypropylene proved to be an appropriate material. The system design is made of various connected segments allowing an easy harvest and replacement of all parts of the construction. However, more technical engineering research is required to find the most cost-effective mode of construction and the best choice of materials (e.g., little corrosion, longevity in spite of mechanical stress) so that easy handling can be guaranteed under relatively harsh weather conditions (cf. construction, deployment, retrieval, service, repairs).

The experimental design also allowed work on such issues as the efficiency of the collecting devices themselves. Healthy mussels will reach market size in offshore conditions only if they are firmly attached to their artificial substrate. As mussels growing on suspended substrates need about 15 months [8, 9] on average to reach market size, they must survive one winter and withstand storm events producing wave heights up to several meters. Continuing investigations on the health and quality of market-sized mussels would be moot if mussels failed to stay attached to substrate gear.

To date, most available substrates are designed and deployed for nearshore use under calm water condition. However, it was found that improvement for construction of new collectors that are feasible for offshore cultivation is in mandate. Research showed that new substrates should have felt-like structures around the core of a collector for larval attraction and long appendices in high density to interweave the mussel conglomerates with the substrate [80]. Future investigations should focus further on the fabrication and testing of a prototype of this collector, concerning the results of this study. Besides providing optimal larval attraction and attachment for juvenile mussels even under winter conditions, any new substrate should proof its durability under conditions of a daily farming routine. This would include mechanical thinning, harvesting processes, and tests on the reusability of the material.

The technical realization and the implications of aquaculture technical requirements on design and construction of the grounding construction of offshore wind turbines were considered. So far, modeling and experimental validation of a submerged 50 m longline aquaculture construction mounted between two steel piles, 17 nautical miles off the coast, show significant



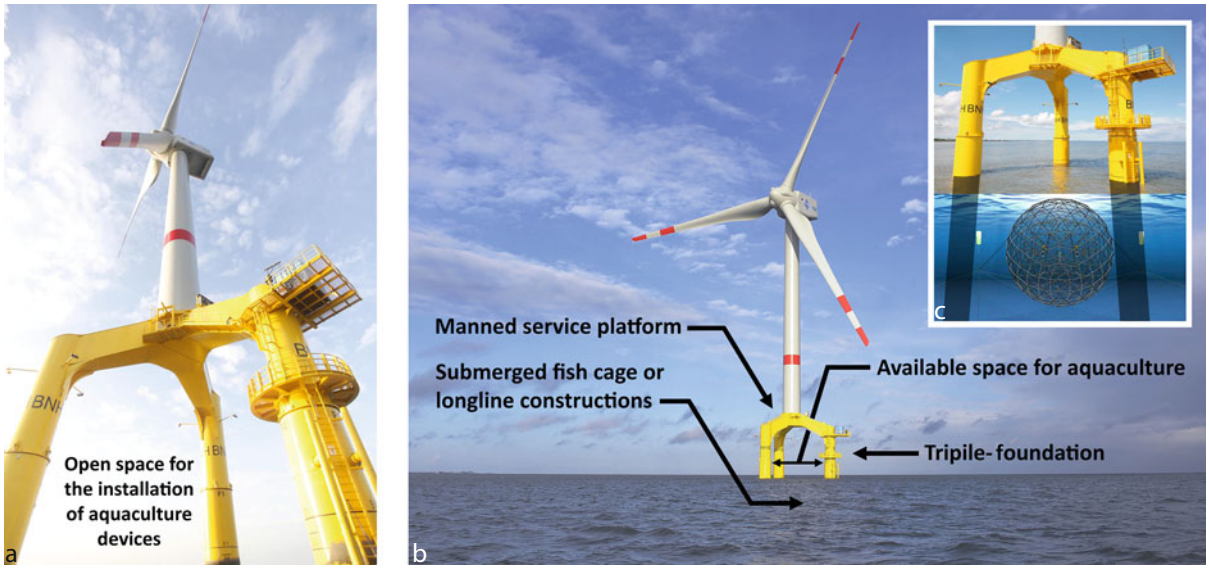
Aquaculture and Renewable Energy Systems, Integration of. Figure 7
Modeling of potential attachment points for the combination of longline connections to a tripod foundation. (**a**) Displays alternative connection points, (**b**) shows the generation of representative loads on the wind-energy installation, including vibrations, (**c**) shows the respective tripod foundation for offshore use in depths of about 20–50 m, and (**d**) shows the development of a static model (3–5 MW class) [47]

forces of up to 90 kN (equivalent to 9 t) induced by waves of up to 1.8 m significant wave height and tidal currents of up to 1.0 m/s [81]. Given the high-energy environment in the North Sea and the non-linear relationship between water movement and its resulting forces, even higher mechanical loads are to be expected within the life cycle of such an arrangement. These must be taken into account when developing techniques for larger-scale offshore cultivation within wind farms.

Finally, a new cage design project has been initiated, where it will be investigated whether aquaculture of fish in between a tripile construction below a windmill has the potential to enlarge the diversity of candidates to be grown offshore (next to bivalve and seaweed) as well as widening the potential of offshore farming within wind farms. First insights are shown in Fig. 8 [82, 83].

Management and Institutional Considerations

From a spatial planning perspective, the ocean space in the Exclusive Economic Zones (EEZ) cannot be considered any more as "commons" in the sense of Ostrom et al. [84] wherein individuals or groups have the right to freely consume and return any kind of resources.



Aquaculture and Renewable Energy Systems, Integration of. Figure 8
Tripile construction for the secondary use for fish cages. (**a**) Shows the open space within a tripile foundation to be used for aquaculture purposes, (**b**) displays a lateral view of the Bard Windmill and the access to the fish cage, and (**c**) is a photo animation and gives an idea how a fish farm, such as an aquapod, could be moored below [82, 83]

As a matter of fact, the "tragedy of the commons" situation Hardin described in 1968 [85], has already been reached for most of the oceans today. Offshore waters are in a process of transition, revealing diverse and heterogenic interests in marine resources. For instance, the development of offshore renewable-energy systems is an international priority driven by the need to reduce the dependence on fossil fuels and decrease human impacts on the global climate regime. Simultaneously, the demand for high-quality seafood is accelerating globally. This leads to an increased complexity and thus to limitations in developing and managing the different and often spatially overlapping maritime activities independently of one another. The upcoming new utilization patterns of the German North Sea, such as wind farms, but also Marine Protected Areas (MPAs) reported to the EU commission in Brussels for the "European Natura 2000 network," reveal a trend toward the development of permanent constructs. Both are examples for new forms of use with a high spatial demand [86]. Not all uses are compatible with each other and user conflicts with existing activities, such as fisheries, maritime traffic, or military missions are preordained. The planned large-scale offshore wind farms as well as designated MPAs are prime examples for the development of lasting marine structures that take up a surface area of several square kilometers each [55].

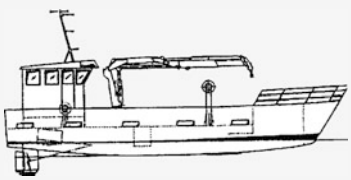
At the same time, the increasing demand for high-quality foods worldwide accelerates the development of marine aquaculture. This potential newcomer can be expected to become an additional competitor in offshore waters [87], contributing to the increase in spatial competition and complexity in the ocean [20]. Conflicts among the respective user groups are inevitable. The growing competition for space represents a major challenge for further developing or even maintaining all forms of marine aquaculture, as well as freshwater fish farming. However, area choice is crucial and spatial planning has a key role to play in providing guidance and reliable data for the location of an economic activity, giving certainty to investors, avoiding conflicts, and finding synergies between activities and environments with the ultimate aim of sustainable development [88]. The inclusion of all stakeholders in this process to find synergies in the open ocean is crucial.

Ongoing multidisciplinary social-science research in Europe shows that it is feasible to establish spatially

efficient and effective wind farm–mariculture co-management regimes. A window-of-opportunity has opened as both groups have realized that they may benefit through the integration of operation and maintenance (O&M) activities vis á vis gaining support in collaborative action by the current impetus of the new EU Maritime Policy. The operation and maintenance of any offshore installation is a major challenge due to restricted logistics and accessibility, forming a large part of the overall costs. A five-to-ten time more expensive scale of operation and more difficult logistics for maintenance and/or harvesting compared to nearshore or onshore sites have to be taken into account [89–91]. Experiences with existing wind farms and mariculture sites off the coast show that work at the sea is not only significantly more cost-intensive, but also more time consuming than on land [92].

There are certain rights and duties involved if prospective spatial and organizational interaction of O&M activities of offshore wind turbines and mariculture installations are to be combined [20]. Different values, perspectives, and demands of the stakeholder groups need to be harmonized [93]. So far, disagreements on the distribution of entitlements to benefits and profits between the different stakeholder groups can be observed (Table 1). The two potential adopters of such a multi-use scheme illuminate different sets of skills and capacities in terms of offers, needs, and constraints characteristics. These are vital resources, which provide the basis for forming any sustainable offshore

Aquaculture and Renewable Energy Systems, Integration of. Table 1 Offers, needs, and constraints characteristics of mariculture operators and offshore wind farmers concerning O&M activities. Interrelated aspects between the two actor groups are indicated in bold (modified after [86])

Characteristics	Actor groups	
	Wind farmers	Mariculture operators
		
Offers	• Fixed offshore infrastructure • Logistic platform • Financial support (EEG amendment)	• Upgradeable sea-going vessels • Offshore mentality • Offshore skills and experience
Needs	• Specialization of equipment (construction vs hire; "marinization" of onshore equipment) • Specialization of personnel • Sea-going vessels • Service demands (man-hours) • Suitable O&M pattern (corrective vs preventive maintenance) • Suitable O&M pattern (opportunity vs periodic maintenance)	• Specialization of equipment (construction vs alteration of existing oil industry/fishery vessels) • Specialization of personnel • Fixed offshore infrastructure • Technical and logistic support • Service demands (man-hours) • Offshore skills and experience • Offshore mentality
Constraints	• Operation costs • Technical challenges • Distance to farm site • Available working days (estimated 100/year) • Difficult logistics for O&M • Reliability of offshore wind turbines	• Access to farm site (uncertain regulatory and permit requirements) • Distance to farm site • Available working days (estimated 30–100/year) • Difficult logistics for maintenance and harvesting • Reliability of culturing devices

co-management arrangements [85]. Hereby, a fair negotiation and bargaining process is the most essential component to effectively orchestrate co-management of offshore wind farmers and future mariculture operators, such as mussel harvesters. The latter already dispose vital skills and experiences for working in the open sea. Still, working methods have to be adjusted to the offshore culture production mode.

If such an offshore co-management is considered as a network activity between private actors, such as wind farmers or mariculture operators/fishermen and public authorities, one of its basic characteristics is the fact that a third party can coordinate the activities of formally separated parties [94]. Ways and means have to be developed that balance the respective interests of dominant and politically supported wind farming participants with small-scale entrepreneurial mariculturists. The key question is how institutional arrangements could act as "boundary organizations" [95] in an offshore co-management process. Such a process is more likely to develop and succeed if an interface management that acts as moderator, disclosing the interests of the actor groups and offering possibilities for concerted action, guides it. With respect to the decision-making arrangements at the three levels (operational level, organizational level, and legislative level), the interface management would thus help to determine the rules for interaction among the actor groups and state authorities at the organizational level. Besides, it would facilitate organizing and decision making of the day-to-day activities at the operational level. However, to authorize and legitimize new co-management arrangements for interacting offshore O&M activities, new policies must be developed or existing laws amended. Following a dynamic process of forming new institutional structures, the establishment of a communication arena may (a) support a common understanding of the entire co-management process, (b) provide the overall framing for an improved communication among the participating actor groups, (c) increase the level of trust among the actor groups, and (d) promote sustainability and efficiency in times of scarcity of spatial resources [85]. However, top–down induced management schemes by, e.g., the national government, hold a high potential for failure. Involving the relevant actors improves the social acceptability of innovative concepts and their applicability [96]. Consequently, it appears that for developing and implementing a wind farm–mariculture multiple-use concept, co-management, such as that described by Carlsson and Berkes [94], should ideally be carried out with the participation of different actors that typically try to find ways to learn from their actions and adapt the behavior to the consequences of their own and other's actions. This must be supported by the relevant authorities at all levels and must find its way into the legislative framework at the EU and national level.

On EU level, the issue of access to space for maritime activities, including aquaculture, has been recognized in several communications over the past years, e.g., in 2007 pertaining to the Integrated Maritime [97] Policy or in 2009 concerning a new impetus for sustainable aquaculture in Europe. In the latter, all Member States are asked to develop marine spatial planning systems, in which they fully recognize the strategic importance of aquaculture. This Strategy also aims at providing EU leadership and guidance to both stakeholders and administrations to ensure consistency and clarity in designing the necessary policies for the future sustainable development of European aquaculture. In this context, a partnership between public authorities and interested parties at EU, national, and local level play a crucial role.

Hence, European aquaculture should benefit from an improved framework for governance; however, it is stressed that the national authorities have a primary role in shaping aquaculture development in their territory. While in some countries aquaculture is defined and regulated under the agricultural laws, in other countries regulations are dispersed, and consequently the responsibilities are in the hand of several agencies with no clearly defined lead agency. So far, a number of important challenges that limit the development of European aquaculture directly depend on policies and actions taken at national or regional level. A bottom–up approach is therefore needed so that the public authorities can establish an appropriate framework for the vision of multiple-use of offshore areas to become operational. A participatory approach contributes to lifting bottlenecks in national legislation. This framework needs to be transparent, consistent and cost-effective in order to allow the industry to realize its potential. Unless these and other regulative issues pertaining to multi-use offshore conditions remain unresolved, an

establishment of offshore co-management arrangements may be very difficult. Still, the current lack of legislation in the EEZ holds the potential to implement concerted innovative concepts of offshore constructions and thereon-interacting activities.

However, several restrictions are still needed to be resolved. Questions pertaining to access rights within the wind farm area have to be deciphered. So far, approved offshore wind farm territories in, e.g., the German EEZ, are designated as restricted area, prohibiting any kind of public access [98, 99]. However, conveying access rights to a second party is inevitable if wind farm O&M is performed by a commissioned subcontractor and not by the licensee itself. In a wind farm–mariculture multiple-use arena, the same access and user rights have to be guaranteed to a mariculture operator who enters the territory for purposes not related to wind farming but for maintaining culturing devices and harvesting procedures. In this case, precise positioning of aquaculture installations within the wind farm territory as well as access lanes for both parties have to be specified.

In addition, the question of harmonizing the tenure or duration of a lease for offshore resources has to be tackled. If there is, i.e., significant discrepancy in the length of lease tenures between the two uses to be combined, the resource users may not be inclined to create long-term co-management arrangements. Furthermore, cooperative management structures also benefit if the leasing process was combined and/or effectively coordinated, since it facilitates, i.e., integrating O&M within a co-management scheme once the projects are operational.

Yet, in order to define the functional structure of such a co-management regime in detail, reliable outcomes on economic and technical integration prospects of a joint wind farm–mariculture venture have to be produced. The latter is a major research demand, which was voiced by most of the interview partners along the North Sea coast so far [93]. Cumulative impacts of different economic sectors, such as offshore wind farms and mariculture need to be addressed, which provides an opportunity to create synergies between different industrial sectors prior to their installation.

Outcome of Economic Studies

The *Feasibility Study* [13, 24] provided a general overview on market prices, market demands, classification of candidate species as high-value products, and the cost of some infrastructure. The study showed possible market value of offshore aquaculture products in comparison to the performance of existing conventionally operated farms in coastal waters.

Basic data for offshore mussel cultivation in close vicinity to a designated offshore wind farm in the open sea of the German Bight were compiled. It contained different case-scenario calculations to illustrate the impact of changing parameter values on overall profitability or non-profitability of this activity. Primary focus was placed on the production of consumer mussels, but seed mussel cultivation is also taken into consideration. This study concluded with providing some recommendations on how favorable terms or actions could further improve profitability of offshore mussel cultivation. Results intended to shed some light on business management topics that future offshore mariculture operators should follow in order to be efficient [100].

Nontheless, the economics of a joint offshore wind farm–mariculture utilization scheme still remain to be evaluated in more detail.

Future Directions

By setting higher value on an inclusion of stakeholder knowledge and opinions, the initiation of the *Coastal Futures Project* resulted in a stronger focus on the practicability of multifunctional use of offshore areas. It can be shown that such innovative new concepts are highly complex and interdependent. First, results indicate that secure technical and economic feasibility appears to be a basic prerequisite to assure that both offshore wind farm operators and aquaculturists will support the multi-use concept, especially as far as the management of joint activities is concerned.

This suggests that as soon as technical and economic aspects are evaluated in more detail, it is important to initialize a comprehensive communication program to provide information to the key public and private actor groups (stakeholders). Furthermore, effective and continuous participation of all stakeholders on all levels from the very beginning of the multi-use approach must be ensured. This supports the orchestration of scientific and local user knowledge in an overall approach to combine different offshore uses. In addition, it contributes to adding a joint wind farm–mariculture venture to their future portfolio.

More detailed data are needed to calculate the economic potentials and risks of a co-used wind farm area for the production of seafood. Apart from the principal feasibility of an area as an aquaculture site, growth rates and product quality must be predictable. First, results on blue mussels from test areas show that highest product qualities can be expected from testing areas offshore. A proven product quality ensures higher market prizes, should compensate for higher investment costs for the culture systems, and help to install a functioning offshore aquaculture system in the German Bight.

Generall, science for open ocean aquaculture needs a transformative moment. It seems necessary to learn the skills to interact constructively with different scientific disciplines and different stakeholders. This will require a new science for managed marine seascapes [101]. Creating a system biology paradigm in ecosystem science and aquaculture will require a multidisciplinary input, with scientific interactions not just at the margins of each discipline, but focused collaboratively on the realization of a vision of multifunctional, spatially effective, and sustainable use of ocean space. This will require new kinds of scientists (with new kinds of career structures) who are trained to work in multidisciplinary teams. The need for such training is now widely recognized and is reflected in the emerging curricula's of many new MSc courses.

It is mandatory to discover what to do, at what scale, in what modality – engineering, farming, legislation, social organization, economic initiatives, etc. – and how to do it. Since the activities in the ocean realm are concerted in integration, future activities must also be integrated over all these modalities.

Bibliography

1. FAO/FIDI (1989) Aquaculture production (1984–1986) Food and Agriculture Organisation of the United Nations. Rome Fisheries Circular 815, 106
2. FAO (1997) Aquaculture development. Technical guidelines for responsible fisheries. Food and Agriculture Organisation of the United Nations, Rome, pp 5–40
3. EU Commission (2000) On integrated coastal zone management: a strategy for Europe. Communication from the Commission to the Council and the European Parliament, COM (2000) 547 final
4. Garvine R, Kempton W (2008) Assessing the wind field over the continental shelf as a resource for electric power. J Mar Res 66(6):751–773
5. Pauly D, Christensen V, Guénette S, Pitcher TJ, Sumaila RU, Walters CJ, Watson R, Zeller D (2002) Towards sustainability in world fisheries. Nature 418:689–695
6. FAO (2004) The state of world fisheries and aquaculture-2004 (SOFIA). Fisheries Department. Food and Agriculture Organisation of the United Nations, Rome, Italy
7. FAO (2009) The State of World Fisheries and Aquaculture (SOFIA) 2008, FAO Fisheries and Aquaculture Department. Food and Agriculture Organisation of the United Nations
8. Buck BH (2007a) Experimental trials on the feasibility of offshore seed production of the mussels *Mytilus edulis* in the German Bight: installation, technical requirements and environmental conditions. Helgol Mar Res 61:87–101
9. Buck BH (2007b) Marikultur als Co-Nutzung in Offshore-Windparks: Status Quo, Probleme und Perspektiven, Meeresumwelt-Symposium 2006: 16. Symposium, 13. bis 14. Juni 2006, CCH – Congress Center Hamburg/Bundesamt für Seeschifffahrt und Hydrographie in Zusammenarbeit mit dem Bundesumweltamt im Auftrag des Bundesministeriums für Umwelt, Naturschutz und Reaktorsicherheit, pp 167–179
10. FAO (2006) Fishery information, data and statistics unit. Aquaculture production: values 1984–2004. FISHSTAT Plus – universal software for fishery statistical time series. Food and Agriculture Organization of the United Nations, Rome, Italy
11. Burbridge P, Hendrick V, Roth E, Rosenthal H (2001) Social and economic policy issues relevant to marine aquaculture. J Appl Ichthyol 17:194–206
12. Rawski TG, Xiao W (2001) Roundtable on Chinese economic statistics: introduction. Chin Econ Rev 12:298–302
13. Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MCM, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405:1017–1024
14. Roth E, Ackefors H, Asche F, Balnath C, Black E, Black K, Boghen A, Browdy C, Burbridge P, Castell JD, Chamberlain G, Dabrowski K, Davies I, Dosdat A, Eleftheriou A, Ervik A, Gordin H, Heinig CS, Hilge V, Karakassis I, Kuhlmann H, Landry T, von Lukowicz M, McGlade J, Price A, Rheault RB, Rosenthal H, Saint-Paul U, Sandifer PA, Saroglia M, Silvert W, SteVens W, Soto D, Varadi L, Verreth J, Verdegem M, Waller U (2002) An intellectual injustice to aquaculture development: a response to the review article on "Effect of aquaculture on world fish supplies", Report of the ICES working group on environmental interactions of mariculture, F:04 REF ACME, Annex 4, pp 83–89
15. Buck BH (2004) Farming in a high energy environment: potentials and constraints of sustainable offshore aquaculture in the German Bight (North Sea). Dissertation. University of Bremen, Germany
16. Davis A, Bailey C (1996) Common in custom, uncommon in advantage: common property, local elites, and alternative approaches to fisheries management. Soc Nat Resources 9:251–265
17. Adger WN, Luttrell C (2000) Property rights and the utilisation of wetlands. Ecol Econ 35:75–89

18. Rana KJ (1997) Aquatic environments and use of species subgroups. In: Review of the state of world aquaculture. FAO Fisheries Circular FIRI/C886 (Rev. 1). Food and Agriculture Organisation (FAO), Rome, Italy, pp 7–16
19. Lee PG, Turk PE (1998) Overview of a modern, shore-based hatchery for offshore mariculture support. In: Stickney RR (ed) Joining forces with industry – open ocean aquaculture. Proceedings of the third annual international conference, May 10–15, Texas Sea Grant College Program, Corpus Christi, pp 87–102
20. Krause G, Buck BH, Rosenthal H (2003) Multifunctional use and environmental regulations: potentials in the offshore aquaculture development in Germany. Proceedings of the multidisciplinary scientific conference on sustainable coastal zone management "Rights and duties in the coastal zone", 12–14 June 2003. Stockholm, Sweden
21. Buck BH, Krause G, Rosenthal H, Smetacek V (2003) Aquaculture and environmental regulations: the German situation within the North Sea. In: Kirchner A (ed) International marine environmental law: institutions, implementation and innovation, vol. 64. International Environmental. Law and Policies Series of Kluwer Law International, The Hague, pp 211–229
22. Firestone J, Kempton W, Krueger A, Loper CE (2004) Regulating offshore wind power and aquaculture: messages from Land and sea. Cornell J Law Public Policy 14(1):71–111
23. Langan R (2009) Co-location of offshore energy and seafood production: potential synergies, compatibilities and conflicts. Conference paper, the ecology of marine wind farms: perspectives on impact mitigation, siting, and future uses, 8th annual Ronald C. Baird Sea Grant Science Symposium, November 2–4 2009, Newport, Rhode Island
24. BMU (2006) Integriertes Küstenzonenmanagement in Deutschland (IKZM). Nationale Strategie mit Bestandsaufnahme nach der EU-Empfehlung 2002/413/EG. Bundesregierung für Umwelt, Naturschutz und Reaktorsicherheit (Federal Ministry for the Environment, Nature Conservation and Nuclear Safety)
25. Kannen A (2004) Holistic systems analysis for ICZM: the Coastal Futures approach. In: Schernewski G, Dolch T (eds) Geographie der Meere und Küsten. Coastline reports, vol 1, pp 171–181
26. Buck BH (2002) Open Ocean Aquaculture und Offshore-Windparks: Eine Machbarkeitsstudie über die multifunktionale Nutzung von Offshore-Windparks und Offshore-Marikultur im Raum Nordsee. Reports on Polar and marine research. Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, pp 412–252
27. Ryan J (2005) Offshore aquaculture – do we need it, and why is it taking so long? International Salmon Farmers Association (Ireland). Expert workshop on sustainable aquaculture, DG JRC European Commission, Institute for Prospective Technological Studies, 17–18 January 2005. Seville, Spain
28. Dahle LA, DePauw N, Joyce J (1991) Offshore aquaculture technology – possibilities and limitations. Aquacult Environ 14:83–84
29. Eleftheriou M (1997) Aqualex: a glossary of aquaculture terms. Wiley-Praxis series in aquaculture and fisheries, Aqualex Multimedia Consortium Ltd
30. Chopin T (2009) Let's not move just the fish to the open ocean. . . an integrated multi-trophic aquaculture approach should not be an afterthought for 2050. Conference paper, the ecology of marine wind farms: perspectives on impact mitigation, siting, and future uses, 8th annual Ronald C. Baird Sea Grant Science Symposium, November 2–4, 2009, Newport, Rhode Island
31. Dena (2007) Renewable energies: Germany's first test Weld for offshore wind energy. Dena fact sheet. German Energy Agency, Berlin
32. Tiedemann A (2003) Windenergieparke im Meer – Perspektiven für den umweltverträglichen Einstieg in eine neue Großtechnologie. In: Lozán J, Rachor E, Reise K, Sündermann J, Westernhagen HV (eds) Warnsignale aus Nordsee & Wattenmeer: Eine aktuelle Umweltbilanz. Wissenschaftliche Auswertungen, Hamburg, pp 142–148
33. BMU/SOW (2007) Offshore wind power deployment in Germany. In: Rehfeldt K, Paschedag U, Bömer J (eds) Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) and Stiftung der Deutschen Wirtschaft zur Nutzung und Erforschung der Windenergie auf See/Offshore Wind Energy Foundation (SOW). Scholz-Druck GmbH, Dortmund, pp 31
34. Gierloff-Emden HGR (2002) Wandel der Umwelt der See-und Küstenlandschaft der Nordsee durch Nutzung von Windenergie. Mitteilungen der Österreichischen Geographischen Gesellschaft 144:219–226
35. Dahlke V (2002) Genehmigungsverfahren von Offshore-Windenergieanlagen nach der Seeanlagenverordnung. Natur und Recht 24:472–479
36. BWE (2008) Datenblatt 2007: Datenblatt Windenergie 2007. Bundesverband WindEnergie
37. GWEC (2007) Continuing boom in wind energy – 20 GW of new capacity in 2007. Press release 15/02 200. Global Wind Energy Council
38. BSH (2010) Wind farms. Bundesamt für Seeschifffahrt und Hydrographie. Federal Maritime and Hydrographic Agency, Hamburg/Rostock
39. Ventus A (2010) Der erste deutsche Offshore-Windpark (first german offshore wind farm). Deutsche Offshore-Testfeld und Infrastruktur. GmbH & Co. KG, Oldenburg
40. BWE (2007) Offshore Windenergie. BWE Hintergrundpapiere. Bundesverband WindEnergie
41. Wirtz KW, Tol RSJ, Hooss KG (2003) Mythos "Offene See": Nutzungskonflikte im Meeresraum. In: Lozan L et al (eds) Warnsignale aus Nordsee & Wattenmeer. Eine aktuelle Umweltbilanz. Wissenschaftliche Auswertungen, Hamburg, pp 157–160
42. Buck BH, Krause G, Rosenthal H (2004) Multifunctional use, environmental regulations and the prospect of offshore co-management: potential for and constraints to extensive open ocean aquaculture development within wind farms in Germany. Ocean Coast Manage 47:95–122

43. Jentoft S (2000) Co-managing the coastal zone: is the task too complex? Ocean Coast Manage 43:527–535
44. Michler-Cieluch T (2009) Co-Management processes in integrated coastal management: the case of integrating marine aquaculture in offshore wind farms. PhD thesis, University of Hamburg, Germany
45. Führböter A, Dette HH (1983) Wasserstände, Wind und Seegang im Seegebiet um Helgoland in den Jahren von 1969 bis 1983. Leichtweiß-Institut für Wasserbau der TU Braunschweig, Bericht Nr, 577
46. Becker G, Dick S, Dippner J (1992) Hydrography of the German Bight. Mar Ecol Prog Ser 91:9–18
47. Buck BH, Berg-Pollack A, Assheuer J, Zielinski O, Kassen D (2006) Technical realization of extensive aquaculture constructions in offshore wind farms: consideration of the mechanical loads. In: Proceedings of the 25th international conference on offshore mechanics and Arctic engineering, OMAE 2006: presented at the 25th International conference on offshore mechanics and Arctic engineering, 4–9 June 2006, Hamburg, Germany/American Society of Mechanical Engineers, pp 1–7
48. Takayanagi K (1998) Water quality guidelines for aquaculture: an example in Japan. In: Howell WH, Keller BJ, Park PK, McVey JP, Takayanagi K, Uekita Y (eds) Nutrition and technical development of aquaculture. Proceedings of the 26th US–Japan aquaculture symposium, Durham/New Hampshire/USA September 16–18, 1997. UJNR technical report No. 26, Durham, University of New Hampshire Sea Grant Program, pp 247–254
49. BSH (2006) MURSYS – marine environment reporting system information from the North Sea and Baltic Sea. Bundesamt für Seeschifffahrt und Hydrographie (BSH), Hamburg
50. Stickney RR (1998) Joining forces with industry – open ocean aquaculture. Proceedings of the 3rd annual international conference, May 10–15, Corpus Christi. TAMU-SG-99–103, Corpus Christi, Texas Sea Grant College Program
51. Bridger CJ, Costa-Pierce BA (2003) Open ocean aquaculture: from research to commercial reality. The World Aquaculture Society, Baton Rouge
52. Michler-Cieluch T, Kodeih S (2008) Mussel and seaweed cultivation in offshore wind farms: an opinion survey. Coast Manage 36(4):392–411
53. Buck BH, Buchholz CM (2004) The offshore-ring: a new system design for the open ocean aquaculture of macroalgae. J Appl Phycol 16:355–368
54. Buck BH, Walter U, Liebezeit G (2011) Larval occurrence and settlement in the German Bight: A trial to estimate potentials for *Mytilus edulis* culture in offshore areas, Helgoland Marine Research (in press)
55. BSH (2009) Bundesamt für Seeschifffahrt und Hydrographie – BSH (Federal Maritime and Hydrographic Agency), CONTIS-Geodata. Hamburg/Rostock
56. Hickman RW (1992) Mussel cultivation. In: Gosling E (ed) The mussel *Mytilus*: ecology, physiology, genetics and culture, vol 25, Development in aquaculture and fisheries science. Elsevier, Amsterdam, pp 465–510
57. Critchley AT, Ohno M, Largo DB (2006) World seaweed resources. An authoritative reference system. ETI BioInformatics. DVDROM
58. Tseng CK (1989) *Laminaria* mariculture in China. In: Doty MS, Caddy JF, Santelices B (eds) Case studies of seven commercial seaweed resources. FAO Fisheries Technical Paper – 281. Food and Agriculture Organization of the United Nations (FAO), Rome
59. Strathmann MF (1987) Reproduction and development of marine invertebrates of the northern Pacific Coast. University of Washington Press, Seattle, pp 670
60. Pulfrich A (1997) Seasonal variation in the occurrence of planktic bivalve larvae in the Schleswig-Holstein Wadden Sea. Helgol Wiss Meer 51:23–39
61. Walter U, Liebezeit G (2001) Abschlußbericht des Projektes: Nachhaltige Miesmuschel-Anzucht im niedersächsischen Wattenmeer durch die Besiedlung natürlicher und künstlicher Substrate. Forschungszentrum Terramare, pp 1–97
62. Walter U, Liebezeit G (2003) Efficiency of the blue mussel (*Mytilus edulis*) spat collectors in highly dynamic tidal environments of the Lower Saxonian coast (Southern North Sea). Biomol Eng 20:407–411
63. Scarratt D (1993) A handbook of northern mussel culture. Island Press, Montague, pp 167
64. Gosling E (2003) Bivalve molluscs: biology, ecology and culture. Blackwell Publishing/MPG Books, Bodmin/Cornwall, pp 443
65. Brenner M (2009) Site selection criteria and technical requirements for the offshore cultivation of blue mussels (*Mytilus edulis* L.). PhD thesis, School of Engineering and Science, Jacobs University, Bremen, pp 151
66. Langan R, Horton F (2003) Design, operation and economics of submerged longline mussel culture in the open ocean. Bull Aquacult Assoc Can 103:11–20
67. Neushul M, Harger BWW (1985) Studies of biomass yield from a near-shore macroalgal test farm. J Solar Energy Eng 107:93–96
68. Neushul M, Benson J, Harger BWW, Charters AC (1992) Macroalgal farming in the sea: water motion and nitrate uptake. J Appl Phycol 4:255–265
69. Turner R (2001) Offshore mariculture: site evaluation. In: Muir J, Basurco B (eds) Options méditerranéennes – Mediterranean offshore mariculture. Etudes et recherches, Serie B, Numéro 30, Zaragoza, CIHEAM, INO Reproducciones, pp 141–157
70. Pérez OM, Telfer TC, Ross LG (2003) On the calculation of wave climate for offshore cage culture site selection: a case study in Tenerife (Canary Islands). Aquacult Eng 29:1–21
71. Dalton R (2004) Fishing for trouble. Nature 431:502–504
72. Naylor R, Burke M (2005) Aquaculture and ocean resources: raising tigers of the sea. Annu Rev Environ Res 30:185–218
73. Allen JI, Moore MN (2004) Environmental prognostics: is the current use of biomarkers appropriate for environmental risk evaluation. Mar Environ Res 58:227–232

74. Buck BH, Walter U, Rosenthal H, Neudecker T (2006) The development of mollusc farming in Germany: past, present and future, World Aquaculture 37(2):6–11 & 66–69
75. Buck BH, Thieltges D, Walter U, Nehls G, Rosenthal H (2005) Inshore–offshore comparison of parasite infestation in *Mytilus edulis*: implications for open ocean aquaculture. J Appl Ichthyol 21:107–113
76. Brenner M, Buck BH, Koehler A (2007) New concept combines offshore wind farms, mussel cultivation. Global Aquacult Advocate 10:79–81
77. Buck BH, Buchholz CM (2005) Response of offshore cultivated *Laminaria saccharina* to hydrodynamic forcing in the North Sea. Aquaculture 250:674–691
78. Manefeld T (2006) Ansiedlungspotential von Miesmuschellarven (*Mytilus edulis*) an verschiedenen Substraten freihängender Brutkollektoren. Thesis, University of Applied Sciences Bremerhaven, Germany
79. Voss D (2006) Parasitierungsgrad der Miesmuschel (*Mytilus edulis*) in Abhängigkeit zur Tiefenzonierung und Entfernung zur Küste. Thesis, University of Applied Sciences Bremerhaven, Germany
80. Brenner M, Buck BH (2010) Attachment properties of blue mussel (*Mytilus edulis L.*) byssus threads on culture-based artificial collector substrates. Aquacult Eng 42:128–139
81. Zielinski O, Assheuer J, Berg-Pollack A, Buck BH, Geisen M, Henkel R, Kassen D (2006) Assessment of mechanical loads and environmental conditions for extensive aquaculture constructions within offshore wind farms: First results from the AquaLast study site. Proceedings of DEWEK 2006: presented at the DEWEK 2006, 22–23 November 2006, Bremen, Germany, pp 1–4
82. Bard (2010) Offshore wind farms. Bard Engineering GmbH. Emden, Germany
83. OFT (2010) *Aquapod* – a submergible fish cage. Ocean Farm Technologies, Searsmont
84. Ostrom E, Burger J, Field CB, Norgaard RB, Policansky D (1999) Revisiting the commons: local lessons, global challenges. Science 284(4512):278–282
85. Hardin G (1968) The tragedy of the commons. Science, 162. Reprinted in Dryzek JS, Schlosberg D (1999) Debating the Earth: the environmental politics reader. Oxford University Press, New York
86. Michler-Cieluch T, Krause G, Buck BH (2009) Reflections on integrating operation and maintenance activities of offshore wind farms and mariculture. Ocean Coast Manage 52(1):57–68
87. Buck BH, Krause G, Michler-Cieluch T, Brenner M, Buchholz CM, Busch JA, Fisch R, Geisen M, Zielinski O (2008) Meeting the quest for spatial efficiency: progress and prospects of extensive aquaculture within offshore wind farms. Helgol Mar Res 62:269–281
88. EU Commission (2009) Building a sustainable future for aquaculture. A new impetus for the Strategy for the Sustainable Development of European Aquaculture. The European Parliament and the Council, COM(2009) 162 final
89. McVey JP (1996) Overview of offshore aquaculture. In: Polk M (ed) Open ocean aquaculture. Proceedings of an international conference, May 8–10, Portland, Maine. New Hampshire/Maine Sea Grant College Program, pp 13–18
90. Braginton-Smith B, Messier RH (1997) Design concepts for integration of open ocean aquaculture and Osprey TM Technology. In: Howell WH, Keller BJ, Park PK, McVey JP, Takayanagi K, Uekita Y (eds) Proceedings of the twenty-sixth US–Japan aquaculture symposium, Durham, New Hampshire. UJNR Technical Report No. 26. University of New Hampshire Sea Grant Program, Durham, pp 239–245
91. Bussel van GJW, Zaaijer MB (2007) Reliability, availability and maintenance aspects of large-scale offshore wind farms, a concepts study. In: Proceedings of MAREC 2001. Delft University of Technology, The Netherlands, pp 119–126
92. Musial W, Butterfield S, Ram B (2006) Energy from offshore wind. In: Proceedings of the offshore technology conference (OTC), 1–4 May, Houston, 11 pp
93. Michler-Cieluch T, Krause G (2008) Perceived concerns and possible management strategies for governing 'wind farm–mariculture integration'. Marine Policy 32(6): 1013–1022
94. Carlsson L, Berkes F (2005) Co-management: concepts and methodological implications. J Environ Manage 75: 65–76
95. Cash DW, Moser SC (2000) Linking global and local scales: designing dynamic assessment and management processes. Global Environ Change 10:109–120
96. Heinelt H (2002) Achieving sustainable and innovative policies through participatory governance in a multi-level context. In: Heinelt H, Getimis P, Kafkalas G, Smith R, Swyngedouw E (eds) Participatory governance in multi-level context. Leske + Budrich, Opladen, pp 17–32
97. EU Commission (2007) An Integrated Maritime Policy for the European Union. Commission Staff Working Document, Accompanying document to the Communication from the Commission to the European Parliament, the Council, The European Economic and Social Committee and the Committee of the Regions, SEC (2007) 1278
98. Dietz T, Ostrom E, Stern PC (2003) The struggle to govern the commons. Science 302(5652):1907–1912
99. Steins NA, Edwards VM (1999) Synthesis: platforms for collective action in multiple-use common-pool resources. Agric Hum Values 16(3):309–315
100. Buck BH, Ebeling M, Michler-Cieluch T (2010) Mussel cultivation as a co-use in offshore wind farms: potential and economic feasibility. Aquacult Econ Manage 14(4): 1365–7305
101. Bradbury RH, Seymour RM (2009) Coral reef science and the new commons. Coral Reefs 28:831–837

Aquaculture, Ecological

Barry A. Costa-Pierce
University of New England, Marine Science Center, Biddeford, ME, USA

Article Outline

Glossary

Biofloc A mixture of detritus, bacteria, and other microscopic organisms that aggregates in flocs, which are used for controlling water quality and enhancing the delivery of natural foods to omnivorous species in aquaculture.

Ecosystem An area of the natural environment in which the structure and functions of the physical (rocks, soil, etc.) and natural (all living organisms) environments are considered together in interacting food webs.

Escapees The unintended releases of cultured organisms from captivity into the wild.

Polyculture The practice of making compatible the culture of multiple species in the same physical space by stocking or planting organisms having different food, spatial, or temporal niches.

Resilience The ability of a natural or aquaculture system to absorb abrupt changes or disturbances without collapsing. A resilient aquaculture ecosystem can withstand physical and economic shocks and rebuild itself.

Stewardship An ethic that engages all affected stakeholders in the cooperative planning and management of the environmental quality to prevent degradation and facilitate recovery in the interest of long-term sustainability.

Watershed An area of land where all of the water that is under it or drains off of it goes into the same place.

Definition of Ecological Aquaculture

Ecological aquaculture is an alternative model of aquaculture development that uses ecological principles as the paradigm for the development of aquaculture [1, 2]. Ecological aquaculture plans, designs, develops, monitors, and evaluates aquatic farming ecosystems that preserve and enhance the form and functions of the natural and social environments in which they are situated. Ecological aquaculture farms are integrated "aquaculture ecosystems" designed to deliver both economic and social profit (Fig. 1).

Ecological aquaculture incorporates at the outset – and not as an afterthought – planning for not only the sustainable production of aquatic foods, but also for innovation [3], community development, and the wider social, economic, and environmental contexts of aquaculture at diverse scales, both large and small, and at the commercial, school, and homeowner scales [4, 5]. Ecological aquaculture also uses the "aquaculture toolbox" [6] to play vital roles in non-food, natural ecosystem rehabilitation, reclamation, and enhancement.

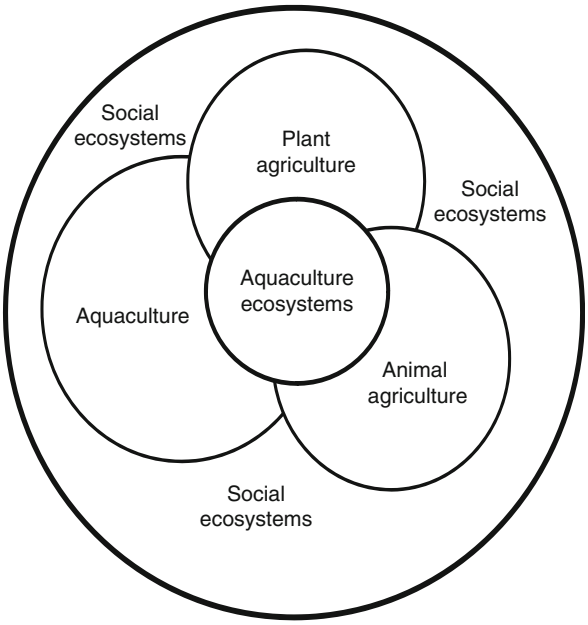
Introduction

The roots of ecological aquaculture are in Asia [7, 8]. In this century, however, Asia, especially China, during the period from 1980s to present has chosen the industrial model of aquaculture development, and has dismantled much of its rich ecological aquaculture heritage, and choosing instead to intensify and import vast quantities of feedstuffs. As a result of intensification and the use of imported feeds, freshwater aquaculture yields from China have increased 10X in just 20 years, and comprise the world's largest aquaculture industries [9].

The FAO ecosystems approach to aquaculture [10] creates a new code for global aquaculture development, combining into one common framework the two most

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Aquaculture, Ecological. Figure 1
Aquaculture ecosystems mimic the form and functions of natural ecosystems, but are a sophisticated, knowledge-based designed farming ecosystem that are planned as combinations of land and water-based aquatic plant, agronomic, algal, and animal subunits, which are embedded into the larger context of human social systems

important social-ecological trajectories for global aquaculture – aquaculture for the world's rich, and aquaculture for the world's poor. Knowledge of the rich archeology and anthropology of aquaculture connects this FAO code to antiquity, creating a single development pathway for aquaculture throughout human history.

Key Principles

There are seven principles of ecological aquaculture:

1. **Ecological aquaculture systems are "aquaculture ecosystems" that mimic the form and functions of natural ecosystems.**

   Ecological aquaculture farms are designed, farming ecosystems. Sophisticated site planning occurs so that farms "fit with nature" and do not displace or disrupt invaluable natural aquatic ecosystems or conservation areas. If localized displacement or degradation does occur, active support of innovative, collaborative research and development programs for ecosystems redesign, relocation, rehabilitation, and enhancement efforts are initiated and supported by the ecological aquaculture farms throughout the life of their farming operations.
2. **Ecological aquaculture is integrated with communities to maximize not only local but also regional economic and social multiplier effects in order to provide maximal job creation and training, and create "aquaculture communities" that are an essential part of vibrant, working waterfronts.**

   Ecological aquaculture operations export to earn profits but also promote and market products locally to contribute to the development of society. Ecological aquaculture operations are committed to building the "culture" of aquaculture in order that "aquaculture communities" can develop and evolve as a source of innovation, education, and local pride. Aquaculture development as a means of community development can result in numerous, innovative economic and social multiplier effects such as aquaculture restaurants, marketing of "sustainable seafoods" that are branded as local and bioregional, and aquaculture tourism.
3. **Ecological aquaculture results in economic profit by practicing trophic efficiency to ensure that aquaculture is humanity's most efficient protein producer.**

   Non-fed, shellfish and algae culture are preferred choices for ecological aquaculture developments. In fed aquaculture, fish meals/oils are not used as either the major protein or energy sources, but are included in animal diets to solve issues of diet palatability only; and, if used, fish meals and oils originate from certified, sustainable fishmeal/oil fisheries only. Fed aquaculture ecosystems rely on protein and oil sources from agricultural sources and seafood processing wastes, and include science innovations such as the development of detrital food webs ("bioflocs") to feed cultured, aquatic organisms.
4. **Ecological aquaculture results in social profit by integrating aquaculture developments into global fisheries, food, and poverty alleviation programs.**

   Ecological aquaculture is part of the global movement to eliminate extreme hunger and

starvation (Millennium Development Goal #1) by being a part of comprehensive plans for sustainable fisheries for poverty alleviation. Ecological aquaculture uses alternative feeds to support programs to deliver more of the world's feed fisheries (sardines, anchovies, mackerels, etc.) away from aquaculture to the world's poor.

5. **Ecological aquaculture practices nutrient management by using ecosystems design, reuse, and recycling, and does not discharge any nutrient or chemical pollution causing irreversible damage to natural aquatic or terrestrial ecosystems.**

   No harmful metals, chemicals, or pharmaceuticals potentially harmful to long-term human or ecosystem health are used in the ecological aquaculture production processes. Ecological aquaculture farms have "sustainability strategic and implementation plans" in place to develop comprehensive, full cycle reuse, and recycling systems for all farming operations.

6. **Ecological aquaculture uses native species/strains, and does not contribute to "biological" pollution.**

   Escapees from aquaculture, especially aquarium operations, have severely impacted aquatic ecosystems worldwide. Exotics species/strains can be good choices only if long-term monitoring data and scientific research indicate that exotic species are unlikely to establish; if exotic species are widely established and provide economic and social profit without irreversible environmental harm; or, the use of native species puts at risk indigenous genetic diversity. Ecological aquaculture operations ensure that innovative engineering and complete escapement technologies are used; that control and recovery procedures are in place; that active research and development programs provide alternatives and new options; and that complete, transparent, public documentation and information are available.

7. **Ecological aquaculture is a global partner, producing information for the world, avoiding the proprietary.**

   Ecological aquaculture farms are aquaculture ecosystems that go beyond "meeting the regulations." They are sites of collaboration, leadership development, and innovation. They are outstanding community citizens and models of stewardship [4]. Successful leadership development triggers developments of innovation and more efficient aquaculture-related technology, and more ecologically appropriate legislation and regulations.

The FAO Ecological Approach to Aquaculture (EAA)

In 2006, the Fisheries and Aquaculture Department of Food and Agriculture Organization (FAO) recognized the need to develop an ecosystem-based management approach to aquaculture similar to the Code of Conduct for Responsible Fisheries. FAO [10] suggested that an ecological approach to aquaculture (EAA) would have three main objectives: human well-being, ecological well-being, and the ability to achieve both via effective governance, within a hierarchical framework that was scalable at the farm, regional, and global levels.

In 2008, FAO defined an EAA as: A strategy for the integration of the activity within the wider ecosystem such that it promotes sustainable development, equity, and resilience of interlinked social-ecological systems. An ecosystem approach to aquaculture, similar to other systems approaches to management, accounts for a complete range of stakeholders, spheres of influences, and other interlinked processes. Applying an ecosystem-based approach must plan for physical, ecological, social, and economic systems as a part of community development, taking into account stakeholders in the wider social, economic, and environmental contexts of aquaculture [10]. FAO developed three principles and key issues at different scales of society:

Principle 1: Aquaculture development and management should take account of the full range of ecosystem functions and services, and should not threaten the sustained delivery of these to society.

The key issue is to estimate ***resilience capacity***, or the limits to "acceptable environmental change." A range of terms has been used to estimate the limits to environmental change, including "environmental carrying capacity," "environmental capacity," "limits to ecosystem function," "ecosystem health," "ecosystem integrity," "fully functioning ecosystems," all of which are subject to a specific social/cultural/political context [11]. Conventional

environmental impact assessments touch on just some of these issues. Application of the precautionary approach is important but inadequate in aquaculture; use of aquaculture risk assessment is becoming widespread [12].

Principle 2: Aquaculture should improve human well-being and equity for all relevant stakeholders.

Aquaculture should provide equal opportunities for development, which requires its benefits to be more widely shared especially locally so that it does not bring detriment to any sector of society, especially the poor. Aquaculture should promote both food security and safety as key components of human well-being.

Principle 3: Aquaculture should be developed in the context of other sectors, policies, and goals.

Interactions between aquaculture and its influences on the surrounding natural and social environment must be recognized. Aquaculture often has a smaller impact than other human activities, e.g., agriculture and industry, but it does not take place in isolation. There are many opportunities to couple aquaculture activities with other primary producing sectors in order to promote materials and energy recycling, and the better use of resources in general.

Applying an Ecological Aquaculture Approach at Different Scales of Society

There are three physical scales important in the planning for and assessment progress toward an ecosystem approach to aquaculture: farm scale, watershed/aquaculture zone, and global. Each of these has important planning and assessment needs.

Farm Scale

Planning for aquaculture farms is easily defined physically and could be few meters beyond the boundaries of farming structures; however, the increasing size and intensity of some farms (e.g., large-scale shrimp farming or salmon farming) could affect an entire water body or watershed. Assessment of an EAA at the farm scale entails an evaluation of planning and implementation of "triple bottom line" programs – ecological, economic, and social programs – that in a comprehensive manner account for impacts to the wider ecosystem and social impacts of farm-level aquaculture developments, including use of better ("best") management practices, and use of restoration, remediation, and mitigation methods. Proper site selection, levels of production intensity, use of species (exotic vs. native), use of appropriate farming systems technologies, and knowledge of economic and social impacts at the farm level should be considered.

For fed aquaculture, there are many concerns as the current trajectory and growth of the large-scale aquaculture industries. Current aquaculture development models are being modified rapidly by advances that will affect the widespread adoption of ecological aquaculture, which, if projected to 2050, confirm that large-scale aquaculture may move fully toward ecological aquaculture approaches (Table 1). There are a growing number of well-documented success stories in ecological aquaculture (Table 2).

Watershed/Aquaculture Zone Scale

Planning for an EAA at watersheds/aquaculture scale is relevant to common ecosystem and social issues such as diseases, trade in seed and feeds, climatic and landscape conditions, urban/rural development, etc. Assessment of an EAA at this scale is a two-phase process and will include, at phase I, assessments of

1. Inclusion of aquaculture as a part of regional governance frameworks, e.g., the overall framework of integrated coastal zone management or integrated watershed, land-water resource management planning, and implementation. Assessments take into account existing scenarios, user competition, and conflicts for land and water uses, and comparisons of alternatives for human development.
2. Impacts of aquaculture on regional issues such as escapees, disease transmission, and sources of contamination to/from aquaculture.
3. Social considerations such as comprehensive planning for all of the possible beneficial multiplier effects of aquaculture on jobs and the regional economy, and considerations of aquaculture's impacts on indigenous communities.

At phase II, progress toward a full implementation of an EAA at watersheds/aquaculture zone scale can be assessed by measuring the

Aquaculture, Ecological. Table 1 Major issues with fed aquaculture today (2010) and projections of these to 2050

Issues	Concerns	Modern developments (2010)	Trajectory of issues to 2050
Feeds/no net gain	Schroeder [28] documents pond can be a net consumer rather than a producer of animal protein. Fishing down and farming up marine food webs (Naylor et al. [29]; Pauly [30])	Food conversion rates improve to ~1:1; fish in/out (FIFO) ratios drop to ~1.7; domestication of farmed species turns carnivores into domesticated omnivores	FIFO ratios drop to 1 or less; aquaculture uses ~50% of world's fish meal and oil with balance met by agricultural meals/oils
Feeds/ocean sustainability	Integrity of marine ecosystems damaged by high removal rates of feed species	Aquaculture use dropping due to rapid cost increases in meals/oils; poverty/social concerns recognized	Ecosystem modeling parcels out science-based removal rates/allocations for aquaculture and ecosystems
Feeds/poverty	Massive poverty and hunger in fish meal/oil producing countries	New recognition in Peru; new international attention to role of meal/oil fisheries & fed aquaculture in poverty alleviation	Governments move to develop food products/prioritize human needs
Habitat destruction	Mangrove destruction and water diversions disrupt nearshore and riverine ecosystems (Macintosh and Phillips [31]; Pullin et al. [32]	Some nations (ex. Thailand) develop policies to prevent damage by proper siting and to rehabilitate damage of shrimp farms	Governments worldwide ban developments in sensitive conservation areas; widespread use of carrying capacity models (McKindsey et al. [33]) and ecological valuation for decision-making
Eutrophication	Intensive aquaculture operations are feedlots producing nutrient pollution loads comparable to human sewage (Folke et al. [21]; Costa-Pierce [34])	Complete feeds, automated feed delivery systems, and nutrition research deliver less pollution; wastes are primarily in the form of soluble nutrients and feces, not waste feeds	Development of land-based recirculating systems; widespread use of land-based integrated aquaculture and water-based IMTA systems
Energy	Intensive aquaculture operations are energy intensive comparable to industrial agriculture and fisheries (Weatherly and Cogger [35])	Scattered R&D in energy use, mostly Life Cycle Analyzes in aquaculture; little/no movement toward use of renewables	Renewable energy systems used

1. Abilities of governments to implement new methods of coastal and water governance to include ecological aquaculture
2. Development of ecological aquaculture approaches that allow agencies responsible for permitting aquaculture to consider and manage activities impacting aquaculture and aquatic ecosystems (e.g., capture fisheries, coastal zone development, watershed management organizations, agriculture, forestry, and industrial developments) more holistically, such as new mechanisms to communicate, cooperate, and collaborate across sectors
3. Design of ecological aquaculture management zones and parks that encourage aquaculture education, research, and development innovations and partnerships, and also emphasize streamlined permitting of integrated aquaculture, polyculture, or innovative, integrated aquaculture–fisheries businesses and initiatives

Global Scale

Planning for an EAA at a global scale considers aspects of transnational and multinational issues for global commodities (e.g., salmon and shrimp). Assessment

Aquaculture, Ecological. Table 2 Global success stories in ecological aquaculture

Region/countries	Aquaculture ecosystems	References
Asia (China, Vietnam, Indonesia)	Rice-fish culture benefits millions of rural people; rice-fish aquaculture ecosystems have been designated as a "Globally important Agricultural Heritage System" (GIAHS)	FAO [9]; Dela Cruz et al. [36]
Asia (China, Thailand, Cambodia, Vietnam, Indonesia)	Integrated aquaculture benefits millions of rural people	Edwards [8]
Asia (China)	Integrated Multi-trophic Aquaculture (IMTA) of fish, shellfish, and seaweeds bioremediates and increases total yields up to 50%	Zhou et al. [37]
Egypt	Integrated aquaculture produced over 650,000 tons of tilapia in 2008, ~60% of total fish production; provision of cheap source of fish at approx. same cost as poultry	McGrath [38]
Canada	IMTA has been adopted by Cooke Aquaculture, the largest salmon aquaculture company in eastern Canada	Chopin et al. [39]; Chopin [40]; Ridler et al. [41, 42]
Canada & USA	Shellfish aquaculture has become widely accepted as environmentally friendly and socially acceptable	National Academy [43, 44]
Tanzania	Seaweed and shellfish aquaculture	Seaweed grown by ~2,000 producers most women; new half-pearl industry growing

of progress toward an EAA at the global level entails evaluation of issues such as: availabilities of fisheries and agriculture feedstocks for formulating aquaculture feeds and impacts on distant marine and social ecosystems, the economic and social impacts of aquaculture on fisheries and agriculture resources, impacts of aquaculture on markets, and impacts of globalization on social sustainability (social capital, goods, and social opportunities). Applications of tools such as lifecycle assessments of aquaculture commodities and the use of innovative social enterprise management guidelines and tools are useful to determine impacts at the global scale.

Social Ecology of Aquaculture

Many analysts are calling for more integrated, multidisciplinary ways of developing ecologically and socially responsible food, energy, water, and waste systems to meet society's needs [13]. Among the first was Lubchenco [14] who called for a new social contract for science and society. Industrial aquaculture in its current development phase does not have a social contract or social license to expand in many areas of the world, especially at the watershed/aquaculture zone and global scales.

Just as important are social investments in aquaculture at the individual level. Aquaculture has an urgent need for developing and engaging leaders who are well trained and experienced decision-makers who are "honest brokers of policy alternatives" [15]. Keen et al. [16] believe transformation toward more sustainable practices will be much more likely if the individuals who make up society can accept change and modify their personal behaviors [17]. Changes in the behavior of individuals can "scope up" and result in larger changes at the community and societal scales by employing a combination of trust-building, favorable performance, accountability, flexibility and innovation, and the inclusion of stakeholders in strategic planning [18, 19].

Folke et al. [20] challenge our education system to continually adapt to the emergence of such new questions and changing social compacts as aquaculture. Any rapid progress toward an ecological approach to aquaculture will require development of education programs that promote broad awareness, recognition, and implications of new approaches to aquaculture, and the creation of new institutions. Bransford et al. [22] suggest that for such subfields of sustainability science as aquaculture more attention needs to be given to educating the next generation of leaders by teaching metacognitive skills such as practicing different ways of thinking in a variety of contexts, with less emphasis being placed on trying to fill students with a large volume of facts and knowledge.

An Ecological Aquaculture Strategy for the "Triple Bottom Line"

Aquaculture development plans will be incomplete unless both economic and social goals are articulated, and agreed upon, at the outset, in transparent, participatory processes. Only then can aquaculture can "evolve" as an integral part of – not separate from – farmers, fishermen, sustainable community development, and the future of "working waterfronts." Aquaculture's success cannot simply be defined as having successfully developed the hatchery, feed, and marketing components of a business plan – the old alignment of the "seed, feed, and the need." Rather, sustainable, ecological aquaculture nurtures "society's success" for the "triple bottom line" of economic, environmental, and social profit [23].

Adversarial social processes occur in jurisdictions where aquaculture is not being developed using a social-ecological "ecosystem approach." In these places, the blue revolution is being televised, tweeted, and blogged. Adversarial processes (conflicts) occur when stakeholders do not recognize each other's interests as legitimate. These processes increase conflict; thrive on uncertainty; have poor communication; are exclusive, divisive, opaque, and closed, and lack trust. Collaborative processes must be created that create trust through shared learning and ownership, creative problem solving, joint fact finding, and employ adaptive management. Robertson and Hull [24] call this a "public ecology" that has both process and content that emphasizes the participation of extended peer communities of research specialists, policy-makers, and concerned citizens. Dasgupta and Maler [25] have used tools developed by economists and ecologists to valuate choices in the midst of this complexity. In general, since aquaculture is such a dynamic, evolutionary field, managers, policy-makers, and community leaders need to participate to allow understanding of new and emerging problems and to stimulate multidisciplinary research; as analysts report that such work is the highest impact science being published today [26].

Clear, unambiguous linkages between aquaculture and the environment must be created and fostered, and the complementary roles of aquaculture in contributing to environmental sustainability, rehabilitation, and enhancement must be developed and clearly articulated to a highly concerned, increasingly educated, and involved public. New aquaculture operations must plan, at the outset, to:

1. Become an integral part of a community and a region.
2. Plan for community development by working with leaders to provide needed inputs and recycle wastes.
3. Create a diversity of unprocessed and value-added products, and provide local market access, since in rich societies, aquaculture products are high-value discretionary purchases that can easily be rejected by the public.
4. Plan for job creation and environmental enhancement on both local and regional scales.

It is well documented that most aquaculture jobs are not directly in production, rather in the affiliated service industries. In the USA, Dicks et al. [27] found that aquaculture production accounted for just 8% of the income and ~16,500 jobs. Aquaculture goods and services accounted for 92% of the income and ~165,500 jobs (most jobs were in equipment, supplies, feeds, fertilizers, transport, storage, processing). However, most aquaculture development plans focus almost exclusively on production concerns and have little/no comprehensive plans for localization of seed, feed, markets, or other aquaculture service industries that produce the most benefits to local economies – to say nothing about employing local professionals (most industrial aquaculture operations import high paying professionals from the outside). In the vast majority of

cases, feed and services are imported to sites, and local people cannot even buy the produce!

An ecological aquaculture development model will create new opportunities for a wider group of professionals to get involved in aquaculture since new advances will be needed not only in technology but also in information, community development, and facilitation. Ecological aquaculture as a "new" field, and one that is important for the future food security and environment of the planet, requires the much more comprehensive planning in order to evolve a new social contract with society.

Future Directions

By 2030, fed aquaculture will turn from the ocean to land-based agriculture to provide its feeds and oils. As such, more sophisticated, ecologically designed and integrated aquaculture systems will become more widespread because they better fit the social-ecological context of both rich and poor countries. Ecological aquaculture provides the basis for developing a new social contract for aquaculture that is inclusive of all stakeholders and decision-makers in fisheries, agriculture, ecosystems conservation, and restoration.

The wildly optimistic scenarios for aquaculture's expansion will not occur unless alternative ecological approaches and ecological intensification of aquaculture are widely adopted. Aquaculture needs to be better integrated into overall fishery societal plans for securing sustainable seafood supplies and restoring damaged, supporting fisheries ecosystems.

The overuse and degraded state of nearly all of the world's aquatic ecosystems, combined with public concerns about adding any "new" uses or sources of aquatic pollution to already overburdened natural and human systems, require aquaculture to develop ecosystems approaches; sustainable operating procedures; and to articulate a sustainable, ecological pedagogy. The fact that an ecological aquaculture approach can ensure aquaculture is a net gain to humanity; and it could be the key organizing paradigm to form a new social contract for aquaculture worldwide.

The massive globalization of seafood trade has meant less dependence on local natural and social ecosystems, and has resulted in some virulent opposition to aquaculture development, especially as industrial aquaculture has removed the local sources of production and markets, and jobs have been externalized. One major consequence of this globalization has been the increased dependence of industrial, "fed" aquaculture on the southeastern Pacific Ocean marine ecosystem for fish meals and oils. The global implications for the Humboldt ecosystem, for local poverty, and the scoping of this unsustainable situation to the entire global protein food infrastructure are profound, and are still largely unrealized.

Aquaculture sites are not only economic engines of primary production that meet the regulations of a society, but can be sites of innovation and pride if they can be well designed as community-based, aquaculture farming ecosystems. A review of the progress toward such an ecosystems approach to aquaculture is necessary to inspire planners and environmental decision-makers at many societal scales (national, regional, local) to make use of such innovative approaches. Sophisticated site planning of aquaculture can occur so that farms "fit with nature" and do not displace or disrupt invaluable natural, aquatic ecosystems, or conservation areas; but contribute to the local economy and society [5].

An ecological aquaculture approach to comprehensive planning for aquaculture at many different scales will integrate aquaculture into plans for not only environmental benefit and the restoration of coastal ecosystems, but also local market developments and the future of coastal communities. As such, ecological aquaculture can move aquaculture beyond endless user conflicts, and could stabilize the regulatory environment and ensure a more equitable process of eco-social design of aquaculture for the future.

Bibliography

Primary Literature

1. Costa-Pierce B (2002) Ecological aquaculture. Blackwell, Oxford
2. Costa-Pierce B (2003) Use of ecosystems science in ecological aquaculture. Bull Aquacul Assoc Canada 103(2):32–40
3. Culver K, Castle D (2008) Aquaculture, innovation and social transformation. Springer, New York
4. Costa-Pierce B (2008a) An ecosystem approach to marine aquaculture: a global review. In: Soto D (ed) Building an ecosystem approach to aquaculture, vol 14, FAO fisheries and aquaculture proceedings. FAO, Rome, pp 81–116

5. Costa-Pierce B (2008b) Epilogue. In: Culver K, Castle D (eds) Aquaculture, innovation and social transformation. Springer, New York, pp 315–325
6. Costa-Pierce B, Bridger C (2002) The role of marine aquaculture facilities as habitats and ecosystems. In: Stickney R, McVey J (eds) Responsible marine aquaculture. CABI, Wallingford, pp 105–144
7. Ruddle K, Zhong G (1988) Integrated agriculture-aquaculture in South China: the Dike-Pond system in Zhujiang Delta. Cambridge University Press, Cambridge
8. Edwards P (2009) Traditional Asian aquaculture: definition, status and trends. In: Burnell G, Allan G (eds) New technologies in aquaculture, improving production efficiency, quality and environmental management. Woodhead Publishing Ltd., Cambridge, UK, pp 1029–1063
9. FAO (Food and Agriculture Organization) (2009) The state of world fisheries and aquaculture 2008. Fisheries Department, Food and Agriculture Organization of the United Nations, Rome
10. Soto D et al (2008) Applying an ecosystem-based approach to aquaculture: principles, scales and some management measures. In: Soto D, Aguilar-Manjarrez J, Hishamunda N (eds) Building an ecosystem approach to aquaculture, vol 14, Rome: FAO fisheries and aquaculture proceedings. FAO, Rome
11. Hambrey J, Senior B (2007) Taking forward environmental carrying capacity and ecosystem services. Recommendations for CCW. CCW Policy Research Report No. 07/22
12. Black E (2006) A risk analysis approach to assessment of the potential genetic interactions of non-salmonid marine finfish escapes from aquaculture with local native wild stocks, pp 93–105. In: ICES WGEIM report on the environmental impacts of mariculture, 24–28 April 2006, Narragansett, Rhode Island, USA. ICES, Copenhagen, Denmark. (Available at www.ices.dk/reports/MCC/2006/WGEIM06.pdf)
13. Dees J, Backman E (1994) Social enterprise: private initiatives for the common good. Harvard Business School Publishing, Harvard University, Boston
14. Brown L (2009) Plan B 4.0: mobilizing to save civilization. W. Norton, New York
15. Lubchenco J (1998) Entering the century of the environment: a new social contract for science. Science 279:491–497
16. Pilke R (2007) The honest broker: making sense of science in policy and politics. Cambridge University Press, Cambridge
17. Keen M, Brown V, Dyball B (2005) Social learning in environmental management. Earthscan, London
18. Huckle J, Sterling S (1996) Education for sustainability. Earthscan, London
19. Brehm J, Rahn W (1997) Individual level evidence for the causes and consequences of social capital. Am J Pol Soc 41:999–1023
20. Knack S (2002) Social capital and the quality of government: evidence for the states. Am J Pol Sci 46:772–785
21. Folke C, Hahn T, Olsson P, Norberg J (2005) Adaptive governance of social-ecological systems. Ann Rev Environ Res 30:441–473
22. Bransford J, Brown A, Cocking R (2000) Hoe people learn: brain, mind, experience, and school. National Academy Press, Washington
23. Savitz A (2006) The triple bottom line: how today's best-run companies are achieving economic, social and environmental success – and how you can too. Jossey-Bass/Wiley, New York
24. Robertson D, Hull R (2003) Public ecology: an environmental science and policy for global society. Environ Sci Policy 6:399–410
25. Dasgupta P, Maler K (2004) The economics of non-convex ecosystems. Springer, New York
26. Jones B, Wuchty S, Uzzi B (2008) Multi-university research teams: shifting impact, geography, and stratification in science. Science 322:1259–1262
27. Dicks M, McHugh R, Webb B (1996) Economy-wide impacts of U.S. aquaculture. Oklahoma Agricultural Experiment Station, Norman, 946p
28. Schroeder G (1980) Fish farming in manure loaded ponds. In: Pullin R, Shehadeh Z (eds) Integrated agriculture-aquaculture farming systems. ICLARM, Manila, pp 73–86
29. Naylor R, Goldburg R, Primavera J, Kautsky N, Beveridge M, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405:1017–1024
30. Pauly D (2000) Global change, fisheries, and the integrity of marine ecosystems: the future has already begun. In: Pimental D, Westra L, Noss R (eds) Ecological integrity. Island Press, Washington, DC, pp 227–244
31. Macintosh D, Phillips M (1992) Environmental considerations in shrimp farming. Infofish Int 6:38–92
32. Pullin R (1993) Environment and aquaculture in developing countries. International Center for Living Aquatic Resources Management, Manila
33. McKinsey C, Thetmeyer H, Landry T, Silvert W (2006) Review of recent carrying capacity models for bivalve culture and recommendations for research and management. Aquaculture 261(2):451–462
34. Costa-Pierce B (1997) Environmental impacts of nutrients discharged from aquaculture: towards the evolution of sustainable, ecological aquaculture systems. In: Baird D, Beveridge M, Kelly L, Muir J (eds) Aquaculture and water resource management. Blackwell, Oxford, UK, pp 81–113
35. Weatherly A, Cogger B (1977) Fish culture: problems and prospects. Science 197:427–430
36. Dela Cruz C, Lightfoot C, Costa-Pierce B, Carangal V, Bimbao M (1992) Rice-fish research and development in Asia. ICLARM, Manila
37. Zhou Y, Yang H, Hu H, Liu Y, Mao Y, Zhou H, Xu X, Zhang F (2006) Bioredimation potential of the macroalga Gracilaria lemaneiformis (Rhodophyta) integrated into fed fish culture in coastal waters of north China. Aquaculture 252:264–276
38. McGrath C (2009) Egypt. A big catch feeds millions. Global geopolitics Net Sites/IPS http://globalgeopolitics.net/wordpress/2009/11/01/egypt-a-big-catch-feeds-millions
39. Chopin T, Buschmann A, Halling C, Troell M, Kautsky N, Neori A, Kraemer G, Zertuche-Gonzalez J, Yarish C, Neefus C (2001)

Integrating seaweeds into marine aquaculture systems: a key toward sustainability. J Phycol 37:975–986
40. Chopin T (2006) Integrated multi-trophic aquaculture. What it is, and why you should care... and don't confuse it with polyculture. Northern Aquacult 12(4):4
41. Ridler N, Robinson B, Chopin T, Robinson S, Page F (2006) Development of integrated multi-trophic aquaculture in the Bay of Fundy, Canada: a socio-economic case study. World Aquacult 37(3):43–48
42. Ridler N, Wowchuk M, Robinson B, Barrington K, Chopin T, Robinson S, Page F, Reid G, Haya K (2007) Integrated multi-trophic aquaculture (IMTA): a potential strategic choice for farmers. Aquacul Econ Manag 11:99–110
43. National Research Council (2009) Shellfish mariculture in Drakes Estero, Point Reyes national seashore. National Academies Press, Washington, California
44. National Research Council (2010) Ecosystem concepts for sustainable bivalve mariculture. National Academies Press, Research Council, Washington

Books and Reviews

Alder J, Campbell B, Karpouzi V, Kaschner K, Pauly D (2008) Forage fish: from ecosystems to markets. Ann Rev Envir Res 33:71–714

Bell J, Waagbo R (2002) Safe and nutritious aquaculture produce: benefits and risks of alternative sustainable aquafeeds. In: Holmer M, Black K, Duarte C, Marba N, Karqakasis I (eds) Aquaculture in the ecosystem. Springer, New York, pp 185–226

Black K (2001) Sustainability of aquaculture. In: Black K (ed) Environmental impacts of aquaculture. Sheffield Academic Press, Sheffield, pp 199–212

Costa-Pierce B, Desbonnet A, Edwards P, Baker D (eds) (2005) Urban Aquaculture. CABI Publishing, Wallingford, UK

Duarte C, Holmer M, Olsen Y, Stot D, Maba N, Guiu J, Black K, Karakassis I (2009) Will the oceans help feed humanity? Bioscience 59:967–976

Gowen R, Bradbury N (1987) The ecological impact of salmonid farming in coastal waters: a review. Ocean Mar Biol Ann Rev 25:563–575

Stickney R, McVey J (2002) Responsible marine aquaculture. CABI, Wallingford

Tacon A, Hasan M, Subasinghe R (2006) Use of fishery resources as feed inputs for aquaculture development: trends and policy implications. FAO fisheries circular No. 1018. Food and Agriculture Organization of the United Nations, Rome

Tacon A, Metian M (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 285:146–158

Turchini G, Bente E, Torstensen E, Ng W (2009) Fish oil replacement in finfish nutrition. Rev Aquacult 1:10–57

Aquaculture, Integrated Multi-trophic (IMTA)

Thierry Chopin
Canadian Integrated Multi-Trophic Aquaculture Network (CIMTAN), University of New Brunswick, Saint John, NB, Canada

Article Outline

Glossary

Biomitigative services provided by extractive aquaculture The environmental, economic, and societal services and benefits received by ecosystems – in their broad definition which includes humans who depend on them – from the conditions and processes of cultivated species, such as seaweeds extracting inorganic nutrients and suspension- and deposit-feeders extracting organic particles recaptured from the activities of fed aquaculture (e.g., fish or shrimp aquaculture), to maintain their health. Biomitigative services can also be provided by natural populations of similar organisms.

Integrated multi-trophic aquaculture (IMTA) The farming, in proximity, of aquaculture species from different trophic levels, and with complementary ecosystem functions, in a way that allows one species' uneaten feed and wastes, nutrients, and by-products to be recaptured and converted into fertilizer, feed, and energy for the other crops, and to take advantage of synergistic interactions between species. Farmers combine fed aquaculture (e.g., finfish or shrimps) with extractive aquaculture, which utilizes the inorganic (e.g., seaweeds or other aquatic vegetation) and organic (e.g., suspension- and deposit-feeders) excess nutrients from fed aquaculture for their growth. The aim is to ecologically engineer balanced systems for environmental sustainability (biomitigative services for improved ecosystem health), economic stability (improved output, lower costs, product diversification, risk reduction, and job creation in disadvantaged communities) and societal acceptability (better management practices, improved regulatory governance, and appreciation of differentiated and safe products).

Definition of the Subject

Fulfilling aquaculture's growth potential requires responsible technologies and practices. Sustainable aquaculture should be ecologically efficient, environmentally benign, product-diversified, profitable, and societally beneficial. Integrated multi-trophic aquaculture (IMTA) has the potential to achieve these objectives by cultivating fed species (e.g., finfish or shrimps fed sustainable commercial diets) with extractive species, which utilize the inorganic (e.g., seaweeds or other aquatic vegetation) and organic (e.g., suspension- and deposit-feeders) excess nutrients from fed aquaculture for their growth. Thus, extractive aquaculture produces valuable biomass, while simultaneously rendering biomitigative services for the surrounding ecosystem and humans. Through IMTA, some of the uneaten feed and wastes, nutrients, and by-products, considered "lost" from the fed component, are recaptured and converted into harvestable and healthy seafood of commercial value, while biomitigation takes place (partial removal of nutrients and CO_2, and supplying of oxygen). In this way, some of the externalities of fed monoculture are internalized, hence increasing the

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

overall sustainability, profitability, and resilience of aquaculture farms. A major rethinking is needed regarding the definition of an "aquaculture farm" (reinterpreting the notion of site-lease areas) and regarding how it works within an ecosystem, in the context of a broader framework of Integrated Coastal Zone Management (ICZM). The economic values of the environmental/societal services of extractive species should be recognized and accounted for in the evaluation of the true value of these IMTA components. This would create economic incentives to encourage aquaculturists to further develop and implement IMTA. Seaweeds and invertebrates produced in IMTA systems should be considered as candidates for nutrient/carbon trading credits (NTC and CTC) within the broader context of ecosystem goods and services. Long-term planning/zoning promoting biomitigative solutions, such as IMTA, should become an integral part of coastal regulatory and management frameworks.

Introduction: Aquaculture Is Needed But Some Practices Need to Evolve

The global seafood industry is at a crossroads: as capture fisheries stagnate in volume, they are falling increasingly short of a growing world demand for seafood. It is anticipated that by 2030, there will be a 50–80 million ton seafood deficit [1]. This gap will likely not be filled by capture fisheries but by aquaculture operations, which already supply almost 50% of the seafood consumed worldwide [1]. Consequently, it is imperative to design the ecosystem responsible aquaculture practices of tomorrow that maintain the integrity of ecosystems while ensuring the viability of this sector and its key role in food provision, safety, and security.

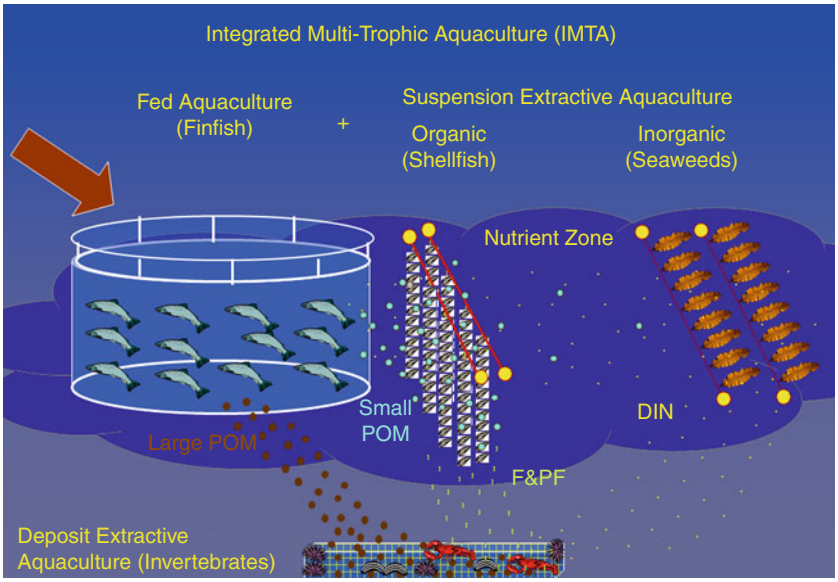
Without a clear recognition of the industry's large-scale dependency and impact on natural ecosystems and traditional societies, the aquaculture industry is unlikely to either develop to its full potential, continue to supplement ocean fisheries, or obtain societal acceptance. The majority of aquaculture production still originates from relatively sustainable extensive and semi-intensive systems [2]; however, the rapid development, throughout the world, of intensive marine fed aquaculture (e.g., carnivorous finfish and shrimp) is associated with concerns about the environmental, economic, and social impacts that these, often monospecific, practices can have, especially where activities are highly geographically concentrated or located in suboptimal sites whose assimilative capacity is poorly understood and, consequently, prone to being exceeded. There are also some concerns with shellfish aquaculture, especially at high density, as shellfish occupy an intermediate trophic level and often play a dual role of organic filtering organisms and waste/nutrient generating organisms [3].

For many marine aquaculture operations, monoculture is, spatially and managerially, often the norm. Species are cultivated independently in different bays or regions. Consequently, the two different types of aquaculture (fed versus extractive) are often geographically separate, rarely balancing each other out at the local or regional scale, and, thus, any potential synergy between the two is lost. To avoid pronounced shifts in coastal processes, the solution to nutrification by fed aquaculture is not dilution, but extraction and conversion of the excess nutrients and energy into other commercial crops produced by extractive aquaculture.

To continue to grow, while developing better management practices, the aquaculture sector needs to develop more innovative, responsible, sustainable, and profitable technologies and practices, which should be ecologically efficient, environmentally benign, product-diversified, and societally beneficial. Maintaining sustainability, not only from an environmental, but also from economic, social, and technical perspectives, has become a key issue, increased by the enhanced awareness of more and more demanding consumers regarding quality, traceability, and production conditions. Integrated multi-trophic aquaculture (IMTA) has the potential to play a role in reaching these objectives by cultivating fed species (e.g., finfish or shrimps fed sustainable commercial diets) with extractive species, which utilize the inorganic (e.g., seaweeds or other aquatic vegetation) and organic (e.g., suspension- and deposit-feeders) excess nutrients from aquaculture for their growth (Fig. 1).

IMTA: A Flexible and Functional Concept

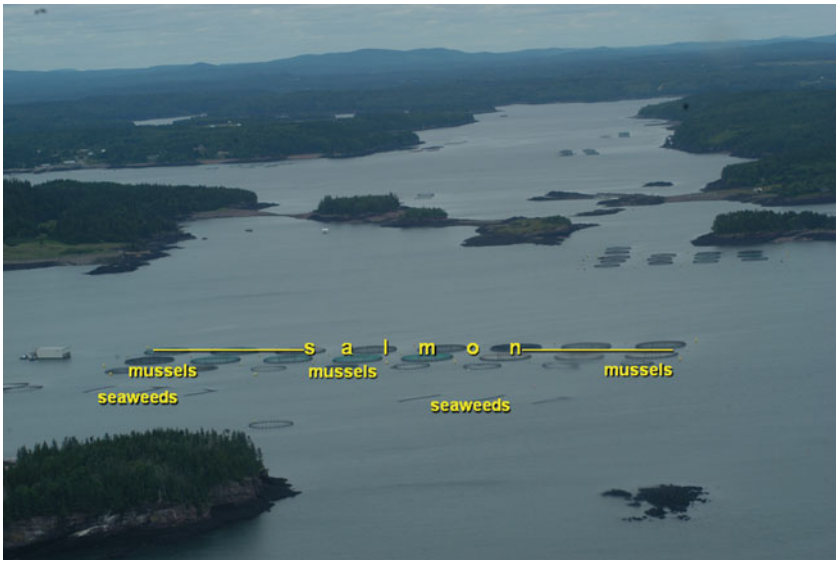
The IMTA concept is extremely flexible [4]. To use a musicology analogy, IMTA is the central/overarching theme on which many variations can be developed according to the environmental, biological, physical,



Aquaculture, Integrated Multi-trophic (IMTA). Figure 1
Conceptual diagram of an Integrated Multi-Trophic Aquaculture (IMTA) operation including the combination of fed aquaculture (e.g., finfish) with suspension organic extractive aquaculture (e.g., shellfish), taking advantage of the enrichment in small particulate organic matter (POM); inorganic extractive aquaculture (e.g., seaweeds), taking advantage of the enrichment in dissolved inorganic nutrients (DIN); and deposit organic extractive aquaculture (e.g., echinoids, holothuroids, and polychaetes), taking advantage of the enrichment in large particulate organic matter (POM) and feces and pseudo-feces (F&PF) from suspension-feeding organisms. The bioturbation on the bottom also regenerates some DIN, which becomes available to the seaweeds

chemical, societal, and economic conditions prevailing in parts of the world where the IMTA systems are operating. It can be applied to open-water or land-based systems, marine or freshwater systems (sometimes called "aquaponics"), and temperate or tropical systems. What is important is that the appropriate organisms are chosen at multiple trophic levels based on the complementary functions they have in the ecosystem, as well as for their economic value or potential. In fact, IMTA is doing nothing other than recreating a simplified, cultivated ecosystem in balance with its surrounding instead of introducing a biomass of a single type one thinks can be cultivated in isolation from everything else. Integration should be understood as cultivation in proximity, not considering absolute distances but connectivity in terms of ecosystemic functionalities.

It should be made clear that in the minds of those who created the acronym "IMTA," it was never conceived to be viewed with the minimalist perspective of only the cultivation of salmon (*Salmo salar*), kelps (*Saccharina latissima* and *Alaria esculenta*), and blue mussels (*Mytilus edulis*) within a few hundred meters: this is only one of the variations (Fig. 2) and the IMTA concept can be extended to very large ecosystems like the Yellow Sea (see below). This also means that IMTA variations include integrated agriculture aquaculture systems (IAAS), integrated sylviculture (mangrove) aquaculture systems (ISiAS), integrated green water aquaculture systems (IGWAS), integrated peri-urban aquaculture systems (IPUAS), integrated fisheries aquaculture systems (IFAS), sustainable ecological aquaculture systems (SEAS), integrated temporal aquaculture systems (ITAS), and integrated sequential aquaculture systems (ISAS, also called partitioned aquaculture systems, PAS, or fractionated aquaculture systems, FAS) [5–7]. There is no ultimate IMTA system to "feed the world." There is not one world but climatic, environmental, biological, physical, chemical, economic, societal, and political conditions,



Aquaculture, Integrated Multi-trophic (IMTA). Figure 2
One of the Integrated Multi-Trophic Aquaculture (IMTA) sites in the Bay of Fundy, New Brunswick, Canada, operated by Cooke Aquaculture Inc.: two rows of salmon cages in the background, then a row of mussel rafts and two seaweed rafts in the foreground

each of which can lead to different choices of systems for feeding these microworlds.

The paradox is that IMTA is not a new concept. Asian countries, which provide more than two thirds of the world's aquaculture production, have been practicing IMTA (often described as a type of "polyculture") for centuries, through trial and error and experimentation. Why, then, is this common sense solution not more widely implemented? The reasons for this generally center around social customs and practices, and market-driven economic models not considering externalities that one is already familiar with, even if common sense tells one that one should modify them. Human society does not change quickly unless there are compelling reasons to do so. What to do when early large profit margins create short-term economic booms, followed within a few decades by dwindling meager profit margins? Often, the temptation is to throw more large volume cultivation operations and destructive methods into the mix, without proper regulations and business plans. Pollution, disease and economic busts generally ensue, major restructuring of the industry becomes necessary, and a few clairvoyant visionaries remain afloat and adapt to jump to the next curve to survive. This evolution is not exclusive to the aquaculture industry. Why do humans have such short and selective memories resulting in them repeating mistakes, regularly?

The fact that humans are currently at a crossroad should motivate them to improve current aquaculture practices, without further delay. Fishery management plans in most countries have been single-species approaches, completely neglecting the interactions between species, not understanding the synergies, or antagonisms, between them and how an ecosystem works based on the complementarities of the different functions of the different organisms inhabiting it. It seems that, despite the knowledge of the limitations of mono-agriculture and mono-fisheries, people are ready to develop similar plans for the management of mono-aquaculture. It should be recognized that there is still a chance for incorporating all the learning about the problems of terrestrial monocultures into the relatively new frontier of aquaculture. To better manage marine, brackish, or freshwater environments to the benefits of mankind and the ecosystem, one needs to develop a new science, marine agronomy, learning from the mistakes made in land agriculture over the

centuries to do a better job with aquaculture. It is interesting to note that traditional agricultural practices, such as crop alternation and fallow, are now being transposed to aquaculture practices.

Why, then, is IMTA not more widely adopted, especially in the western world? Paul Greenberg, in his fascinating book "Four Fish" [8], mentioned a very interesting point. In Leviticus, the third book of the Hebrew bible in which, according to the Jewish tradition, God dictated commandments to Moses, one can read (19:19): "You must not sow your field with two different kinds of seed" (also translated as "two kinds of seed" or as "mixed seed"). One can wonder if this represents, in fact, one of the most ancient treatises recommending mono-agricultural practices and if it is not the reason why integrated culture techniques have been ignored for centuries in the Judeo-Christian civilization, while they have flourished in other civilizations, especially in Asia. Moreover, if Asian cultures are accustomed to the concept of considering wastes from farming practices as resources for other crops rather than pollutants, this attitude still has a long way to progress in the western world where aquaculture is a more recent development.

The Need for Diversifying Responsible Aquaculture Systems and for an Ecosystem Approach

The common old saying "Do not put all your eggs in one basket," which applies to agriculture and many other businesses, should also apply to aquaculture. Having excess production of a single species leaves a business vulnerable to sustainability issues because of fluctuating prices in what has become commodity markets and potential oversupply, and the possibility of catastrophic destruction of one's only crop (diseases, damaging weather conditions). Consequently, diversification of the aquaculture industry is advisable for reducing economic risk and maintaining sustainability and competitiveness.

From an ecological point of view, diversification also means cultivating more than one trophic level, i.e., not just raising several species of finfish (that would be "polyculture"), but adding into the mix organisms of different and lower trophic levels (e.g., seaweeds, shellfish, crustaceans, echinoderms, worms, bacteria, etc.) to mimic the functioning of natural ecosystems. Staying at the same ecological trophic level will not address some of the environmental issues because the system will remain unbalanced due to nondiversified input and output needs. Evolving aquaculture practices will require a conceptual shift toward understanding the working of food production systems rather than focusing on technological solutions.

One of the innovative solutions promoted for environmental sustainability (biomitigative services for improved ecosystem health), economic stability (improved output, lower costs, product diversification, risk reduction, and job creation in disadvantaged communities), and societal acceptability (better management practices, improved regulatory governance, and appreciation of differentiated and safe products) is IMTA. The aim is to increase long-term sustainability and profitability per cultivation unit (not per species in isolation as is done in monoculture), as some of the uneaten feed and wastes, nutrients, and by-products of one crop (fed animals) are not lost but recaptured and converted into fertilizer, feed, and energy for the other crops (extractive plants and animals). These, in turn, can be harvested and marketed as healthy seafood, while feed costs are reduced because of their reuse in multiple niches and biomitigation is taking place (partial removal of nutrients and CO_2, and supply of oxygen). In this way, all the cultivation components have a commercial value, as well as key roles in recycling processes and rendering biomitigative services. Some of the externalities of fed monoculture are internalized, hence increasing the overall sustainability, long-term profitability, and resilience of aquaculture farms. The harvesting of the different types of crops participates in the capture and export of nutrients outside of the coastal ecosystem.

The biomass and functions of the fed and extractive species naturally present in the ecosystem in which aquaculture farms are operating must also be accounted for or this will lead to the development of erroneous carrying/assimilative capacity models. For example, the 158,811 t (fresh weight) of the intertidal seaweed, *Ascophyllum nodosum* (rockweed), in proximity to salmon aquaculture operations in southwest New Brunswick, Canada, are not neutral in the ecosystem and represent a significant coastal nutrient scrubber which should be taken into consideration to

understand the functioning of that part of the Bay of Fundy.

IMTA, While Not the Panacea to and for Everything, Is, Nevertheless, One of the Improvement Options

IMTA has never been portrayed as the solution to and for everything! For example, IMTA does not address the issues of escapees from open-water fish farms. It is, of course, in the interest of everybody, especially the industry (to not lose money) to reduce the number of escapees. This is, however, a question of engineering of the rearing systems (cages, netting material, etc.) and the suitability of the environment to survival should escapes occur. To solve the escapee issue, it has been suggested that fish farms should be pulled from the open water and placed on land or in closed containment. Moving on land is, however, not a guarantee for zero escapees. There are well-known escapee cases from land-based operations, with serious consequences. For example, the bighead carp (*Hypophthalmichthys nobilis*) and the silver carp (*Hypophthalmichthys molitrix*) were brought from Asia to the southern USA in the 1970s to help control algal proliferation in channel catfish (*Ictalurus punctatus*) farms. There are reports of escapees into the lower Mississippi River system, especially associated with flood episodes in the early 1990s. Self-sustaining populations have been able to move northward to enter the Upper Mississippi River system and the Illinois River system. Presently, there are fears that these fish could enter the Great Lakes system through the Chicago Sanitary and Ship Canal and the Des Plaines River to finally reach Lake Michigan, after an escape of around 2,000 km in approximately 20–30 years. Electric fish barriers have been put in place, but their efficiency has been questioned. The use of rotenone, a biodegradable piscicide, was authorized but seemed to have killed more common carps (*Cyprinus carpio*; itself an introduced species from Europe in the 1830s) than bighead and silver carps. On April 26, 2010, the US Supreme Court decided not to get involved in a dispute over how to prevent these carps from making their way into the Great Lakes; it turned down a new request by the State of Michigan to consider ordering permanent closing of the Chicago-area shipping locks. What the impacts on the ecosystems could be, should these fish get into the Great Lakes systems, is unknown, but they are well-known for their ability to consume large amounts of algae and zooplankton, eating as much as 40% of their body weight per day, and they are fierce competitors when it comes to securing their food needs. The silver carp is also a danger to recreational fishers, water skiers, and boaters because of its habit to jump out of the water when startled by boat motors or other noises, creating life-threatening aerial hazards with high speed impacts.

The number of escapees from land-based facilities is not as well documented as with cage-based aquaculture. Perhaps because land-based fish escapes are more likely to occur as a continuous "trickle" instead of a single major event such as a net tear that would lead to "large-scale" escapes. However, reports do surface from time to time in the media, particularly if there is some novelty in the story. A recent example is the report of the cultured salmonid brown trout, *Salmo trutta*, escaping from a pond farm in the UK. A wildlife photographer caught them in action, making large leaps out of the water straight into a metal feed pipe a meter above and connected to a tributary of a river (http://www.telegraph.co.uk/earth/earthnews/3318094/Photographer-captures-trouts-great-escape.html). Ideally, land-based recirculation systems would reduce the potential for escapes. However, most recirculation systems have at least partial water exchange [9] and where there is water exchange and discharge, there is a potential for escapees. These systems are still not widely used and to the authors knowledge there has not been any initiative taken to document escapees, or lack thereof, within these systems. It may, therefore, be premature to classify such systems as "escape proof." It is unlikely that any land-based aquaculture operations could ever be 100% "escapee-proof" and, consequently, they will also need to develop anti-escapee strategies (avoiding flood plains, electric fences, grids of the appropriate mesh, catchment basins, etc.).

Moving to land-based or closed-containment operations is one approach that may help address some sustainability issues but is not without its problems. Large amounts of energy, often diesel or electric power, are required to pump and aerate water. Nutrients are either pumped back into the water or settled somewhere and "trucked" offsite. All of these processes leave a "carbon footprint," and only partly solve the issue of

excess nutrients. IMTA, or its variation called "aquaponics," will have to be added to closed-containment or land-based systems to treat the effluents. One "impact" may simply be traded for another. Ayer and Tyedmers [10], in their life cycle assessment of alternative aquaculture technologies, warned that one could be in a case of environmental problem shifting, not solving, where, while reducing local ecological impacts, the increase in material and energy demands may result in significant increased contributions to several environmental impacts of global concern, including global warming, nonrenewable resource depletion, and acidification.

Land-based or closed-containment operations have also been advocated as a way of controlling diseases and their transmission. However, the proponents very often equate diseases to the sole problem of sea lice, leaving the issues related to viral or bacterial pathogens unaddressed. Some concerns have been expressed that multiple species on the site might increase the risk for disease transmission. It must, however, be realized that sites in the ocean and on land will always have additional unintended species associated with the operation, ranging from microorganisms to marine mammals, depending on the situation. The question is not whether to have only one species on the site, but at what density do negative interactions occur with the unintended ones and whether there are any positive interactions associated with more diversified systems. In fact, two studies [11; Robinson, pers. comm.] have demonstrated in laboratory experiments that the blue mussel, *Mytilus edulis*, is capable of inactivating the infectious salmon anemia virus (ISAV), as well as the infectious pancreatic necrosis virus (IPNV). Mussels are, consequently, not a likely reservoir host or vector for ISAV and IPNV. Put in an IMTA perspective, this could mean that mussel rafts could be strategically placed to serve as a kind of sanitary/biosecurity cordon around salmon cages to combat certain diseases. Pang et al. [12, 13] also reported reduced total bacteria and *Vibrio* counts in a seaweed-abalone IMTA system.

In regard to parasites, two studies [14; Robinson, pers. comm.] indicate that blue mussels can consume copepodids, the planktonic and infectious stage of sea lice, and several studies, in both Europe and New Zealand, have highlighted the fact that mussels can consume small zooplankton. Having a biofilter such as mussels at IMTA sites may decrease the frequency of exposure to pathogens and planktonic parasites. The hope is that having multiple species on a farm will result in some positive interactions between species allowing some biological control of the outbreaks of pathogens and parasites, hence reducing the number of costly chemical treatments required. If this is validated, filter feeders may have additional contributions to sustainability beyond reduction of the particle load. One of the 14 projects of the recently created Canadian Integrated Multi-Trophic Aquaculture Network (CIMTAN) is investigating the role of bivalves in potentially reducing sea lice populations. Most of the work has been conducted in the lab so far, but results are very positive and it has been demonstrated that mussels eat the larval forms. Ongoing work is developing a trap system that exploits various behaviors of sea lice to attract and filter them out of the system. Another CIMTAN project is looking into the possibility that mussels could reduce the horizontal transmission of *Loma salmonae*, responsible for microsporidial gill disease of salmon (MGDS), a serious endemic gill disorder in marine netpen reared, and wild, Chinook (and other Pacific) salmon. Trials will examine the proof of principle that blue mussels remove microsporidial spores from water and to what extent these spores retain short-term infectious potential as determined by branchial xenoma expression in test fish.

IMTA is not entering directly the debate regarding the inclusion of fishmeal and fish oil in commercial feeds (nor are land-based or closed-containment operations). IMTA could, however, provide a partial solution. Modern commercial salmon diets in Canada contain much less fishmeal (about 15–25%) and fish oil (about 15–20%) than they did less than 10 years ago (40–60%). By-products (trimmings, offal) of wild catch fisheries are now used to supply a major portion of the fishmeal ingredients. Finding replacements for marine ingredients is a priority and there are several large research projects worldwide addressing this issue. The feed company Skretting has now produced a salmon feed which includes no marine ingredients. Turning toward land plant proteins is not without its impacts. Extra farmland area (more deforestation) would be needed, which, moreover, would need to be irrigated and fertilized on a planet already suffering from water availability problems and with fertilizer

prices soaring. The price of some staple food crops used in traditional agriculture (corn, soya bean, sugar cane, etc.) would rise considerably due to announced competition for their uses, as recently seen when they were potentially sought out as energy crops for the production of first-generation biofuels [15–17]. Reallocation of acreage for subsidized potential biofuel crops such as corn, sugar cane, oil palm, canola, switch grass, etc., has already had significant ecological and societal costs due to its impacts on ecosystem health, biodiversity, and food security [18–21]. Partial substitution with organisms already living in water and not needing extra fertilization in an IMTA setting, such as seaweeds, could, in fact, be a very interesting option, fitting well within the sustainability and management concept of IMTA, and representing a logical loop for companies developing an IMTA and diversification strategy. If cultivated in the water column in IMTA systems, there would, moreover, be no issue of raking natural beds of seaweeds attached to the bottom of the ocean (destruction of seafloor and impact on ecosystem functions such as nursery ground for animals).

Some environmental nongovernmental organizations arguing for fishmeal/fish oil replacement have also voiced concerns that, after all, marine fish should eat marine ingredients . . . obviously, one cannot have it both ways! There is also the paradoxical situation of farmed freshwater fish, which are being grown less and less on humans and animal wastes and naturally occurring algal blooms, but more and more on already competing staple foods such as corn and soy: they have lost their off-flavored or muddy taste to become tasteless or "unfishy"! So, what does one want to receive in one's kitchen? A flavor-neutral, versatile product easily adapted with numerous sauces, while one is lamenting that farmed salmon or bass are not what wild salmon or bass used to be? Quite an irony, even more so when people learn that these herbivorous whitefish are more and more being fed pellets containing fishmeal and fish oil because they grow faster! What is really important is a balanced diet using balanced sourcing of raw material.

Some will argue that "fish require nutrients, not ingredients." At the same time, there is also the well-known saying "You are what you eat," and in this case, people have to realize and accept that humans are mostly corn, soya, and fishmeal, if they look at what the four mammals (cow, pig, sheep, and goat) and four poultry (chicken, turkey, duck, and goose) that they have selected as their meat choices are eating. Historically, most of the reduction fishery (small fish such as anchovies, herring, sardines, and menhaden) went into the production of pet feeds and farm animal feeds. Subsequently, this fishery supplied a significant part of the marine ingredients for fish feeds. The landing of the reduction fishery has been fairly stable (fluctuating between 15 and 30 million metric tons since the 1970s) and, in the absence of aquaculture, the fishery would likely return to supplying pet and farm animal feeds, and a current resurgence of interest directly by humans. This is not to justify relaxed vigilance for finding replacements for marine ingredients in fish feed, but simply to suggest that an absence of fish farming will not stop the use of this resource. How can one get out of this vicious circle? Cultivating several organisms, at different trophic levels, in proximity so that the food and wastes are utilized efficiently more than once through a cascade of recapturing and remetabolizing is one approach: that is IMTA. The other is to consider that if terrestrial food production systems are close to their limits, one does not have other options but to turn again to the sea, this time not for fish but to have seaweeds and invertebrates entering one's food habits, either directly or delivered through feed given to intermediates to what reach one's plate. The discrepancy between the marine and agricultural production systems has to be reduced: presently, especially in the western world, humans feed approximately two steps higher in the marine food web than in the agricultural food web.

People should continue to eat seafood (fish but also invertebrates and seaweeds), not according to seafood pocket guides which simplistically paint species with one stroke of green (best choice), orange (good alternative), or red (avoid), but according to the fishing and aquaculture practices used to grow, harvest, and process them: an admittedly more complex mosaic, but also much more realistic and attractive to look at than a traffic light!

Interestingly, what is referred to as the fifth tasting sense by Japanese (after sweet, sour, salty, and bitter) and called umami (= savoriness or good flavor) comes from seaweeds. The product responsible for umami was first identified in 1908 by Professor Kikunae Ikeda,

of the Tokyo Imperial University, searching for the chemical reason of the strong flavor in seaweed broth (mostly of the kelp *Saccharina japonica*, formerly *Laminaria japonica*). It is due to the detection in our mouth of the carboxylate anion of the amino acid called glutamic acid and its salts, glutamates, in particular monosodium glutamate (MSG). Inosine monophosphate (IMP) and guanosine monophosphate (GMP), degradation products of the energy-storing molecule adenosine triphosphate (ATP) greatly enhance the perceived intensity of umami. This explains, chemistry displacing romantics, why a dead tuna (once full of energy) served with seaweeds is such a savory delicacy, the very essence of the success of the sushi bar fad gaining the western world.

We have never pretended that IMTA is the solution, the silver bullet, to and for everything. It is now up to us to develop the better aquaculture practices of tomorrow. IMTA is based on several common sense principles:

- The solution to nutrification is not dilution, but extraction and conversion through diversification.
- This is, in fact, a rewording of the first law of thermodynamics "Rien ne se perd, rien ne se crée, tout se transforme" ("Nothing is lost, nothing is created, everything is transformed") as summarized by Antoine Laurent de Lavoisier, the well-known French chemist and physicist (but also tax collector, which explains his premature death at age 51 in 1794 under the Terror period of the French Revolution).
- What is waste for some is gold for others.
- Do not put all your salmon eggs in the same basket.

A lot of common sense, but, unfortunately, common sense is not that common! IMTA is one of the promising options, but, certainly, it needs to be tailored to the location in the world where it is implemented. It should also be developed in association with other practices. Like for energy, not one solution will satisfy all the needs and it is a variety of solutions that will help one secure one's seafood procurement in a responsible manner. The solutions will be at the interfaces of these techniques and will be interdisciplinary. They will embrace both scientific and technological advancements and traditional knowledge. IMTA is exactly at this interface, modernizing traditional practices: combining ecosystem complementary crops, bay management area, and fallowing are nothing new, but revisited and updated, based on what humans have learned from past experience (which includes a lot of mistakes over the centuries, but not assimilated by the characteristically short-term memory of humans!).

Recognizing and Valuing the Biomitigative Services Rendered by the Extractive Components of IMTA: Should a System of Nutrient and Carbon Trading Credits Be Developed?

A few economic analyses have indicated that the outlook for increased profitability through IMTA is promising [22, 23]. However, these analyses were based solely on the commercial values from the sale of biomass – being of fish, shellfish, or seaweeds – and used conservative price estimates for the cocultivated organisms based on known applications. One aspect not factored into these analyses is the fact that the extractive component of an IMTA system not only produces a valuable multipurpose biomass, but also simultaneously renders waste reduction services to society. It is particularly important to recognize that once nutrients have entered coastal ecosystems, there are not many removal options available: the use of extractive species is one of the few realistic and cost-effective options. The economic values of the environmental/societal services of extractive species should, therefore, be recognized and accounted for in the evaluation of the true value of the IMTA components. Further development of economic models is needed to help shed light on the economic (society) and commercial (industry) attractiveness of IMTA.

Ecosystem services have been ignored until recently [24]. To improve the sustainability of anthropogenic nutrient loading practices such as aquaculture, incentives such as Nutrient Trading Credits (NTC) should be established as a means to promote nutrient load reduction or nutrient recovery. During the last few years, there has been much talk and excitement about carbon credits. However, within coastal settings, the concerns have largely been with nitrogen, due to the fact that its typical role as the limiting nutrient is not any longer the case in some regions. Potential effects of carbon loading in the marine environment should also be considered: localized benthic anoxia and, consequently, hydrogen

sulfide release may occur when solid waste deposition rate exceeds aerobic decomposition rate. Ocean acidification due to increased dissolved CO_2 levels has also prompted serious new concerns [25] and a Carbon Trading Credit (CTC) system should also be contemplated. With an appropriate composition of cocultured species, IMTA has the potential to reduce the amounts of dissolved (inorganic) and solid (organic) forms of nitrogen, carbon, phosphorus (more an issue in freshwater environments), etc., making extractive aquaculture a good candidate for a NTC and CTC, or other suitable approaches, to deal with the pressing issues of coastal nutrient loading.

Currently, there are few countries with laws or regulations that require aquaculture operations to responsibly internalize their environmental costs, such as nutrient discharges. There are some precedents, such as where land-based trout farmers in Denmark are allowed to increase their feed quota with documented evidence of reduced effluent discharge [26], but such incentives are not widely spread. In most jurisdictions, adjacent ecosystems are left to accommodate the nutrient load, and performance-based standards are used to determine if farms have exceeded their assimilative capacity.

The implementation of regulations resulting in internalization of environment costs by fish farms, without a direct economic compensatory response such as the Danish feed quota increase, could result in a significant reduction in profitability. In land-based systems, it is relatively easy to quantify nutrient load and concentration via comparison between farm inflows and outflows, thereby creating a benchmark for "economic compensation." Such values are practically impossible to empirically measure in an open-water system, "leaky" by definition, and, consequently, so is the practical implementation of such incentives. However, Troell et al. [27] and Chopin et al. [28] demonstrated that by integrating the seaweed, *Gracilaria*, in the dual role of nutrient scrubber and commercial crop (for agar production), with salmon farms in Chile, the environmental costs of waste discharges would be significantly reduced and profitability significantly increased.

Interestingly, the removal of nitrogen could be much more lucrative, by approximately a factor of 100, than that of carbon. The cost of removing nitrogen is not clearly defined, but there are several interesting studies that may help define a range of possible prices for economic evaluation of the NTC concept. The cost of removing 1 kg of nitrogen varies between US$3 and US$38 at sewage treatment facilities, depending on the technology used and the labor costs in different countries [28]. The municipality of Lysekil, in Sweden, is paying approximately US$10/kg removed by the filter-feeding mussel, *Mytilus edulis*, to the farm Nordic Shell Produktion AB [29, 30]. Ferreira et al. [31, 32], with the development of the Farm Aquaculture Resource Management (FARM) model, determined a net value of €18–26 billion/year of nutrient eutrophication reduction services provided by shellfish aquaculture in the coastal waters of the European Union. Gren et al. [33] calculated that the cleaning costs of nutrients by mussel farming can be considerably lower than other abatement measures and estimated that mussel farming should be credited between €0.1 and €1.1 billion/year in the Baltic Sea.

Using this information, and without presuming what the final design of IMTA sites will be in the future, preliminary calculations for the relatively small-scale IMTA project on the East coast of Canada indicate that the annual harvesting of kelps (Fig. 3) would equate to the removal of 35.75 t of nitrogen from the ecosystem, representing an NTC of between US$357,504 and US$1,072, 512. The same could be applied to another key nutrient, phosphorus. With an annual removal of 4.09 t and a value of US$4/kg removed [28], this would represent another contribution to the NTC of US$16,343, a much smaller amount but it could also be an important way of extracting phosphorus, at a time when some are predicting it to be the next element human society will be short of (in its natural or mined forms).

Carbon Trading Credits (CTC) could also be calculated. There may be some arguments about what is meant by trapping and sequestering carbon. Some may argue that it should be reserved to long/geological term storage (sink) and not to transient storage [34]. This is, in fact, a question of how long one allows the recycling clock to run. There is no permanent storage of carbon; it happened that a particular fossil biofuel, petroleum, has been sequestered over geological time to suddenly be reused at an accelerated rate over the last few centuries. But the first law of thermodynamics,

Aquaculture, Integrated Multi-trophic (IMTA). Figure 3
Harvesting of the kelp, *Saccharina latissima*, at an Integrated Multi-Trophic Aquaculture (IMTA) site in the Bay of Fundy, New Brunswick, Canada. Kelps remove dissolved nutrients from the ecosystem while providing commercial products

as enunciated by Antoine Laurent de Lavoisier more than two centuries ago, still applies: "Rien ne se perd, rien ne se crée, tout se transforme," i.e., "Nothing is lost, nothing is created, everything is transformed." If even temporary removal of carbon from the ocean by biomass harvesting until further transformation (and rerelease of carbon) can be credited for potentially increasing seawater pH and absorbing CO_2 from the atmosphere and/or the cultivated animals, then CTC should be calculated. Marine vegetation is getting more and more recognition as a sink for anthropogenic carbon emissions (the so-called blue carbon [35]). Marine primary producers contribute at least 50% of the world's carbon fixation and may account for as much as 71% of all carbon storage in oceanic sediments. Then, micro-algae, macro-algae, and marine plants, such as mangroves and seagrasses, have a role to play in CO_2 sequestration and removal, and carbon storage [36]. Marine photosynthesis accounts for 50% of the total primary productivity of the planet (54–59 PgC/year from a total of 111–117 PgC/year [37]). Of this, marine macrophytes (seaweeds and seagrasses) account for approximately 1 PgC/year concentrated in coastal regions where they can play a significant role in the sequestration of anthropogenic carbon emissions and the global carbon cycle. Brown marine macro-algae (such as *Macrocystis*, *Saccharina*, *Laminaria*, *Ecklonia*, *Sargassum*, *Ascophyllum*, and *Fucus*), red algae (such as *Porphyra*, *Palmaria*, *Eucheuma* and *Gracilaria*) and green algae (such as *Ulva*), are capable of very high rates of photosynthesis and productivity. These rates of productivity compare very favorably to those of terrestrial crops that have been recommended as possible sources of first-generation biofuels (corn, *Zea mays*) or second-generation biofuels (switch grass, *Panicum virgatus*; E-grass, *Miscanthus giganteus*) and position marine macro-algae very well for being part of the third-generation biofuels [36].

Coming back to the IMTA project on the East coast of Canada, using a value for carbon removal of around US$30/t [34], the annual harvesting of kelps would represent an annual removal of 306.43 t and a CTC of US$9,193: a larger amount of carbon, but for a much smaller value of trading credits, underlining the difficulty in removing dissolved nutrients from aquatic systems and the acute issue of their presence in coastal systems. Similar calculations could be applied to the organic extractive component of IMTA. In the case of shellfish, accumulation of nitrogen, phosphorus, and carbon should be considered both in meat and shells, which are especially rich in calcium carbonates.

At a much larger scale, the occurrence of large and recurrent "green tides" should also be brought into focus. Large proliferations of opportunistic green algae, especially of the genus *Ulva*, in response to large anthropogenic nutrient loading, have been in the news over the last few years in places around the world such as Northern Brittany in France, the southern regions of the UK, and Venice in Italy. The green tide in Qingdao, China, just before the sailing competitions of the 2008 Olympic Games, got a lot of attention (Fig. 4). The following question needs to be asked: Are these green tides a negative media photo opportunity, or are they reminders of the significant role seaweeds play in coastal processes and the services they render? Within 3 weeks, 1 million tons of *Ulva prolifera* were removed from the vicinity of Qingdao to allow the sailors and windsurfers to compete (but it is estimated that approximately 2 million tons of *U. prolifera* sank to the bottom of the Bay; another environmental problem shifting, but not a solution). The harvesting of 1 million tons equated to between 3,000 and 5,000 t of nitrogen removal for a NTC value between US$30 and US$150 millions! Additional NTC of US$1.6 million for the removal of 400 t of phosphorus, and CTC of US$900,000 for the removal of 30,000 t of carbon, should also be factored in.

A smaller green tide occurred in 2007. Large ones were also reported in 2009 and 2010 but they stayed offshore in the Yellow Sea [38, 39]. Out of sight should, however, not mean out of mind. If urgent measures are not taken, this will be a recurrent event for years to come. Is there a solution? Green tides are not the cause, but the unintentional consequence of coastal eutrophication. With the presence of sufficient nutrients and solar energy, these opportunistic species, with a well-adapted anatomy, morphology, and physiology, will proliferate. Obviously, it would be beneficial to reduce nutrient loading at the source, but this may not be possible in the present context of economic development along China's coastal zone. The problem is that *U. prolifera* is presently an unwanted and uncontrolled growing nuisance species of limited commercial value. To control its proliferation, the solution may be to create a competition for nutrients by intentionally cultivating algal species, which not only carry on the biomitigation, but also have a commercial value,

Aquaculture, Integrated Multi-trophic (IMTA). Figure 4
A green tide of *Ulva prolifera* in Qingdao, China, just before the 2008 Olympic Games, triggered a massive cleanup

where *U. prolifera* starts to enter the coastal environment (discharges from juvenile river crab land-based aquaculture ponds along Jiangsu province, south of Shandong province where Qingdao is located). This time, the IMTA concept has to be interpreted as an integrated land pond/coastal aquaculture system in a supra Integrated Coastal Zone Management (ICZM) effort, beyond provincial borders, to address issues at the Yellow Sea scale. It is understood that this "out of the box" approach to ICZM will, initially, raise eyebrows as the idea of growing more seaweeds (but of commercial value) to contain the proliferation of other seaweeds, presently considered nuisances, is not the most intuitive approach for a lot of people or decision makers! The question is simple: what are the best nutrient scrubbers once nutrients are in a dissolved state and have reached coastal waters? The answer is seaweeds, but can people, preferably, grow the ones they have applications for?

At the present time, there seems to be a stage of recognition, awareness, and communication of the concepts of ecosystem services and biomitigative services rendered by extractive aquaculture (the differences between the two not always being clearly identified and explained in some publications). Next will come the time to transform the concepts into biomitigative solutions and then their inclusion in regulatory and management frameworks. Establishing and implementing a structure for the payment schemes (credits or incentives) of these services will be a delicate matter. Will it be one agency, but with funds coming from where? Should it be a regional, national, or international agency(ies), trading at which scale(s)? Will an extractive aquaculture operation in existence for many years receive credits, or will only the new ones? Would a fed aquaculture operation also practicing extractive aquaculture be eligible for credits, or will it be the case for the extractive only aquaculture operations? What about the situation in which people run both types of farms. Moreover, due to complex hydrographic and current patterns, it is obvious that extractive species at a site are not limited to absorbing/sequestering the nutrients generated exclusively at that site. Consequently, is it possible to establish a clear spatial nutrient removal budget which would be associated with the corresponding credits/incentives? Will the sequestration have to be "permanent," or will a temporary removal/storage be acceptable and more realistic? A lot of regulatory details will have to be worked out before this complex scheme becomes a reality.

What Will It Take to Increase the Acceptance and Adoption of IMTA as a Responsible Aquaculture Practice of the Future?

Presently, the most advanced IMTA systems in open marine waters and land-based operations have three components (fish, suspension feeders or grazers such as shellfish, and seaweeds, in cages, rafts, or floating lines), but they are admittedly simplified systems [40]. More advanced systems will have several other components (e.g., crustaceans in mid-water reefs; deposit feeders such as sea cucumbers, sea urchins and polychaetes in bottom cages or suspended trays; and bottom-dwelling fish in bottom cages) to perform either different or similar functions, but for various size ranges of particles, or selected for their presence at different times of the year (e.g., different species of seaweeds). The most advanced IMTA systems, near or at commercial scale, can be found in Canada, Chile, South Africa, Israel, and China [41, 42]. Ongoing research projects related to the development of IMTA are taking place in the UK (mostly Scotland), Ireland, Spain, Portugal, France, Turkey, Norway, Japan, Korea, Thailand, the USA, and Mexico. It will also be interesting to observe how new seaweed cultivation initiatives in different parts of the world for biofuel production could be an additional driver to adopt IMTA practices.

For IMTA to develop to a commercial scale, appropriate regulatory and policy frameworks need to be put in place. Present aquaculture regulations and policies are often inherited from previous fishery frameworks and reasoning, which have shown their limitations. It is, therefore possible that some of the existing regulations and policies could impose unintentional constraints on the future growth of IMTA. To develop the aquaculture of tomorrow, current governance structures pertaining to aquaculture need to be revisited and reviewed with the aim of identifying changes in the regulatory/policy environment that are needed to facilitate the operation of IMTA farms. Adaptive regulations need to be developed by regulators with flexible and innovative minds, who are not afraid to put in place mechanisms that allow the testing of innovative

practices at the R&D level, and, if deemed promising, mechanisms that will take these practices all the way to C (commercialization). As the IMTA concept continues to evolve, it is important that all sectors of the industry are aware of the implications of the changes involved, so that they can adapt in a timely and organized manner.

To move research from the "pilot" scale to the "scale up" stage, some current regulations and policies may need to be changed or they will be seen as impediments by industrial partners who will see no incentive in developing IMTA. For example, an earlier version of the Canadian Shellfish Sanitation Program (CSSP) prevented the development of IMTA because of a clause that specified that shellfish could not be grown closer than 125 m of finfish netpens. This paragraph was clearly not written with IMTA in mind, but it seriously impinged its development. After 4 years (2004–2008), it was amended so that IMTA practices could develop to commercial scale legally, based on recent, reliable, and relevant data and information provided by three government departments and the IMTA project on the east coast of Canada. While 4 years may seem long, it is a relatively short delay considering that regulations and legislations require thorough review with due governmental process involving several federal and provincial departments. This suggests that new aquaculture practices should be accompanied by timely regulatory review to avoid market delays for new products. As governments move to revise current regulatory regimes, it will be necessary to press the importance of accommodating and indeed encouraging new sustainable solutions such as IMTA. IMTA also requires approaching aquaculture development and management with a holistic approach and not one species, or group of species, at a time. It is known that this approach has led to many failures in the management of the fisheries; vigilance is required so that the same flaw is not repeated in the management of aquaculture.

Most current aquaculture business models do not consider or recognize the economic value of the biomitigative services provided by biofilters, as there is often no cost associated with aquaculture discharges/effluents in land-based or open-water systems. In order to ensure further development of IMTA systems worldwide, from the experimental concept to the full commercial scale, defining and implementing the appropriate regulatory and policy frameworks, and financial incentive tools such as NTC and CTC, may therefore be required to clearly recognize the benefits of the extractive components of IMTA systems. Better estimates of the overall costs and benefits to nature and society of aquaculture waste and its mitigation would create powerful financial and regulatory incentives to governments and the industry to jointly invest in the IMTA approach, as the economic demonstration of its validity would be even more obvious. Moreover, by implementing better management practices, the aquaculture industry should increase its societal acceptability, a variable to which it is very difficult to give a monetary value, but an imperative condition for the development of its full potential. Reducing environmental and economic risk in the long term should also make financing easier to obtain from banking institutions [43].

The determination to develop IMTA systems will, however, only come about if there are some visionary changes in political, social, and economic reasoning. This will be accomplished by seeking sustainability, long-term profitability, and responsible management of coastal waters. There is still a large amount of education required to bring society into the mindset of incorporating IMTA into their suite of social values. Some of the attitudinal surveys conducted in Canada [23, 44] and the USA [45] indicate that the general public is in favor of practices based on the "recycling concept." Consumers' perceptions and attitudes may also have to change. Why is recycling and the concept of "what is waste for some is gold for others" well accepted in agricultural practices, but is not yet acquired when transposed to aquaculture practices? Will consumers come to accept eating products cultured in the marine environment in the same way they accept eating products from recycling and organic agricultural practices, for which they are willing to pay a higher price for the perceived higher quality or ethical premiums? After all, regulations require mushrooms to be specifically grown on farmyard manure and animal excrements to receive organic certification (European Community Regulations No 2008R0889 – Article 6). Will a greater appreciation of the sustainable ecological value of the IMTA concept, a willingness to support it tangibly with shopping money, and an increased pressure on elected representatives emerge? This will be the ultimate test.

The degree to which researchers and extension people become creatively involved with this educational component will be vital to the success of IMTA practices. The differentiation of IMTA products through traceability and eco-labeling will also be key for their recognition and command of premium market prices.

Some have argued that the adoption of IMTA in the western world is slow. For example, on the east coast of Canada, there were obviously no IMTA sites in the Bay of Fundy in 2001 when IMTA research started. Nine years later, 8 of the 96 finfish sites in southwest New Brunswick have the combination salmon (or cod)/mussels/kelps and 8 other sites have been amended to develop IMTA. This is a respectable conversion of almost 16% in 9 years. Moreover, it would not be reasonable to anticipate an instant conversion, as the industry needs to develop markets to absorb the cocultured biomass: this also takes time and can only be progressive.

Future Directions: The Path Forward

Several IMTA projects, worldwide, have now accumulated enough data to support the proof of concept at the biological level. The next step is the scaling up of more experimental systems to commercial scale to further document the economic and social advantages of the concept, which will be key to offering IMTA to practitioners of monospecific aquaculture as a viable option to their current practices. Emerging responsible aquaculture approaches must generate net economic benefits for society if they are to be advocated. Working on appropriate food safety regulatory and policy frameworks in the respective countries will be essential for enabling the development of commercial scale IMTA operations in a more universal fashion.

It has taken decades to reach current finfish aquaculture production levels and learn new species husbandry. A major rethinking is, however, needed regarding the definition of an "aquaculture farm" by reinterpreting the notion of site-lease areas and regarding how it works within an ecosystem, in the context of a broader framework. Within Integrated Coastal Zone Management (ICZM), integration can range from the small scale (a leased site with its spatial limits) to a Bay Management Area (BMA) and to the larger scale of a region connected by the functionalities of the ecosystem. Amending regulations to allow a new type of aquaculture systems will not occur overnight. This should, however, not discourage the finfish aquaculture industry from practicing IMTA, as even small amounts of cocultured species production are useful at the initial stage of development.

Selecting the right combination of species will be critical. They will have to be appropriate for the habitat, the available culture technologies and labor forces, and the environmental, climatic, and oceanographic conditions. They will have to be complementary in their ecosystem functions, growing to a significant biomass for efficient biomitigation, commanding an interesting price as raw material or presenting an interesting added value for their derived products. Their ecological interactions and synergies within an IMTA system will have to be identified and understood to take full advantage of them. Their commercialization should not generate insurmountable regulatory hurdles.

Optimal design will not only facilitate nutrient recovery, but should also promote augmented growth beyond what would be expected were these species cultured in isolation. In addition to the obvious economic return from increased growth rates from additional species, some less tangible benefits should also be factored in, such as the biomitigative services rendered by the extractive species. Economic analyses will have to recognize and account for the values of the environmental/societal services of extractive crops to estimate the true value of these IMTA components. Economic analyses will need to be part of the overall modelling of IMTA systems, as they get closer to commercial scale and their economic benefits and costs, as well as their impacts on coastal communities, are better understood. It will then be possible to add profitability, resilience, social/economic desirability, and economic impacts to the comparison between IMTA and monoculture settings. They will have to include the pricing and marketing potential and impact of organic and other eco-labellings, the value of biomitigative services for enhanced ecosystem resilience, the savings due to multi-trophic conversion of feed and energy which would otherwise be lost, and the reduction of risks through crop diversification and increased societal acceptability of aquaculture (including food safety, food security, and consumer attitudes toward buying sustainable seafood products). This would create

economic incentives to encourage aquaculturists to further develop and implement sustainable marine agronomy practices such as IMTA, and would increase the societal acceptability of aquaculture by the general public. Seaweeds and invertebrates produced in IMTA systems should be considered as candidates for a variety of regulatory measures that internalize these benefits. For example, nutrient and carbon trading credits (NTC and CTC) could be used to promote nutrient removal, CO_2 sequestration, oxygen provision, and coastal eutrophication reduction within the broader context of ecosystem goods and services. Long-term planning/zoning promoting biomitigative solutions, such as IMTA, should become an integral part of coastal regulatory and management frameworks.

Nutrient extractive aquaculture appears to be a viable ecological engineering option for managing/internalizing some of the externalities generated by aquaculture operations. Effective government legislation/regulations and incentives to facilitate the development of IMTA practices and the commercialization of IMTA products will be necessary. The development and adoption of technology often depends in part on the level of legislative pressure from a nation's government, itself reacting to pressures from consumers, environmental nongovernmental organizations, and the public at large. If environmental legislation remains a low priority with government, then little progress toward the use of biofilters (as a means of effluent mitigation) will occur. The only motivator will be profits obtained from additional product growth and regulatory incentives. Therefore, if governments put legislative pressure on the proper management of wastewater effluent, openly support the use of biomitigation for effluent management, and put in place the appropriate corresponding financial tools (funding for IMTA Research & Development, outreach and technology transfer, and NTC and CTC incentives), then the development of IMTA will be encouraged.

Caution: Let's Not Promise the Moon and Let's Be Conscious of Societal Constraints, Particularly in the Western World

During the last few years, there has been a renewed interest in the mariculture of seaweeds and their uses, something that should make phycologists and ecologists rejoice, as this group of organisms, never clearly systematically circumscribed, has been misunderstood, unappreciated and under/misused over the centuries. There is now an opportunity to explain what seaweeds are, and the many applications, benefits, and services they can provide. However, how can people do that appropriately and responsibly, without "promising the moon" that they will not necessarily attain, and risking another "purgatory period" in between each energy crisis?! Seaweeds (and algae in general) made the news in the 1970s–1980s; they are back in the news now (2000s–2010s). If people are not careful to distance themselves from charlatanistic claims, which abound in the media and even in certain scientific circles, they could be in a situation of not developing a sustained public interest and use of these organisms, but be in another phase of denial until the next fad cycle (2030s–2040s?), which is not productive for the acquisition of still much needed scientific knowledge, nor the teaching of our discipline or the placement of our in-between fashion students. While everyone wants the seaweed sector to develop, some biotechnological issues and societal constraints, particularly in the Western World, should be recognized and a responsible and gradual implementation strategy for the long term should be adopted.

The western marine biology community has been dominated by people who have received a mostly zoological training from kindergarten to high school, very often reinforced by a monospecific (or monogrouping) specialization at university, instead of receiving and developing an ecosystem approach to knowledge and issue solving, which are then sadly missing when concepts of ecosystem-based management, species cocultivation, and interdisciplinarity are mentioned. Not surprisingly, the knowledge of seaweeds and their functions and services in/to the ecosystem is reduced and remains at universities and research institutions that have been wise in keeping their diverse expertise, instead of succumbing to fad cycles, which, then, force them to periodically reinvent the wheel. The consequence is that every time one wants to raise the possibility of using seaweeds in research and development and commercialization (R&D&C), one has to go through a lonely period of "preaching in the desert" before facts and common sense start to prevail.

One key, common, and deeply rooted misunderstanding to shake from the minds of people is that there is more than fish in the ocean! Over the centuries humans have been quite minimalist in their meat choices: four mammals (cow, pig, sheep, and goat) and four poultry (chicken, turkey, duck, and goose), hence, Paul Greenberg's idea of four fish (salmon, sea bass, cod, and tuna) for the title of his book [8]. However, the ocean cannot function with only fish, and the seafood solutions cannot come from within only this group of organisms. Maybe the problem resides deeply among the English-speaking people with this overuse of "fish": fish is a noun, which can even encompass shellfish and seaweeds in its general use, and fish is a verb... if you go harvest seaweeds along the shore you could be paradoxically fishing seaweeds, which for a Cartesian French-speaking person does not make much sense! In French, there is "poisson" as a noun and "pêcher" as a verb, even if both come from "pisces" in Latin. So, when a French person "va à la pêche," it is not necessarily to get a fish, but also to go "à la pêche aux moules" (mussels), "aux oursins" (sea urchins), or "aux algues marines" (seaweeds, for which many languages also have a higher opinion, as marine algae, instead of weeds of the sea!). To function, IMTA requires, in fact, not four components but five: the fed organisms (e.g., fish or shrimps), the extractive inorganic component (e.g., seaweeds or other aquatic vegetation), the extractive small organic component (e.g., suspension feeders such as shellfish), the extractive large organic component (e.g., deposit feeders such as sea-urchins, sea cucumbers, or sea worms), and certainly a fifth component, the microbial component, of which presently not much is known. So, if people want aquaculture to work, they have to stop being obsessed with fish aquaculture! Paradoxically, it is interesting to know that fish aquaculture, of which so much is heard, represents, in fact, only 9% of the total mariculture (aquaculture in the marine environment). Shellfish aquaculture represents 43%. Seaweed aquaculture represents even more (46%), but 99.8% of it is carried out in Asia, hence the ignorance in the western world [46, 47].

It is also important to understand that sustained successful ventures rarely happen overnight and that more than a 3 year grant is generally necessary to successfully take a concept along the R&D&C continuum. For example, the IMTA program on the east coast of Canada is starting to collect the fruits of its tireless efforts as it enters its 15th year of activities, which so far could be divided into four periods: (1) the "preaching in the desert" period from 1995 to 2000 [48], (2) the R&D proof of concept period from 2001 to 2006, (3) the R&D&C pilot scale period from 2006 to 2012, overlapping with (4) the R&D&C industrial-scale and networking period with the establishment of CIMTAN since 2009. People, consequently, have to stay away from claims of solving hunger in the world, converting everybody into frequent direct "seaweedivores," 100% biomitigation (which, in fact, is not necessarily the goal), renewing energy at unbelievable rates that defy the rules and equations governing photosynthesis, and all that within the next 5 years with the almighty, miraculous seaweeds and micro-algae!

If there is no shortage of interesting ideas that work at the small demonstration scale, the problems generally appear when scaling up is contemplated and people start to realize what the consequences will be and, especially, the realistic, or unrealistic, deployment footprints required to implement these experimental ideas to commercial-market scales, which should make sense from environmental, economic, and production perspectives and also have an acceptable societal impact.

People should also stay away from the cliché that around 71% of this planet is covered by oceans and that, consequently, there is a lot of space for aquaculture development. If aquaculture will most probably expand into more exposed and open ocean locations in the future, due to the reduced availability of new and appropriate sheltered nearshore sites, it is doubtful that one will see farms in the middle of the Atlantic, Pacific, and Indian Oceans, due to simple logistics and weather issues. Moreover, the present international law of the sea is not that comforting for privately owned equipment (farms in this case) found at sea. The vagueness of territorial jurisdictional competence in the Exclusive Economic Zone (EEZ) in different countries, and certainly in international waters, has been a major impediment to progress of the so-called offshore aquaculture. Moving to the open ocean has been considered a means for moving away from environmental and public perception issues in the coastal zone. However, this move should not encourage an "out of sight, out of mind" attitude, as open ocean development will also come

under scrutiny by a more and more educated public. Even if greater residual currents, deeper waters, and lower nutrient baselines are expected to reduce impacts from open ocean operations through wider dispersion plumes of nutrients, as compared to similarly sized nearshore operations, there will be a point when reasonably accessible and manageable open ocean ecosystems will eventually reach their assimilative carrying capacities. Why should one think that open ocean aquaculture, the "last frontier," will be without its own border/limits? Despite the sea being so immense, one is now learning the hard way the concept of overfishing . . . Instead of taking the position that in open ocean environments the hydrodynamic conditions will be appropriate for dispersion (a way of exporting problems, not solving them) and reduced environmental impacts (but at a significant cost in lost food), the open ocean aquaculture sector will also have to capitalize on recapturing the by-products of fed aquaculture and, hopefully, engineer, right from the beginning, efficient open ocean IMTA systems with their built-in biomitigative functions. The solution to nutrification in the open ocean environment, like in the nearshore environment, should not be dilution, but extraction and conversion through diversification. Why repeat what was done with the development of nearshore aquaculture (fish aquaculture development in the 1970s and IMTA development in the 2000s) with open ocean aquaculture (moving the fish to the open ocean in the 2010s . . . oh, the extractive species should have also been moved in the 2050s!)? These open ocean systems will also require trophic diversification from an environmental and economic perspective, with "service species" from lower trophic levels (mainly seaweeds and invertebrates) performing ecosystem balancing functions while representing value-added crops [49, 50]. Open ocean IMTA should not be an afterthought for 2050.

For some, the ecological, engineering, economic, and social challenges remaining to be solved may be daunting. However, our goal is to develop modern IMTA systems, which are bound to play a major role worldwide in sustainable expansions of the aquaculture operations of tomorrow, within their balanced ecosystem, to respond to a worldwide increasing seafood demand with a new paradigm in the design of the most efficient food production systems. There are no simple solutions, but one thing is certain – the human population is increasing on this planet and as people get richer, and their standards of living increase, they want more meat and dairy products in their diet, the temptation of the "western diet," while, ironically, Westerners aspire to change their diets! Will terrestrial agriculture be able to continue to supply most of this food? A balanced and responsible diet is required, and some of this food will have to come, increasingly, from aquatic food production systems, be them in seawater, brackish water, or freshwater. As was the case on land, where the acquisition of food by hunter/gatherer societies had to evolve toward agricultural practices, humans will have to accept an evolution in seafood procurement. It has to be understood, particularly in the western world, that "the modern global supermarket has a basic internal ecology" [8]. The average consumer is not a "foodie" and is not that interested in or cannot afford local, seasonal, less-than-100-miles food if not rich enough or not living within a region graced by a clement climate year long. The modern supermarket wants guaranteed supply on a 12 month basis, with limited variability in seasonality and quality. Most of the time, agricultural products can provide that comfort, barring the occurrence of an unexpected disease, contamination, drought, flood, economic protectionism, or political barrier. The seafood counter is a much more variable department to manage, at the present time, with a convoluted succession of many intermediates before seafood arrives on ice at a supermarket. It is interesting to note that the aquaculture industry's ability to provide 12 month availability of its products, moreover of consistent quality, is improving.

People are presently witnessing the emergence of a plethora of organizations developing their own standards and eco-label/certification schemes as they jockey for position in the global marketplace. The problem is that there are presently too many possible horses to ride and nobody really knows which one(s) will cross the finish line and, consequently, which one (s) to bet on as worth being associated with. One can only wonder what will happen when so many fisheries and aquaculture operations will be eco-certified. If everything is certified, nothing will be certified . . . and certification will lose its aura the same way some argue organic labelling is losing its significance, after having been used and overused. All that, of course,

to the great confusion of the consumers, who cannot follow this contradictory debate/competition among standard setters, and may decide to simply stay away from seafood all together when, in fact, seafood products are healthy [51]. One of the problems is that some of these standards are passing or failing grades, with no incentives for continuous improvement from a minimal baseline to be decided, followed by a tiered approach. Some argue that it would give accreditation to companies at a very low level. However, putting the bar so high is not a recipe for gradual improvement of everybody involved, to progress and gradually reach the ultimate goal, although admittedly not overnight. If 20% of the global farmed seafood producers are certified at the highest threshold, what happens to the remaining 80% and the chance of incentivizing them to improve their practices? What happens when, in a bay management area, several aquaculture companies have taken the appropriate measures to be certified, but a "black sheep" (should it be a black cod?!) makes the whole certification scheme crumble once the hydrodynamics of the bay are considered? By analogy, in which the vector this time is not water but wind, one sees the same dilemma in parallel agriculture situations where conventional and organic agriculture practices are separated by illusionary buffer zones. On one hand, one can understand the desire by suppliers and retailers to see a hard to meet certification scheme so as to differentiate themselves from the others (most probably amounting to the privilege of displaying a sticker or logo on the packaging); on the other hand, too high a certification carrot, or moving goalposts, may not be the best strategy if progress toward overall better and more responsible aquaculture practices is the goal. The market will ultimately decide who remains in the competition and which logo(s) will be trusted by the general public, but there still are several years of confusion ahead.

Agricultural development has been associated with significant changes in landscape and land use; one can expect that the evolution of sourcing one's seafood more and more through aquaculture will also trigger significant "seascape" and "sea use" modifications, all the way to one's deepest human social structures and governance. The transformation from hunters/gatherers to farmers happened many centuries ago on land. Humans are in the middle of this transformation at sea and that is maybe why they are so uncomfortable with this evolution they are part of, and not able to sit back and analyze without being emotional. It is up to them to be a link in the chain, which will hopefully lead to fishing and aquaculture practices done right, enabling them to become herders and farmers of the sea. It should not be forgotten that they are still in the infancy of modern, intensive aquaculture and that some agricultural practices have taken centuries to develop into better, not necessarily yet best, management practices.

Beyond the market and marketing issues and the biological, environmental, economic, technological, engineering, and regulatory issues of aquaculture developments, the basic question will be that of societal acceptance. Are humans ready to evolve in their use of the "last frontier" of this planet and consider not only the challenges of the physical forces at sea (wave exposure, winds, currents, depth, etc.) but also those of shipping routes, fishing zones, offshore gas and mineral extraction areas, migration routes for marine mammals and birds, recreational uses, and then finally deal with the concept of zoning some portions of the oceans for large aquaculture parks, as sustainable food production systems for an ever-seafood-hungry human population? Despite all the campaigns, boycotts, documentaries, books, seafood pocket guides, scare tactics, sustainable/local/seasonal movements among affluent restaurant goers in weather clement regions and western world well offs, one can only admit that the global human population continues to grow and eat more seafood than ever per capita per year. So, where does that leave people? Paul Greenberg wrote that very often people consider fish as "a crop, harvested from the sea that magically grew itself back every year. A crop that never required planting" [8]. But are they investing in the principal, being in fisheries or in aquaculture, to only harvest the interests every year so as to not reduce/eat the capital for long-term sustainability? Are people ready to put some savings aside in the form of Marine Protected Areas (MPAs), not only for their natural beauty, but also for their functions in the ecosystem such as breeding grounds, nursery habitats, and food production areas? It seems that the concept of zoning the sea, or what is now called, in a softer terminology, "marine spatial planning" (MPS), is finally starting to be legislated in some countries, notably in the UK and the USA.

The same question of readiness for marine spatial planning could also be applied to emerging projects of wind farms and biofuel farms at sea. In fact, combining IMTA open-ocean farms with wind, underwater turbine, and/or biofuel farms into large multipurpose integrated food and renewable energy parks (IFREP) could be a means for reducing their cumulative footprint, while integrating green energy with food and fuel production and processing [52]. Our business models will have to change from "one species – one process – one product" to a streamed bioeconomic chain, or web, approach among different industry sectors for the production, on one hand, of a wide range of bio-based, high-valued food and feed products/ingredients/supplements, specialty fine and bulked chemicals, agrichemicals, biostimulants, pharmaceuticals, nutraceuticals, functional foods, cosmeceuticals, botanicals, pigments and, on the other hand, lower-valued commodity energy carrying molecules/biofuels, all of them produced within reduced footprint requirements. The synergies and the services rendered by cultivating organisms of different trophic levels in an integrated manner will have to be understood and valued. The physiological, biochemical, and production performances of the different organisms will have to be improved to make the systems even more efficient, profitable, and competitive. The aquaculturists and different multi-sector end users will need to become interdisciplinary in their approach and learn to collaborate and share/integrate the biomass cultivation and processing steps (production, harvesting, pretreatment and transportation, separation and fractionation, and sequential biomass processing), while aiming at the lowest resource and energy inputs. Culture diversification into species that might otherwise be inappropriate for food markets fits well within the sustainability and management concept of IMTA. Functionalities will have to be maintained, as much as possible, along the process for optimal use/valorization of the multipurpose biomass, and not necessarily the maximization of just one end product, as some coproducts will, in fact, reveal themselves as the real drivers of the emerging integrated sequential bio-refinery (ISBR) concept [53], extended to macro-algae instead of only considering micro-algae. Market volumes/values, biomitigative services, and public acceptance will have to be included and fit into the models.

If the "Not In My BackYard" (NIMBY) and the "Build Absolutely Nothing Anywhere Near Anything" (BANANA) attitudes continue to prevail, especially in the western world, then humans will not be able to secure their food, chemicals, and energy in an intricately interconnected ecosystem responsible manner, despite all the rhetoric heard today regarding alternative technologies and solutions (the so-called "greenwash"). Self-sufficiency of humans will not be ensured but will become dependent on other food, chemicals, and energy "masters," who may no longer be in the Middle East but instead in the Far East (99.8% of the 15.8 million tons of cultivated seaweeds come from China, Indonesia, the Philippines, Korea, and Japan [46, 47]). It is time to walk the talk and recognize the implications – notably regarding marine spatial planning and our societal production and food habits – of the policies elaborated for the future.

The 1960s were the time of the "Green Revolution" on land, but some would question if it was really "green" (increased dependence on synthetic fertilizers and irrigation to increase crop yields per hectare at the expense of long-term soil health and yields per unit of input; increased dependence of indebted farmers on multinational producers of seeds, increasingly genetically modified, and which have not always delivered the touted benefits). It was thought that the sea was so immense that one needed not to worry about fishery limits, but now it is known that it is not always the case with many examples of overfishing of some populations. The 1980s were the time of the "Blue Revolution" of aquaculture development at sea, but it is also known that it is not always "green." It is, consequently, time to make the "Blue Revolution" greener; it is time for the "Turquoise Revolution" to move aquaculture to a new ERA of Ecosystem Responsible Aquaculture at sea and on land, in seawater and freshwater, and in temperate and tropical regions.

Acknowledgments

I greatly appreciate the support this work received from the Natural Sciences and Engineering Research Council of Canada (NSERC) strategic Canadian Integrated Multi-Trophic Aquaculture Network (CIMTAN) in collaboration with its partners, Fisheries and Oceans

Canada, the University of New Brunswick, Cooke Aquaculture Inc., Kyuquot SEAfoods Ltd. and Marine Harvest Canada Ltd.

Bibliography

Primary Literature

1. FAO (2009) The State of World Fisheries and Aquaculture 2008. FAO of the United Nations, Rome, xvi+176 p
2. Tacon AGJ, Metian M, Turchini GM, De Silva SS (2010) Responsible aquaculture and trophic level implications to global fish supply. Rev Fish Sci 18(1):94–105
3. Troell M, Halling C, Neori A, Chopin T, Buschmann AH, Kautsky N, Yarish C (2003) Integrated mariculture: asking the right questions. Aquaculture 226:69–90
4. Chopin T (2006) Integrated multi-trophic aquaculture. What it is and why you should care ... and don't confuse it with polyculture. North Aquacult 12(4):4
5. Edwards P (1993) Environmental issues in integrated agriculture-aquaculture and wastewater-fed fish culture systems. In: Pullin RSV, Rosenthal H, Maclean JL (eds) Environment and aquaculture in developing countries. ICLARM Conference Proceedings, vol 31, pp 139–170
6. Hambrey J, Edwards P, Belton B (2008) An ecosystem approach to freshwater aquaculture: a global review. In: Soto D, Aguilar-Manjarrez J, Hishamunda N (eds) Building an ecosystem approach to aquaculture. FAO/Universitat de les Iles Balears Experts Workshop. 7–11 May 2007, Palma de Mallorca Spain. FAO Fisheries and Aquaculture Proceedings 14. FAO, Rome, pp 117–221
7. Troell M (2009) Integrated marine and brackishwater aquaculture in tropical regions: research, implementation and prospects. In: Soto D (ed) Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper 529. FAO, Rome, pp 47–131
8. Greenberg P (2010) Four fish – the future of the last wild food. Penguin Press, New York, 285 p
9. Timmons MB, Ebeling JM, Wheaton FW, Summerfelt ST, Vinci BJ (2002) Recirculating aquaculture systems (2nd edn). NRAC Publication No. 01-002. Cayuga Aqua Ventures, Ithaca, 769 p
10. Ayer NW, Tyedmers PH (2009) Assessing alternative aquaculture technologies: life cycle assessment of salmon culture systems in Canada. J Cleaner Prod 17:362–373
11. Skår CK, Mortensen S (2007) Fate of infectious salmon anaemia virus (ISAV) in experimentally challenged blue mussels *Mytilus edulis*. Dis Aquat Org 74:1–6
12. Pang SJ, Xiao T, Bao Y (2006) Dynamic changes of total bacteria and *Vibrio* in an integrated seaweed-abalone culture system. Aquaculture 252:289–297
13. Pang SJ, Xiao T, Shan TF, Wang ZF, Gao SQ (2006) Evidences of the intertidal red alga *Grateloupia turuturu* in turning *Vibrio parahaemolyticus* into non-culturable state in the presence of light. Aquaculture 260:369–374
14. Molloy SD, Pietrak MR, Bouchard DA, Bricknell I (2011) Ingestion of *Lepeophtheirus salmonis* by the blue mussel *Mytilus edulis*. Aquaculture 311:61–64
15. Johansson DJA, Azar CA (2007) A scenario based analysis of land competition between food and bioenergy production in the US. Clim Change 82:267–291
16. Naylor RL, Liska AJ, Burke MB, Falcon WP, Gaskell JC, Rozelle SD et al (2007) The ripple effect: biofuels, food scarcity and the environment. Environment 49:30–43
17. SOFA (2008) The state of food and agriculture. Biofuels prospects, risks and opportunities. FAO of the United Nations, Rome
18. Jordan N, Boody G, Broussard W, Glover JD, Keeney D, McCown BH, McIsaac G, Muller M, Murray H, Neal J, Pansing C, Turner RE, Warner K, Wyse D (2007) Sustainable development of the agricultural bio-economy. Science 316:1570–1571
19. Sawyer D (2008) Climate change, biofuels and eco-social impacts in the Brazilian Amazon and Cerrado. Phil Trans R Soc B 363:1747–1752
20. Koh LP (2007) Can palm oil plantations be made more hospitable for forest butterflies and birds? J Appl Ecol 44:703–713
21. Fargione J, Hill J, Tilman D, Polasky S, Hawthorne P (2008) Land clearing and the biofuel carbon debt. Science 319:1235–1238
22. Whitmarsh DJ, Cook EJ, Black KD (2006) Searching for sustainability in aquaculture: an investigation into the economic prospects for an integrated salmon-mussel production system. Mar Policy 30:293–298
23. Ridler N, Wowchuk M, Robinson B, Barrington K, Chopin T, Robinson S, Page F, Reid G, Szemerda M, Sewuster J, Boyne-Travis S (2007) Integrated multi-trophic aquaculture (IMTA): a potential strategic choice for farmers. Aquac Econ Manage 11:99–110
24. Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O'Neill RV, Paruelo J, Raskin RG, Sutton P, van den Belt M (1997) The value of the world's ecosystem services and natural capital. Nature 387:253–260
25. Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, Fabry VJ, Miller FJ (2004) Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 305(5682):362–366
26. Thomsen B (2006) Freshwater aquaculture in Denmark: a new platform for sustainable growth. Proceeding of the Canadian Freshwater Aquaculture Symposium, Aquaculture Canada 2004. Aquaculture Association of Canada Special Publication vol 11, pp 26–30
27. Troell M, Halling C, Nilsson A, Buschmann AH, Kautsky N, Kautsky L (1997) Integrated marine cultivation of *Gracilaria chilensis* (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. Aquaculture 156:45–61
28. Chopin T, Buschmann AH, Halling C, Troell M, Kautsky N, Neori A, Kraemer GP, Zertuche-Gonzalez JA, Yarish C, Neefus C (2001) Integrating seaweeds into marine aquaculture systems: a key towards sustainability. J Phycol 37:975–986
29. Lindhal O, Kolberg S (2009) Can the EU agri-environmental aid program be extended into the coastal zone to combat eutrophication? Hydrobiologia 629:59–64

30. Lindahl O, Hart R, Hernroth B, Kollberg S, Loo L-O, Olrog L, Rehnstam-Holm A-S, Svensson J, Svensson S, Syversen U (2005) Improving marine water quality by mussel farming: a profitable solution for Swedish society. Ambio 34:131–138
31. Ferreira JG, Hawkins AJS, Bricker SB (2007) Management of productivity, environmental effects and profitability of shellfish aquaculture – the Farm Aquaculture Resource Management (FARM) model. Aquaculture 264:160–174
32. Ferreira JG, Sequeira A, Hawkins AJS, Newton A, Nickell TD, Pastres R, Forte J, Bodoy A, Bricker SB (2009) Analysis of coastal and offshore aquaculture: application of the FARM model to multiple systems and shellfish species. Aquaculture 289:32–41
33. Gren IM, Lindhal O, Lindqvist M (2009) Values of mussel farming for combating eutrophication: an application to the Baltic Sea. Ecol Eng 35:935–945
34. Lackner KS (2003) A guide to CO_2 sequestration. Science 300:1677–1678
35. Nellemann C, Corcoran E, Duarte CM, Valdés L, De Young C, Fonseca L, Grimsditch G (2009) Blue carbon. A rapid response assessment. United Nations Environment Program, GRID-Arendal. www.grida.no
36. Chung IK, Beardall J, Mehta S, Sahoo D, Stojkovic S (2010) Using marine macroalgae for carbon sequestration: a critical appraisal. J Appl Phycol. doi:10.1007/s10811-010-9604-9
37. Beardall J, Raven JA (2004) The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia 43:26–40
38. Liu F, Pang SJ, Chopin T, Xu N, Shan TF, Gao SQ, Sun S (2009) The dominant *Ulva* strain of the 2008 green algal bloom in the Yellow Sea was not detected in the coastal waters of Qingdao in the following winter. J Appl Phycol. doi:10.1007/s10811-009-9489-7
39. Pang SJ, Liu F, Shan TF, Xu N, Zhang ZH, Gao SQ, Chopin T, Sun S (2010) Tracking the algal origin of the *Ulva* bloom in the Yellow Sea by a combination of molecular, morphological and physiological analyses. Mar Env Res 69:207–215
40. Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Shpigel M, Yarish C (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361–391
41. Chopin T, Robinson SMC, Troell M, Neori A, Buschmann AH, Fang J (2008) Multitrophic integration for sustainable marine aquaculture. In: Jørgensen SE, Fath BD (eds) The encyclopedia of ecology, ecological engineering, vol 3. Elsevier, Oxford, pp 2463–2475
42. Barrington K, Chopin T, Robinson S (2009) Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. In: Soto D (ed) Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper 529. FAO, Rome, pp 7–46
43. Brzeski V, Newkirk G (1997) Integrated coastal food production systems – a review of current literature. Ocean Coast Manage 34:55–71
44. Barrington K, Ridler N, Chopin T, Robinson S, Robinson B (2010) Social aspects of the sustainability of integrated multi-trophic aquaculture. Aquac Int 18:201–211
45. Shuve H, Caines E, Ridler N, Chopin T, Reid GK, Sawhney M, Lamontagne J, Szemerda M, Marvin R, Powell F, Robinson S, Boyne-Travis S (2009) Survey finds consumers support Integrated Multi-Trophic Aquaculture. Effective marketing concept key. Global Aquaculture Advocate 12(2):22–23
46. Chopin T, Sawhney M (2009) Seaweeds and their mariculture. In: Steele JH, Thorpe SA, Turekian KK (eds) The encyclopedia of ocean sciences. Elsevier, Oxford, pp 4477–4487
47. FAO (2010) The State of World Fisheries and Aquaculture 2010. FAO of the United Unions, Rome, xv + 197 p
48. Chopin T (1995) Mixed, integrated, poly-, or multi-level aquaculture – whatever you call it, it is time to put seaweeds around your cages! In: Proceedings of the Conference on cold water aquaculture to the Year 2000, St. Andrews, Canada, 6–8 September 1995. Aquaculture Association of Canada, Special Publication No. 2 (1997):110
49. Chopin T (2008) Integrated multi-trophic aquaculture (IMTA) will also have its place when aquaculture moves to the open ocean. Fish Farmer 31(2):40–41
50. Troell M, Joyce A, Chopin T, Neori A, Buschmann AH, Fang JG (2009) Ecological engineering in aquaculture – potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. Aquaculture 297:1–9
51. Lee JH, O'Keefe JH, Lavie CJ, Harris WS (2009) Omega-3 fatty acids: cardiovascular benefits, sources and sustainability. Nat Rev Cardiol 6:753–758
52. Chopin T (2009) Let's not move just the fish to the open ocean... an integrated multi-trophic aquaculture approach should not be an afterthought for 2050. In: The ecology of marine wind farms: Perspectives on impact mitigation, siting, and future uses. 8th Annual Ronald C. Baird Sea Grant Science Symposium. 2–4 November 2009, Newport, USA. http://seagrant.gso.uri.edu/baird/2009_windfarms/abstracts/chopin.pdf
53. Subhadra B, Grinson G (2010) Algal biorefinery-based industry: an approach to address fuel and food insecurity for a carbon-smart world. J Sci Food Agric. doi:10.1002/jsfa.4207

Books, Reviews, and Websites

Costa-Pierce BA (2002) Ecological aquaculture: the evolution of the blue revolution. Blackwell Science, Oxford, 382 p

Soto D, Aguilar-Manjarrez J, Hishamunda N (2008) Building an ecosystem approach to aquaculture. FAO/Universitat de les Iles Balears Experts Workshop. 7-11 May 2007, Palma de Mallorca, Spain. FAO Fisheries and Aquaculture Proceedings 14. FAO, Rome, 221 p

Web site of Thierry Chopin: www.unbsj.ca/sase/biology/chopinlab/

Web site on IMTA on Wikipedia: http://en.wikipedia.org/wiki/Integrated_Multi-trophic_Aquaculture

Aquaculture, Sustainability Science in

Barry A. Costa-Pierce[1], Glenn G. Page[2]
[1]University of New England, Marine Science Center, Biddeford, ME, USA
[2]SustainaMetrix, LLC, Johns Hopkins University, Baltimore, MD, USA

Article Outline

Glossary

Stewardship Ecosystem stewardship is an ethic practiced by aquaculture practitioners, organizations, communities, and societies who strive to sustain the qualities of healthy and resilient ecosystems and their associated human communities. Stewardship takes the long-term view and promotes activities that provide for the well-being of both the present and future generations.

Nested systems of governance Environmental and societal issues relating to sustainable aquaculture impact, and are influenced by, conditions and actions (at both higher and lower levels) in an ecosystem governance hierarchy. Some issues can be addressed more effectively at one level, and less effectively at another. The choice of the issue or set of issues to be addressed within a given site must therefore be made in full knowledge of how responsibility and decision-making authority are distributed within a layered governance system. Planning and decision-making for aquaculture at one scale, for example, within a municipality or province, should not contradict or conflict with planning and management at another scale, for example, planning for large-scale aquaculture at the nation-state scale. The reality is that such contradictions and conflicts are common. A major challenge for the aquaculture practitioner is to recognize these differences and work to either change them or select goals and strategies that recognize that such contradictions must be accommodated or resolved. In practical terms, this means that a central feature of ecosystem-based aquaculture is that all planning and decision-making must recognize and analyze conditions, issues, and goals in respect to the next higher level in a governance system. Thus, ecosystem-based aquaculture at the municipal scale must – at a minimum – be placed within the context of governance at the scale of the province.

Participation One of the defining characteristics of the practice of the ecosystem approach to aquaculture is its emphasis on participation and its relevance to the people affected. The emphasis upon participation recognizes that if an aquaculture program is to be successful, those whose collaboration and support is needed must be involved in the processes of defining the issues that the program will address, and in selecting the means by which goals and objectives will be achieved. Both individuals and members of communities and institutions are more likely to comply with a management program when they feel that it is consistent with their values, responds to their needs, and to their beliefs of how human society should function. Voluntary compliance by a supportive population lies at the heart of the successful implementation of a program. A participatory approach helps stakeholders and the public to see the efforts of an aquaculture program as a whole.

Area of focus The area of focus (AoF) is the geographically defined area that an ecosystem-based aquaculture project or program has decided to address and that therefore is the focal point for a baseline.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

The term "area of focus" is a geographic limit set to model the choices available to the aquaculture practitioner and allows for a dialogue between stakeholders as to the influence of the production. The AoF is a simplification of the far more complex concept of an "action arena" put forward by Ostrom [1] to model the choices of individuals when studying the behavior of institutions.

Adaptive management A central feature of the practice of any form of ecosystem-based aquaculture is that it must respond positively to changing conditions within its AoF (and to its own experience). In other words, the practice of aquaculture must be grounded in a process of learning and adaptation (the "evolution of the blue revolution" [2]). Adaptive management is not reactive management, but proactive thinking and acting. This does mean that the aquaculture practitioner simply responds to the unexpected. Adaptive management in aquaculture is a conscious process of examining the course of events as these events are revealed by preselected indicators of changes in an aquaculture ecosystem (both its social and environmental components), and by events occurring at differing spatial scales.

Capacity building There is growing international recognition that the lack of human capacity to practice an ecosystem approach to aquaculture is a key factor in limiting forward progress in the conservation and sustainable use of aquatic systems [3, 4]. To date, however, no accepted performance standards have been developed for assessing the effectiveness and impacts of aquaculture projects and programs that have adopted the ecosystem approach. Conceptual frameworks and methods for assessing the maturity of aquaculture development and management initiatives, and gauging their impacts upon the condition of coastal ecosystems are offered herein. These are the core ingredients for an ecosystem's approach to aquaculture that builds the capacity of local populations and leaders to identify forces that shape the coastal ecosystems of which they are a part, and to select the actions that can maintain and enhance qualities that are critical to a desirable future.

Carrying capacity The carrying capacity is the number of organisms or farming operations that the environment can sustain indefinitely without environmental harm, given the food, habitat, space, water, and other requirements from the environment.

Precautionary principle A principle that states that if an action or policy has a suspected risk of causing harm to the public or to the environment that in the absence of scientific consensus the burden of proof rests on those who advocate taking the action.

Sustainable development The management and conservation of the natural resource base and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Sustainable development conserves resources, is environmentally non-degrading, and is technically appropriate, economically viable, and socially acceptable [5].

Transdisciplinary A modern research strategy that crosses many disciplinary boundaries to create a holistic approach. Transdisciplinary research efforts are focused on problems that cross the boundaries of two or more disciplines, and develop new or reframe old concepts, methods, and findings that were originally developed by one discipline, but are now used by several others.

Definition

There is no one definition of "sustainability" as the concept applies to aquaculture. Most aquaculture scientists define sustainability as synonymous with "environmental sustainability." Sustainable aquaculture is however a concept broader than determinations of site-specific environmental impacts since it embodies a scientific knowledge of systematic impacts of aquaculture off-site, and impacts to combined human-environmental systems. Sustainable aquaculture incorporates the concepts of "stewardship," "design with nature," the "precautionary principle," "risk analysis," and "carrying capacity." Sustainability science in aquaculture is used to undertake more comprehensive planning for multiple impacts on multiple time and spatial scales to better understand and plan for the consequences of aquaculture development options.

Introduction

- "The changes taking place [on planet Earth] are, in fact, changes in the human-nature relationship. They are recent, they are profound, and many are accelerating. They are cascading through the Earth's environment in ways that are difficult to understand and often impossible to predict. Surprises abound" [6].

There are many definitions of "sustainability" as the concept applies to aquaculture. The most popular definition of sustainable development is to "meet present needs without compromising the ability of future generations to meet their needs" adopted at a United Nations conference in 1987. Most definitions of sustainability are synonymous with "environmental sustainability" of air, water, and land systems. Sustainability is however a concept broader than examining the site-specific environmental impacts of externalities in planning for site-specific developments; it also accounts for systematic impacts off site, and impacts to combined human-environmental systems for food, water, waste, energy, and shelter. The many definitions of sustainability all embody in common, the concepts of "stewardship," "design with nature," plus incorporate recent concepts of the "precautionary principle," and "carrying capacity." Sustainability science uses the wisdom from multiple disciplines in decision-making (e.g., it is "transdisciplinary"). In aquaculture, it is used to undertake more comprehensive planning for multiple impacts on multiple time and spatial scales to better understand and plan for the consequences of development options.

The emerging fields of ecological aquaculture [2,3] and agroecology [7, 8] recognize that the implementation of more sustainable food production systems require knowledge about how ecosystems are utilized and how conflicts among social groups are addressed. A baseline of response to social–ecological changes is the foundation for the implementation of more sustainable food systems, and the practice of adaptive management must be included as responses to changes in the condition of ecosystems in which new food production is conducted requires incorporation of an iterative learning process.

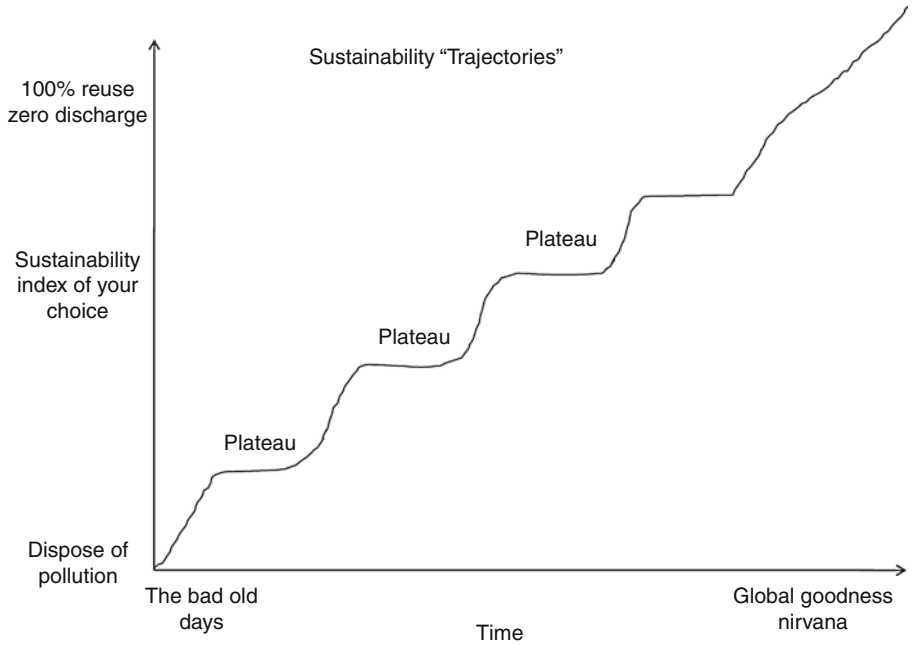
The use of sustainability science in aquaculture marks the path toward encouraging a long-term perspective and an appreciation of the roles played not only by ecologists, but also by civil societies, markets, and governments in adapting to food systems and ecosystems changes. The use of sustainability science in aquaculture is an approach that is fundamentally a knowledge-based enterprise that incorporates baseline information on natural and human ecosystems, then develops, evaluates, encourages, and communicates imagination, ingenuity, and innovation at both the individual and institutional levels [9].

This information is designed for use by teams of aquaculture professionals working to apply the principles of ecosystem-based management. Information obtained is typically cross sectoral as interdisciplinary groups are needed that are educated in such diverse fields as the natural and social sciences, law, and business. Applying the notions of sustainability science in aquaculture is intended to inspire engagement of governmental agencies, businesses, nongovernmental groups, and academics to achieve the highest form of sustainable development in any known protein production food system by using the concepts of ecological design and through the many forms of stewardship. At present, there is a paucity of information targeted specifically for those engaged in aquaculture programs and projects in places where the ability of government to regulate and direct the processes of ecosystem change is weak or severely constrained.

Sustainability Strategic and Implementation Planning for Aquaculture

The concept of sustainability and the methods to measure the evolutionary progress toward more sustainable systems are limited, but have become a necessity. Wurts [10] stated that "Whether the word sustainability has become overused or not, it has catalyzed a forum for oversight of the growth and development of aquaculture on a global scale."

Sustainability is not a "black/white" phenomena; rather, it is many "shades of gray," an evolutionary process that is called the "sustainability trajectory" (Fig. 1). To measure and evaluate progress along a trajectory requires establishment of baselines for the main issues of public concerns, then developing a diverse but targeted set of resource and social indicators. These indicators are then used to report progress on and analyze interactions between social, environmental,



Aquaculture, Sustainability Science in. Figure 1
Sustainability is neither a "black or white," nor an "either or" concept. It is the evolution of practices and principles over time toward ameliorating environmental and social impacts, with plateaus along the way in changed states. In many cases, these "pauses" are done to ensure economic viability. In this diagram, one example indicator (water use, other important indicators have been proposed [17]) is plotted along such a "sustainability trajectory"

and economic impacts (both positive and negative ones). It is important to note that sustainability science as applied to aquaculture is driven as much by social as by environmental/ecological concerns; thus, sole involvement of technical experts in sustainability plans and assessments is insufficient.

Developing an operational framework for how the sustainability of aquaculture operations operates is the first step. Having such a blueprint is rare for aquaculture businesses and management entities, and is very much needed. There are numerous certification bodies that are vying for the opportunity to use their labels/logos to claim ownership of the sustainability rubric in aquaculture. An overall sustainability science approach is proposed, which can step above the cacophony of approaches and assist in developing a common language and can be used by international and national, non-advocacy organizations such as the FAO, ICES, or governments and industry.

The approach used here is based upon the development of a baseline that has two parts and then follows a sequence of five steps:

The *first part of a baseline* is an ecosystem audit of the AoF that defines the natural and social systems within which aquaculture is planned.

This involves the documentation and analysis of both natural and social systems, draws upon case studies of other aquaculture systems in the region and how the governance system in a specific place has responded – or failed to respond – to the trajectories of ecosystem change. It examines the long-term trends in both human well-being and the environmental conditions in the AoF and examines responses to the issues raised by past and current expressions of food production systems.

The *second part of the baseline* is an outline of the strategic approach to designing a new aquaculture program, or adapting an ongoing program, to address the ecosystem management issues of the place in terms of economic, environmental, and societal benefits. Together, these parts form the reference points against which future changes in the aquaculture ecosystem will be gauged. These methods encourage a long-term perspective, an appreciation of the roles played by civil

society, markets, and government, and offer a holistic, ecosystem-based, approach to stewardship.

Baselines are not formulaic but are designed planning exercises with buy-in from key stakeholders such as the client, community, regulatory community, or identified group of people involved in the project. While not formulaic, baselines do comprise a set of common metrics to include

- Ecological aquaculture design (or redesign) of production practices (see Ecological Aquaculture chapter in this Encyclopedia)
- Health and quality control standards
- Social goals at both the individual and community levels for local food, job, and regional development (e.g., "green jobs," "local foods")
- Governance goals

The following five steps encompass some essential parts of any baselining process:

1. *Define the sustainability issues*. Aquaculture systems can use environmentally derived feeds, water, and energy, occupy land and water space, and generate wastes. There are at least eight issues of wide public and regulatory concerns regarding aquaculture development:
   - Destruction of habitats
   - No net gain to global seafood supplies
   - Environmental impacts of discharged wastes
   - Impacts of escapees
   - Diseases in farmed fish
   - Chemical use and discharge
   - Impacts on coastal marine mammals
   - Improper siting causes visual pollution

   Once issues are defined, a baseline can be further developed which can measure progress over time by
2. *Completing a sustainability assessment* of these issues by evaluating the status of current aquaculture practices that affect natural and social resource systems (Table 1), which also includes an assessment of governance systems (Tables 2 and 3) [11–13]
3. *Completing a detailed risk analysis* for all components of this comprehensive assessment [14]
4. *Completing a plan for ameliorating identified impacts* by incorporation of better (or best) practices [15–17], and/or enhancing reuse or recycling pathways, and
5. *Completing a plan for communicating* the evolution of operations toward greater stewardship and sustainability [14]

To be effective, sustainable aquaculture initiatives must: (a) be "profitable" over long periods of time – ideally many decades; (b) be capable of being adapted to changing conditions; and (c) provide the mechanisms to encourage both wise resource use and collaborative behaviors. Much of the challenge lies in achieving changes in the behavior of those who may be unaware of the benefits of sustainable aquaculture.

Sustainable aquaculture integrates the best available science with a transparent, equitable, and democratic approach to planning and decision-making. This ecosystem approach to management needs to be carried out in a strategic manner that tailors principles of good practice to the culture and the needs of a specific place. Successful, sustainable aquaculture operations advance through linked cycles of planning, implementation, and reassessment. These features of ecosystem management signal the transition from traditional sector-by-sector planning and decision-making to a more holistic approach based on the interactions between sectors and within and among ecosystems.

Aquaculture that is constructed upon principles that encourages high-energy consumption and the profligate use of natural resources must give way to new locally derived values and new forms of practice. As suggested by Daly [18], qualitative development rather than quantitative growth is the path of future progress. If such ideas are to be made operational at the scale of an aquaculture operation, a trajectory can be established based on goals for profit as well as social and environmental benefit. Once the goals of an aquaculture program or project have been defined as expressions of the ecosystem approach, much of the day-to-day work is concerned with the well-known best practices of aquaculture management.

For example, there has been much debate about the impacts of shrimp pond mariculture on mangrove forests through the Topics. Mangrove ecosystems provide essential goods and services to humanity, harboring an extraordinarily large biodiversity for the small

Aquaculture, Sustainability Science in. Table 1 Important sustainability science tools used to assess and communicate the sustainability of aquaculture[1]

Social Sustainability	Environmental Sustainability	Economic Sustainability
Stakeholder analysis: analysis of attitudes of stakeholders at the initiation of and throughout a project. Allows tracking of how stakeholders change attitudes over time with educational processes [46–50]	**Life cycle analysis**: complete assessment of products from raw material production, manufacture, distribution, use and disposal, including all transportation; used to optimize environmental performance of a single product or a company. A similar analysis called a MET (Materials, Energy, and Toxicity) Matrix is also used [53–55]	**Cost-benefit analysis**: analysis of cost effectiveness of different uses to determine if benefits can outweigh costs [59]
ISO 26000 guidelines for corporate social responsibility [51]	**ISO 14000 certification:** norms to promote more effective and efficient environmental management and provide tools for gathering, interpreting, and communicating environmental information [56]	
ICLEI (International Council for Local Environmental Initiatives) provides software and tools to help local governments achieve sustainability goals [52]	**Environmental impact assessment**: the process of identifying, predicting, evaluating, mitigating biophysical, social, and other effects of development proposals prior to policy decisions [57, 58]	**Triple bottom line or "full cost" accounting:** costs considered for all environmental, economic, and social impacts; costs measured in terms of opportunity costs (the value of their best alternative use); guiding principle is to list all parties affected and place a monetary value on effects on welfare as valued by them [60, 61]
	Environmental indicators: the use of quantitative indicators of resource use, efficiency, and waste production in aquaculture [17]	

[1]This table does not contain a comprehensive list of all available tools; rather, tools selected here were chosen since they appear regularly in the modern sustainable aquaculture research, industry, and management literature. Gibson et al. [62] give a most complete analysis of all of the available tools for sustainability assessments.

areas of the planet that these systems occupy, and provide a sustainable source of timber and charcoal to coastal communities while protecting fragile coastlines from erosion and storms. Establishment of proper scientific baselines to measure the true impacts of mariculture on coastal ecosystems is essential. Pullin [19] cautions that, "Analysis on depletion of mangrove cover in Asia point towards the fact that shrimp ponds have recently been and/or now being constructed either on former mangrove areas that were cleared long ago and considered degraded), or on more recently cleared areas for which the primary purpose of clearance was timber abstraction (logging, wood chip industries or charcoal production) or by adopting traditional trapping ponds. . . . Aquaculturists in Asia are therefore more often than not the end users of already degraded or destroyed mangroves rather an the primary culprits of mangrove destruction."

Good examples globally of an ecosystem approach to aquaculture at the watershed/aquaculture zone scale are found in both Israel and Australia. Both nations face severe land, water, and energy constraints. In Israel, highly efficient, landscape-sized integrations of reservoirs with aquaculture and agriculture have been developed [20, 21], as well as highly productive, land-based aquaculture ecosystems for marine species [22]. These aquaculture ecosystems are productive, semi-intensive enterprises that are water and land efficient, and are net

Aquaculture, Sustainability Science in. Table 2 Sustainability science assessments of aquaculture includes an assessment of governance systems, which examine the three processes of governance: the marketplace, the government, and civil society

Major Expressions of Governance
Government
Laws and regulations
Taxation and spending policies
Education and outreach
Marketplace
Profit seeking
Ecosystem service valuation
Cost-benefit analysis
Ecolabeling and Green Products
Civil Society: Organizations and Institutions
Product choices
Advocacy and lobbying
Vote casting
Comanagement
Stewardship activities

energy and material gains to society. Aquaculture ecosystems are organized following well-established ecological principles similar to the fields of agroecology and agroecosystems [23].

In Australia, an Ecologically Sustainable Development (ESD) framework approach to aquaculture development was used [24]. This ESD framework identified important issues, developed comprehensive reports for each issue, and then prioritized each using risk assessments. The ESD process employed extensive community consultation that considered social and environmental values of all other marine users, and users' management plans for operations and administration as well as environmental administrative attributes, then proposed development and monitoring plans.

As a result of this ESD approach, nine marine aquaculture zones of 2,400 ha in Port Phillip Bay and Westernport, Victoria, Australia were permitted. The Australian ESD approach combined analytical and participatory methods and developed sustainability plans that considered both ecosystem and human well-being, then developed implementation strategies by designing and enhancing effective governance systems for the expansion of aquaculture.

The development of a sustainability baseline should be the responsibility of a lead aquaculture agency. Its full implementation may require alternative methods of governance and employ innovative management approaches. There may be a need to facilitate an operational definition of aquaculture ecosystem boundaries for assessment, or area of focus, to set geographical limits to assess parameters such as carrying capacity or water management needs, and to understand the governance regime within which the area of focus is nested in order to understand and clarify such things as administrative and legal jurisdictions.

Using such guidance and sustainability science frameworks, the possibilities for designing productive aquaculture ecosystems that better fit into the local social and ecological context are many, since aquaculture can encompass the wide availability of species, environments, and cultures.

Improved Governance of Aquaculture Ecosystems

To be effective, ecosystem-based aquaculture initiatives must (1) be sustainable over long periods of time – ideally over many decades, (2) be capable of being adaptable to changing conditions, and (3) provide the mechanisms to encourage or require specified forms of resource use and collaborative behaviors among institutions and user groups that are stakeholders of the aquaculture system. Much of the challenge lies in both understanding and achieving changes in the behavior of the stakeholder groups and institutions associated with the aquaculture production systems. Ecosystem-based aquaculture integrates the best available science with a transparent, equitable, and democratic approach to planning and decision-making. Management needs to be carried out in a strategic manner that tailors principles of good aquaculture practice to the culture and the needs of a specific place. Successful aquaculture programs advance and change through linked cycles of planning, implementation, and reassessment. These features of ecosystem management signal the transition

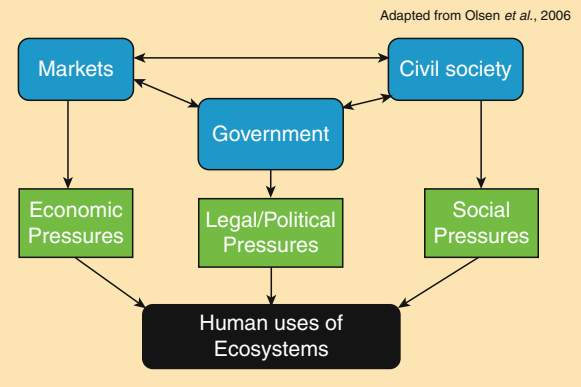
Aquaculture, Sustainability Science in. Table 3 Orders of governance outcomes [12, 13] applied to an ecosystem approach to aquaculture [2, 3]

Orders	Explanations	Indicators
First Order	Government at the national level commits to a plan of action designed to adopt an ecosystem approach to aquaculture (EAA) by issuing a formalized commitment to an EAA, thereby putting in place the "enabling conditions"	New laws, programs, and procedures are initiated that provide the legal, administrative, and management mechanisms to achieve the desired changes in behavior by:
		(i) Building constituencies that actively support EAA with the user groups that will be most affected; with government institutions involved; and with the general public
		(ii) Developing a formal government mandate for an EAA with the authority necessary to implement actions in the form of laws, decrees, or other high-level administrative decisions that create an EAA as a permanent feature of the governance structure of aquaculture; creation of commissions, working groups, user organizations, and nongovernmental organizations (NGOs) dedicated to the advancement of an EAA agenda; designating EAA zones
		(iii) Devoting resources, especially sustained annual funding, adequate to implement an EAA
		(iv) Developing an implementation plan of action for an EAA that is constructed around unambiguous goals
		(v) Creating the institutional capacity necessary to implement the new EAA plan of action
Second Order	Evidence of successful implementation of an EAA	1. Changes in the behavior of institutions and interest groups have occurred such as collaborative planning and decision-making through creation of task forces, commissions, civic associations, etc.
		2. Successful application of conflict mediation activities
		3. Evidence of functional changes such as establishment of new public–private partnerships, new collaborative actions undertaken by user groups, implementation of new school curricula that incorporates an EAA
		4. Changes in behaviors directly affecting ecosystem goods and services, such as the elimination of socially and environmentally destructive aquaculture practices
		5. Investments in infrastructure supportive of EAA policies and plans
Third Order	Evidence of sustained achievements in institutional and behavioral change due to an EAA as seen in the environment and indicators for the quality of life, incomes, or engagement in alternative livelihoods that have improved target communities	1. Improvements in ecosystem qualities, such as sustained conservation of desired ecosystems and habitats, halting or slowing undesired trends such as nutrient releases, feed wastage, diseases, damaged benthic ecosystems, etc.
		2. Improvements in society as evidenced by monitoring of social indicators such as increases in indices of quality of life, reduced poverty, greater life expectancy, better employment opportunities, greater equity in access to coastal resources, and the distribution of benefits from their use, greater order, transparency, and accountability in how planning and aquaculture development decision-making processes occur, greater food security, or greater confidence in the future

from traditional food production sector planning and decision-making to a holistic approach based on the interactions between sectors and within and among ecosystems.

FAO Fisheries and Aquaculture Department [25] found that one of the key trends toward more sustainable forms of aquaculture development and management is enhanced regulation and better governance. Governance is defined as the formal and informal arrangements, institutions, and mores that structure and influence how resources or an environment are utilized, how problems and opportunities are evaluated and analyzed, what behavior is deemed acceptable or forbidden, and what rules and sanctions are applied to affect how natural resources are distributed and used.

As shown in Table 2, there are three mechanisms by which the processes of governance are expressed: the marketplace, the government, and the institutions and arrangements of civil society [11]. These mechanisms interact with one another through complex and dynamic interrelationships that are examined and contrasted and documented in a baseline. Each of the three governance mechanisms influence and can alter patterns of behavior through measures such as those identified in Fig. 2. For sustainable, ecosystems-based aquaculture, it is important to distinguish between management and governance. Management is the process by which human and material resources are harnessed to achieve a known goal within a known institutional structure. Aquaculture business management, park management, personnel management, or disaster management is therefore spoken about. In these instances, the goals and the mechanisms of administration are well known and widely accepted. Governance, in contrast, addresses the values, policies, laws, and institutions by which a set of issues are addressed. It probes the fundamental goals and the institutional processes and structures that are the basis for planning and decision-making. Governance sets the stage within which management occurs [12].

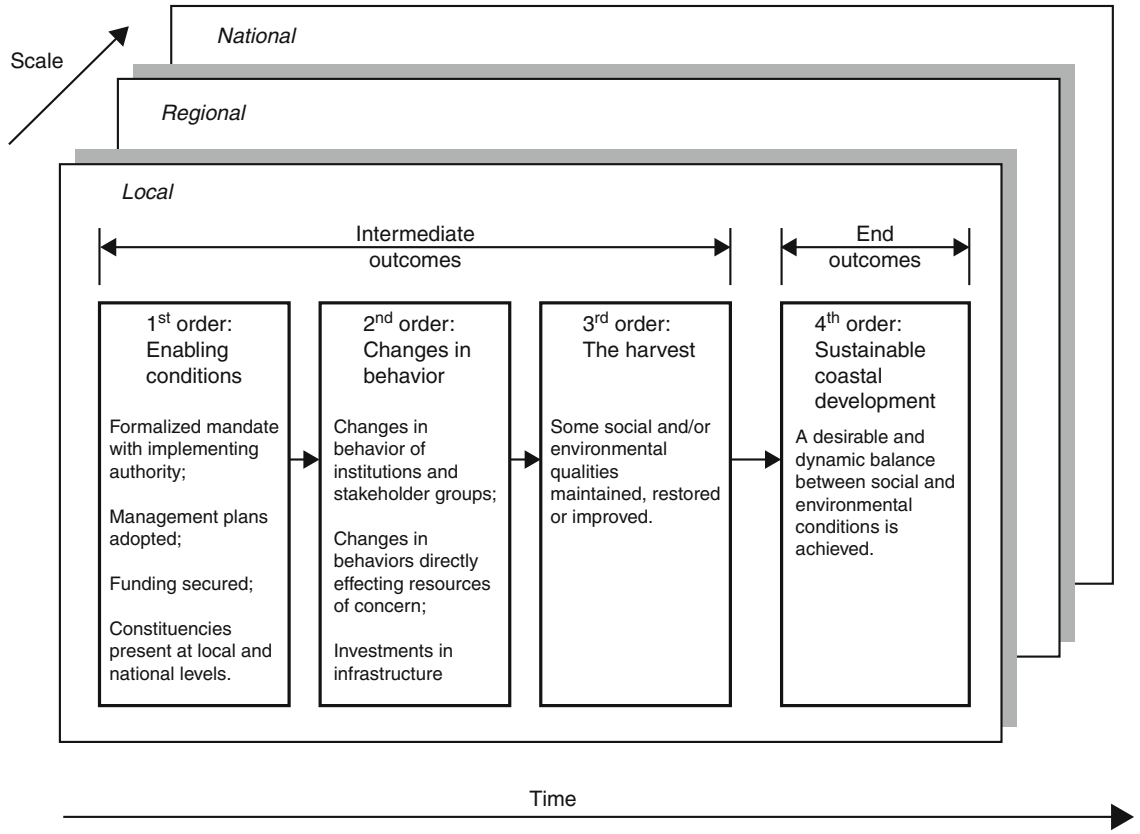


Aquaculture, Sustainability Science in. Figure 2
The three mechanisms by which the processes of governance are expressed interact with one another through complex and dynamic interrelationships that are vital parts of sustainability science assessments of aquaculture as each alter behaviors and decision-making that determine human uses of ecosystems [11–13]

The future of sustainable aquaculture is highly dependent on understanding the response by all three expressions of governance: markets, civil society, and government. For example, Kenya has fostered a participatory policy formulation for aquaculture, providing a legal and investment framework through government, establishing public–private partnerships to engage markets, providing basic infrastructure support, promoting self-regulation, providing a research platform for civil society to be engaged, undertaking zoning for aquaculture, and providing monitoring and evaluation support [25].

Adaptation of sustainability frameworks used to evaluate the needs and progress of governance on coastal management plans are essential to evaluate progress toward an ecosystem approach to aquaculture and build in adaptive learning and action into the strategic planning process. Governance frameworks recognize not only the importance of changes in practices such as changes over time in aquaculture farming ecosystems, but also recognize that for each change, there are correlated changes in the behavior of key partners and stakeholders within the sphere of influence of the management activity, and that these changes can be measured at local, regional, and national levels (Fig. 3, Table 3).

Sectoral agencies responsible for managing activities impacting aquatic ecosystems (e.g., capture fisheries, coastal zone development, watershed management organizations, agriculture, forestry, and industrial developments) will have to develop new ways of interacting to regularly communicate, cooperate, and



Aquaculture, Sustainability Science in. Figure 3
The four orders of coastal governance outcomes. This framework is used to develop governance baselines in environmental programs [12, 13]. An example of how progress toward better governance for sustainable aquaculture is shown in Table 3

collaborate. The need for innovative governance to implement an ecosystem-based approach to aquaculture can be seen as an obstacle but can also be seen as an opportunity to increase the social benefits that are likely to develop through synergies among food production sectors.

Social Ecology of Aquaculture

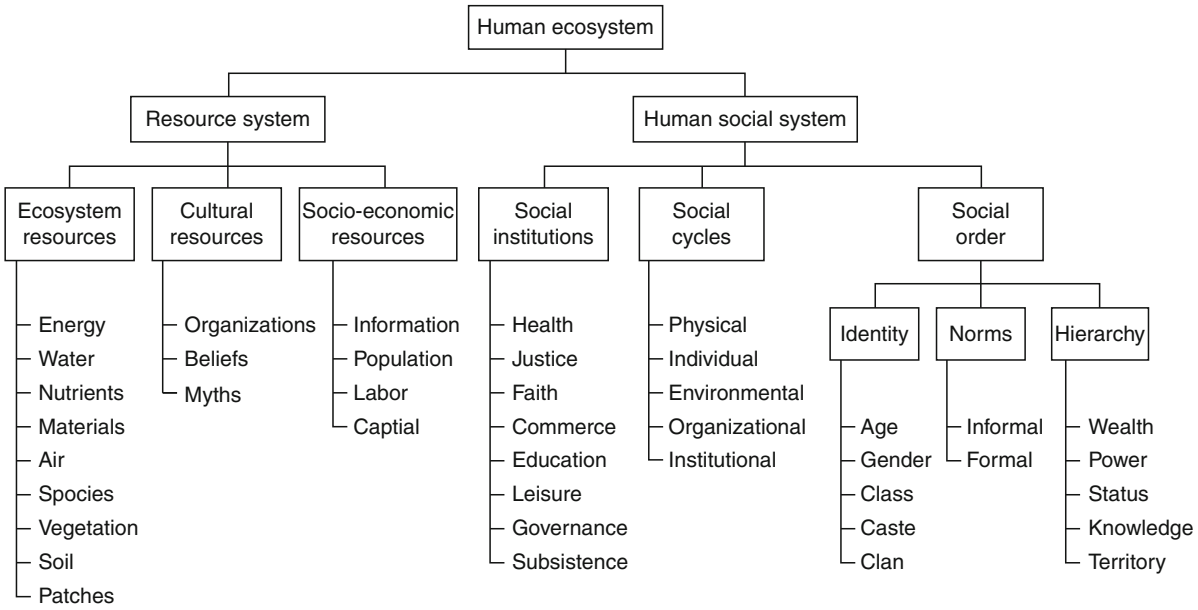
While there is much information on the natural ecology of food-producing ecosystems, there are few comprehensive frameworks for capturing the necessary social ecology of aquaculture.

Cadenasso et al. [26] have developed a "Human Ecosystem Framework" that could contribute to a baseline approach and assist in organizing multidisciplinary, social–ecological approaches to aquaculture development (Fig. 4). The most sustainable growth trajectories for aquaculture are to move toward more sustainable, social–ecological approaches to development; to shift patterns of production and consumption patterns from global to bioregional and local foods production and job creation; and to develop the indigenous human and institutional capacities that clearly demonstrate to society that "aquaculture is culture."

Future Directions: Sustainability Science Opportunities for Aquaculture

There are at least four major opportunities for sustainability science in the field of aquaculture in the

1. Determination of "sustainable aquaculture" for retail seafood companies
2. Growing fields of marine ecosystem and habitat restoration, conservation biology, and ecology



Aquaculture, Sustainability Science in. Figure 4
The Human Ecosystem Framework [26]. Assessment of new interventions such as aquaculture into societies requires knowledge of not only biophysical and natural resource systems but also social resources and human social systems

3. Accelerated use of agricultural meals and oils, and
4. Development of sustainable aquaculture for the poor

Determination of Sustainable Aquaculture for Retailers

Sustainability science approaches to aquaculture can be used to better plan and develop aquaculture production networks for multiple species. Such planning approaches can be used to plan for the creation of highly diversified, segmented aquaculture networks, for maximal job creation at every unit step from "farm to plate" (e.g., seafood value chain planning), by creating numerous interconnections supplying inputs and outputs using local resources and recycled wastes and materials and expertise, and to close "leaky" loops of energy and materials that can potentially degrade natural ecosystems.

Behavioral changes will be required by industry. Social investments, strategic incentives/subsidies, and innovative market mechanisms can help facilitate change in behaviors. Self-regulation by the aquaculture industry has led to codes of practice and better management practices.

Sustainability assessments are predicated upon the fact that the modern aquaculture industry desires to be seen as a responsible steward. This means going beyond "meeting the regulations." There are a cacophony of certification bodies and seafood watch cards – there are an estimated 200 sustainable seafood guides available internationally – which has created a far too complex and sometimes conflicting recommendations to both consumers and retailers on what is "sustainable seafood" [27, 28]. Roheim [27] states that "Shrimp, in some form, appears as a green, yellow, red, and non-consensus list item" in the seafood "watch cards."

The logic behind consumer approaches is that informed consumers who care about sustainable seafood will demand aquaculture products that carry a label or fit into the "green" (buy) area of a watch card, as opposed to those products that do not have the label, sending a market signal back to aquaculture industries that only products from sustainable aquaculture farms are preferred. Many of the independent certification programs that have developed ecolabels and "seafood watch cards" to provide consumers with additional information come from nongovernmental organizations (NGOs) with specific advocacy agendas

and not from neutral, scientific sources, or from regulatory bodies charged with protecting the environment and society. For example, many fisheries and aquaculture scientists are deeply concerned that consumer recommendations of NGOs are moving demand (and use) from farmed stocks to already overburdened wild fisheries. The Monterey Bay Aquarium's (MBA) Seafood Watch Program has produced millions of folding wallet cards featuring a "stop light" system of green (sustainable), yellow (chose with caution), and red (do not choose) recommendations. Farmed shrimp and salmon, two of the world's largest aquaculture industries are on the MBA red list. Roheim [27] mentions that the Compass Group a major food service company has used the MBA cards to decrease purchases of farmed shrimp and salmon, which, in effect, has created additional fishing pressure on wild shrimp and salmon stocks.

Most organizations believe that consumers' increasing awareness of environmental and food safety issues will lead them to accept a wide variety of standards and labels, most of which are specifically intended to allay consumers' concerns about negative environmental consequences.

However, Roheim [27–29] points out concerns over ecolabeling, especially the lack of transparency and opportunity for participation in the development of standards, and concerns of developing countries that ecolabeling schemes are an attempt at disguised protection of domestic industries to restrict market access and erode competitiveness. In addition, Wessels et al. [30] found that successful ecolabeling programs must accelerate consumer education programs so that consumers become more aware of differences in species, geographic regions, and certifying agencies.

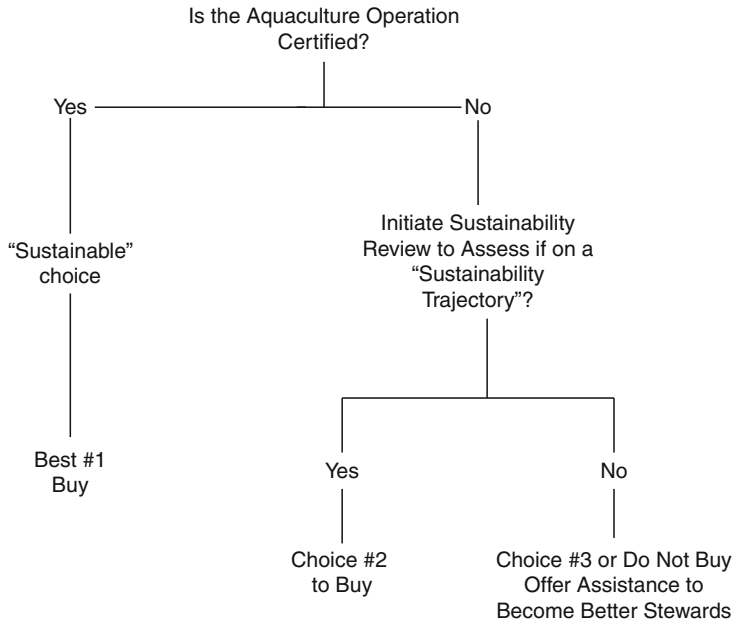
Roheim [29] states that ecolabels require traceability. Traceability is the ability to follow the movement of a food through specified stages of production, processing, and distribution. Essentially, it is a record-keeping system that identifies and tracks products, transportation of products, and ingredients of products from origin to consumption, while providing the ability to quickly trace back products at any point along the supply chain. It is necessary for food safety purposes, in order to track backward in the food chain the source of food that made consumers ill, so that products could be removed from store shelves.

For consumers, Roheim [27] argues the need for ecolabels determined at the larger international levels, such as the Marine Stewardship Council (MSC), so that consumers "do not need to inquire about catch area or gear types, but only need to look for the label." The plethora of efforts has also confused and perplexed retailers who are the main "drivers" of certification at present, not consumers [27–29]. However, even though many buyers wish to purchase sustainable seafood, most seafood products are not certified, and they are very confused by the many NGO efforts. A purchasing policy determined by assessing which seafoods are "sustainable" by making an assessment of the plethora of NGOs, "opinions" seems confusing, risky, and costly. Rather, a simple, buying protocol (Fig. 5) that incorporates a sustainability assessment (where needed) as discussed here is recommended.

Aquaculture and the Restoration of Ecosystems

Aquaculture science can be viewed as a "toolbox" with great potential for restoring aquatic ecosystems. There is an unbalanced focus on marine animal husbandry (e.g., "fed" aquaculture) causing a lack of appreciation for the positive environmental attributes of nonfood aquaculture such as marine agronomy, endangered species aquaculture, and aquaculture for environmental enhancement and rehabilitation, all of which use modern marine hatchery and nursery aquaculture practices [31].

Aquaculture technologies (hatchery, nursery, grow-out) for marine plants are used for the restoration of mangroves, sea grasses, and coastal wetland plants such as *Spartina sp.* In addition, live rock and coral aquaculture facilities are active for not only the aquarium trade, but also for the environmental restoration of coral reefs (liveaquaria.com). In this regard, there is little difference between sustainable aquaculture and the emerging fields of ecological engineering and industrial ecology. Indeed, tidal wetland, mangrove forest, coral, and sea grass restoration aquaculture – in addition to establishment and maintenance of oyster reefs – are important examples of aquaculture creating, enhancing, and maintaining productive marine ecosystems and habitats, and improving water quality.



Aquaculture, Sustainability Science in. Figure 5
A simple decision tree for determinations of the sustainability of aquaculture products by retailers

Aquaculture and Agriculture Science

Here, science questions as to whether aquaculture contributes to the depletion of world fisheries. Fed aquaculture depends on both wild and farmed fish stocks and on intact aquatic habitats and excellent water quality, plus a growing quantity of agricultural resources. There is much ongoing policy, research, and management concerns on the interactions of marine food fish fisheries ("biomass fisheries") with aquaculture and human welfare. There is much less planning and research regarding the future impacts of fed aquaculture on agriculture.

Agricultural meals and oils as alternatives to marine sources are developing rapidly. Current projections forecast that fed aquaculture may use 50% or less of the world's fish meal [32], which would mean a large expansion of use of agricultural and other terrestrial sources of feed proteins and oils. Terrestrial proteins and oils from soybeans, sunflowers, and lupins are available at volumes larger than the available global quantities of fish meal. Soybeans have high protein content of ~28%, peas have ~22%, and these have good amino acid profiles. Other abundant agricultural cereals have lower protein contents of ~12–15%. Processing can create protein concentrates with protein levels of >50% [33]. Vegetable oils have very low EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) levels. However, substitution of plant oils upward of 50% of added dietary oil has not resulted in growth reductions or increased mortalities in fish such as salmon and trout.

If agricultural sources of meals and oils are the future of fed aquaculture, there will be a need for a new sustainability planning and science on the impacts of fed aquaculture as a driver of agriculture production, especially so for soybeans. Increased aquaculture consumption of the world's grains and oils raises the concern over the spread of unsustainable agriculture practices. Brazil has been targeted as one of the world's major soybean suppliers. Costa et al. [34] have demonstrated that soybean farms are causing reduced rainfall in the Amazonian rainforest. About one seventh of the Brazilian rainforest has been cut for agriculture, about 15% of which is soybeans. Soybeans, which are light in color, reflect more solar radiation, heating the surface of the land less and reducing the amount of warm air convected from the ground. Fewer clouds form as a result, and less precipitation falls. In soybean

areas, there was a 16% less rainfall compared to a 4% decrease in rainfall in land areas cleared for pasture.

Aquaculture for the Poor

Approximately 1.3 billion people live on less than a dollar a day, and half of the world's population lives on less than 2 dollars a day. FAO has stated that the world will need to produce 70% more food for an additional 2.3 billion people by 2050 [4]. Scarce natural resources will need to be used more efficiently, and there will be a need for proper socioeconomic frameworks to address imbalances and inequities to ensure that everyone in the world has access to the food they need. Food production will have to be carried out in a way that reduces poverty and takes account of natural resource limitations [4].

The world's population will rise from 6.8 to 9.1 billion in 2050, with nearly all population growth occurring in the economically developing countries. Without additional global food strategies, an estimated 370 million people will be hungry in 2050. The magnitude of the problem is most acute in Africa. In 10 African countries of an estimated 316 million persons where aquatic proteins are an important dietary component, 216 million live on US$2/day, 88 million are undernourished, and 16 million children under 5 are malnourished [35].

Small-scale coastal and inland freshwater fisheries provide more than 90% of the fish consumed in Africa. Over 2.5 million people are involved in fishing and 7.5 million in trading, marketing, and processing. The most important fisheries/aquaculture ecosystems are located on the coasts of west and southern Africa and the river basins of Senegal, Niger, Volta, Congo, Lake Chad, Nile, and Zambezi Rivers. But today, aquaculture provides less than 5% of Africa's fish, with most concentrated in Egypt and Nigeria [35].

Aquaculture is a global enterprise with local roots. There are strong concerns that aquaculture is evolving away from its global responsibility to provide net benefits (additional foods) for a protein-hungry planet [36–38]. Greater than 75% of global fisheries are traded. In 2000, more than 60% of fish meal was traded. Only 7% of meat is traded, 17% of wheat, and 5% of rice. To tackle this huge challenge, the FAO ecosystems approach to aquaculture [39] has created a new code for responsible global aquaculture development, and has combined this into one common development framework for a global implementation strategy for aquaculture that can be used to measure the trajectory of social responsibility for global aquaculture.

If aquaculture is designed, implemented, and evaluated as aquaculture ecosystems, a new social contract would have a close relationship between aquaculture professionals who not only develop an alternative model of aquaculture development but also interact closely with capture fisheries and agriculture but help deliver to the world's poor its needs for nutrient dense, protein-rich seafoods. Components of a global strategy could be to:

1. **Allocate more food fish and oils for poverty alleviation and human needs worldwide, and allocate less marine resources for feed fish for fed aquaculture so as to: (a) increase the ecosystem resilience of the Humboldt ecosystem, and (b) relieve the increasing overdependence of aquaculture countries such as Thailand (shrimp) and Norway (salmon) on this southeastern Pacific Ocean marine ecosystem.**

   Alder et al. [37] estimated that about 36% of the world's fisheries catch (30 million tons) are processed into fish meal and oil, mostly to feed farmed fish, chickens, and pigs. Daniel Pauly of the University of British Columbia has stated that "Globally, pigs and chickens alone consume six times the amount of seafood as US consumers and twice that of Japan." Jacquet et al. [28] reported that Peru exports about half of the world's fish meal from its catch of 5–10 MMT/year of anchovies while half of its population of 15 million live in poverty and 25% of its infants are malnourished. A campaign launched in 2006 combining scientists, chefs, and politicians to demonstrate that anchovies are more valuable to the Peruvian people and its economy as direct foods has resulted in a 46% increase in demand fresh and 85% increase in canned anchovies. One ton of fillets has sold for five times the price of 1 t of meal and requires half the fish (3 t for 1 t fillets vs. 6 t for 1 t meal). Peru has decided to dedicate 30% of its annual food security budget (approx. US$ 80 million) for programs to supply anchovies to its people. Higher prices for

fish used as direct human foods for food security will limit processing of fish to meals for terrestrial animal and aquaculture feeds, thereby decreasing the supply of fish meals and oils for global aquaculture trade and development, but meeting the Millennium Development Goals of eliminating everywhere extreme hunger and starvation.

2. **Accelerate research into the elucidating functional feed ingredients in fish diets that are showing the potential to eliminate the needs for fish meal and oils in aquaculture.**

   Skretting Aquaculture Research Centre [40] reported a research on "functional ingredients" that are contained in fish meals and oils, which contribute to efficient feed conversions and high growth rates, fish health, and welfare. Initial research focused on beta-glucans that not only stimulate the immune system of fish and protect against the effects of bacterial furunculosis but also allow reductions in fish meal contents in diets to 25%. Additional research with phospholipids in meals, triglycerides in fish oil, and antioxidants in 2008 have resulted in excellent fish performances from feeds with almost no marine fish meal and oil. Current research is exploring the extraction of functional ingredients from other non-marine by-products.

3. **Develop alternative ecological aquaculture models that accelerate the movement toward use of agricultural, algal, bacterial, yeasts meals, and oils.**

   Aquaculture uses most of the world's fish meal (68%) and fish oil (88%); however, Tacon and Metian [32] predict that fish meal and oil use in aquaculture will decrease to become high priced, specialty feed ingredients. Currently, about 40% of aquaculture depends on formulated feeds: 100% of salmon, 83% of shrimp, and 38% of carp. As stated previously, research on the use of agricultural meals and oils to replace use of ocean resources especially on the functional components of fish meals/oils needed for fish nutrition is a major subject of aquaculture research and development [41, 42]. Turchini et al. [43] reported that for all of the major aquaculture fish species that 60–75% of dietary fish oil can be substituted with alternative lipid sources without significantly affecting growth performance, feed efficiency, and feed intake. Naing et al. [44] found that palm oil could replace fish oil in rainbow trout diets, and reduce the dioxin contents in fish.

4. **Develop new governance systems that integrate aquaculture, agriculture, and fisheries using ecosystem-based management approaches, which combine production, distribution, and consumption networks that do not institutionalize poverty and hunger, but provide new alternative tools and education in multisectoral ecosystem approaches.**

   The massive environmental change being brought about by the accelerated growth of the world's population has caused profound change to the world's ecosystems. Crutzen and Stoermer [45] have called this new era the "Anthropocene." In this era, massive quantities of additional foodstuffs will be needed to sustain humanity; nutrient-dense, high-quality aquatic proteins will be especially important. The tools and training for creating the next generation of transdisciplinary, sustainability scientists will need to be further developed and well utilized; otherwise, it will result in serious consequences for the Earth's living systems.

Bibliography

Primary Literature

1. Ostrom E (1986) An agenda for the study of institutions. Public Choice 48(1):3–25
2. Costa-Pierce B (2002) Ecological aquaculture. Blackwell, Oxford
3. Costa-Pierce B (2008) An ecosystem approach to marine aquaculture: a global review. In: Soto D (ed) Building an ecosystem approach to aquaculture, pp 81–116. FAO Fisheries and Aquaculture Proceedings 14, FAO, Rome, 221p
4. FAO (Food and Agriculture Organization) (2009) The state of world fisheries and aquaculture 2008. Fisheries Department, Food and Agriculture Organization of the United Nations, Rome
5. FAO (1995) Code of conduct for responsible fisheries. FAO, Rome, 41 pp. Available at ftp://ftp.fao.org/docrep/fao/003/W4493e/W4493e00.pdf
6. Moore B III, Underdal A, Lemke P, Loreau M (2002) The Amsterdam declaration on global change. In: Steffen W, Jäger J, Carson DJ, Bradshaw C (eds) Challenges of a changing earth. Springer, Heidelberg
7. Gliessman S (1998) Agroecology: ecological processes in sustainable agriculture. Ann Arbor Press, Ann Arbor
8. Altieri M (2002) Agroecology: the science of natural resource management for poor farmers in marginal environments. Agr Ecosyst Environ 93:1–24

9. Kates R, Clark W, Corell R (2001) Sustainability science. Science 292:641–642
10. Wurts W (2000) Sustainable aquaculture in the twenty-first century. Rev Fish Sci 8:141–150
11. Olsen S, Sutinen J, Juda L, Hennessey T, Grigalunas T (2006) A handbook on governance and socioeconomics of large marine ecosystems. Coastal Resources Center, University of Rhode Island, Narragansett
12. Olsen S (2003) Frameworks and indicators for assessing progress in integrated coastal management initiatives. Ocean Coast Manage 46(3–4):347–361
13. Olsen S, Page G, Ochoa E (2009) The analysis of governance responses to ecosystem change: a handbook for assembling a baseline. LOICZ Reports & Studies No. 34. GKSS Research Center, Geesthacht, Germany
14. GESAMP (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP Joint Group of Experts on Scientific Aspects of Marine Environmental Protection) (2008) Assessment and communication of environmental risks in coastal aquaculture. FAO, Reports and Studies GESAMP No. 76, Rome, 198 pp
15. Tucker C, Hargreaves J (2008) Environmental best management practices for aquaculture. Wiley-Backwell, Ames, IA
16. National Research Council (2010) Ecosystem concepts for sustainable bivalve mariculture. National Academies Press, Research Council, Washington, DC
17. Boyd C, Tucker C, McNevin A, Bostick K, Clay J (2007) Indicators of resource use efficiency and environmental performance in fish and crustacean aquaculture. Rev Fish Sci 15:327–360
18. Daly HE (1996) Beyond growth: the economics of sustainable development. Beacon, Boston
19. Pullin R (1993) Discussion and recommendations on aquaculture and the environment in developing countries. In: Pullin R, Rosenthal H, Maclean J (eds) Environment and aquaculture in developing countries. ICLARM Conference Proceedings 31, pp 312–338
20. Hepher B (1985) Aquaculture intensification under land and water limitations. GeoJournal 10:253–259
21. Mires D (2009) Development of inland aquaculture in arid climates: water utilization strategies applied in Israel. Fisheries Manag Ecol 7:189–195
22. Neori A, Shpigel M, Ben-Ezra D (2000) A sustainable integrated system for culture of fish, seaweed and abalone. Aquaculture 186:279–291
23. Pimentel D, Pimentel M (2003) Sustainability of meat-based and plant-based diets and the environment. Am J Clin Nut 78(3):660–663
24. Fletcher W, Chesson J, Fisher M, Sainsbury K, Hundloe T (2004) National ESD reporting framework: the 'how to' guide for aquaculture. FRDC Project 2000/145.1, Canberra, 88 pp
25. FAO Fisheries and Aquaculture Department (2006) State of the world's fisheries and aquaculture. FAO Italy, Rome, 162 p. Available at http://www.fao.org/docrep/009/A0699e/A0699E00.htm#Contents
26. Cadenasso M, Pickett S, Grove M (2006) Integrative approaches to investigating human-natural systems: the Baltimore ecosystem study. Nat Sci Soc 14:4–14
27. Roheim C (2009) An evaluation of sustainable seafood guides: implications for environmental groups and the seafood industry. Mar Resour Econ 24:301–310
28. Jacquet J, Hocevar J, Lai S, Majluf P, Pelletier N, Pitcher T, Sala S, Sumaila R, Pauly D (2009) Conserving wild fish in a sea of market-based efforts. Oryx, The Int J Cons. doi:10.1017/S0030605309990470
29. Roheim C (2001) Product certification and ecolabelling for fisheries sustainability FAO Fisheries Technical Paper 422. FAO, Rome, Italy
30. Wessells C, Johnston R, Donath H (1999) Assessing consumer preferences for ecolabelled seafood: the influence of species, certifier, and household attributes. Am J Agr Econ 5: 1084–1089
31. Costa-Pierce B, Bridger C (2002) The role of marine aquaculture facilities as habitats and ecosystems. In: Stickney R, McVey J (eds) Responsible marine aquaculture. CABI, Wallingford, pp 105–144
32. Tacon A, Metian M (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 285:146–158
33. Bell J, Waagbo R (2002) Safe and nutritious aquaculture produce: benefits and risks of alternative sustainable aquafeeds. In: Holmer M, Black K, Duarte C, Marba N, Karqakasis I (eds) Aquaculture in the ecosystem. Springer, New York, pp 185–226
34. Costa M, Yanagi S, Souza P, Ribeiro A, Rocha E (2007) Climate change in Amazonia caused by soybean cropland expansion, as compared to caused by pastureland expansion. Geophys Res Lett 34(L07706). doi:10.1029/2007GL029271
35. Allison E, Beveridge M, van Brakel (2009) Climate change, small-scale fisheries and smallholder aquaculture. In: Culberg M (ed) Fish, trade and development. Royal Swedish Academy of Agriculture and Forestry, Stockholm, pp 73–87
36. Goldburg R, Naylor R (2005) Future seascapes, fishing, and fish farming. Front Ecol Environ 3(1):21–28
37. Alder J, Campbell B, Karpouzi V, Kaschner K, Pauly D (2008) Forage fish: from ecosystems to markets. Ann Rev Environ Res 33:71–714
38. Naylor R, Hardy R, Bureau D, Chiu A, Elliott M, Farrell A, Forster I, Gatlin D, Goldburg R, Hua K, Nichols P (2009) Feeding aquaculture in an era of finite resources. Proc Nat Acad Sci 106(36): 15103–15110
39. Soto D, 21 co-authors (2008) Applying an ecosystem-based approach to aquaculture: principles, scales and some management measures. In: Soto D, Aguilar-Manjarrez J, Hishamunda N (eds) Building an ecosystem approach to aquaculture. Rome: FAO Fisheries and Aquaculture Proceedings 14. FAO, Rome, 221 pp
40. Skretting Aquaculture Research Centre (2009) Revealing the essential functions of vital feed ingredients. Accessed at

http://www.skretting.com/Internet/SkrettingGlobal/webInternet.nsf/wPrId/D30B62DD93F65CFEC1257663004DA962!OpenDocument
41. Watanabe T (2002) Strategies for further development of aquatic feeds. Fish Sci 68:242–252
42. Opstvedt J, Aksnes A, Hope B, Pike I (2003) Efficiency of feed utilization in Atlantic salmon (Salmo salar L.) fed diets with increasing substitution of fish meal with vegetable proteins. Aquaculture 221:365–379
43. Turchini G, Bente E, Torstensen E, Ng W (2009) Fish oil replacement in finfish nutrition. Rev Aquacult 1(1):10–57
44. Naing A, Satoh S, Tsuchida N (2007) Effect of replacements of fishmeal and fish oil on growth and dioxin contents of rainbow trout. Fish Sci 73:750–759
45. Crutzen P, Stoermer E (2000) The "Anthropocene". Glob Change Newslett 41:17–18
46. Fletcher A (2003) Mapping stakeholder perceptions for a third sector organization. J Intell Cap 4(4):505–527
47. Savage G, Nix T, Whitehead B (1991) Strategies for assessing and managing orgnaizational stakeholders. Acad Manage Exec 5(2):61–75
48. Hemmati M, Dodds F, Enayti J, McHarry J (2002) Multistakeholder procesess on governance and sustainability. Earthscan, London
49. Dalton T (2005) Beyond biogeography: a framework for involving the public in planning of U.S. Marine Protected Areas. Conserv Biol 19:1392–1401
50. Dalton T (2006) Exploring participants' views of participatory coastal and marine resource management processes. Coast Manage 34:351–367
51. ISSD (International Institute for Sustainable Development) (2004) ISO social responsibility standardization. An outline of the issues. International Institute for Sustainable Development, Winnipeg, Manitoba. http://www.iisd.org/pdf/2004/standards_iso_srs.pdf
52. http://www.iclei.org
53. American Center for Life Cycle Assessment. http://www.lcacenter.org
54. Bartley D, Brugère C, Soto D, Gerber P, Harvey B (2007) Comparative assessment of the environmental costs of aquaculture and other food production sectors: methods for meaningful comparisons. FAO/WFT Expert Workshop, 24–28 April 2006, Vancouver, Canada. FAO Fisheries Proceedings. No. 10. FAO, Rome, 241p
55. Ayer N, Tyedmers P (2009) Assessing alternative aquaculture technologies: life cycle assessment of salmonid culture systems in Canada. J Cleaner Prod 17:362–373
56. International Organization for Standardization (ISO). http://www.iso.org/iso/iso_14000_essentials
57. Environmental Impact Assessment Review. http://www.elsevier.com/wps/find/journaldescription.cws_home/505718/description#description
58. IAIA (International Association for Impact Assessment) (1999) Principles of environmental impact assessment best practices. IAIA, Fargo, ND, USA. http://www.iaia.org/publicdocuments/special-publications/Principles%20of%20IA_web.pdf
59. US Department of Transportation. http://www.fhwa.dot.gov/planning/toolbox/costbenefit_forecasting.htm
60. Savitz A (2006) The triple bottom line: how today's best-run companies are achieving economic, social and environmental success – and how you can too. Jossey-Bass/Wiley, New York
61. McCandless M, Venema H, Barg S, Oborne B (2008) Full cost accounting for agriculture – final report. Valuing public benefits accruing from agricultural beneficial management practices: an impact pathway analysis for Tobacco Creek, Manitoba. International Institute for Sustainable Development, Winnipeg, Manitoba, Canada. http://www.iisd.org/pdf/2008/measure_fca_2008.pdf
62. Gibson R, Hassan S, Holtz S, Tansey J, Whitelaw G (2005) Sustainability assessment: criteria and processes. Earthscan, London

Books and Reviews

Pullin R (1993) Environment and aquaculture in developing countries. International Center for Living Aquatic Resources Management, Manila

Smil V (2000) Feeding the world: a challenge for the twenty-first century. MIT Press, Cambridge MA

Tacon A, Hasan MR, Subasinghe RP (2006) Use of fishery resources as feed inputs for aquaculture development: trends and policy implications. FAO Fisheries Circular No. 1018. Food and Agriculture Organization of the United Nations, Rome

Aquapod Systems for Sustainable Ocean Aquaculture

Stephen H. Page
Ocean Farm Technologies, Inc., Morrill, ME, USA

Article Outline

Glossary

Artisanal fisheries Traditional fisheries involving fishing households (as opposed to commercial companies), using relatively small amount of capital and energy, relatively small fishing vessels or canoes, often beach-based, making short fishing trips, close to shore, mainly for local consumption.

Biofouling The accumulation of living organisms on some surface by bacteria, fungi, protozoa, algae, and invertebrates.

Geodesic The shortest line between two points on a specific surface.

Open ocean aquaculture The culture of marine organisms in exposed ocean locations, not sheltered by islands or embayments.

Definition of the Subject

Open ocean aquaculture is not well defined by the industry, but in general refers to culture of marine fish, invertebrates, or algae in exposed ocean locations. Open ocean aquaculture is contrasted to near-shore marine aquaculture in that it occurs in areas removed spatially from land, typically by 1 km or more; deep water, generally deeper than 20 m; and exposure to wind, waves, and ocean currents without shelter from the mainland or islands. The subject of this entry is principally the design and engineering of containment systems suited for open ocean aquaculture.

Introduction

Near-shore finfish aquaculture worldwide is challenged and constrained by resource user competition, environmental carrying capacity of near-shore environments, and, in most cold-water regions of the world essentially a monoculture of Atlantic salmon. Expansion of marine aquaculture in the next 20 years will happen substantially in offshore, exposed open ocean areas, with a diversification of species.

The patented Aquapod™ is a unique containment system for marine aquaculture, suited for rough open ocean conditions and a diversity of species. The Aquapod is constructed of individual triangular net panels fastened together in a spheroid shape (Fig. 1). The majority of the panels are simply structural members and netting. Some individual panels or groups of panels have other functions, such as access, feeding, fish transfer and grading, harvest, mooring, and mortality recovery. Other individual panels may have pneumatically controlled flotation devices which allow an almost infinite orientation of the Aquapod in the water. The Aquapod functions as a total containment system for finfish while submerged or partially surfaced.

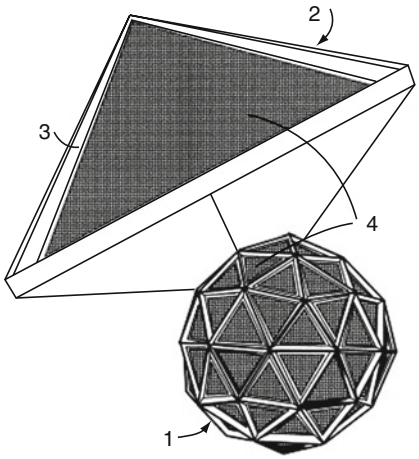
Elements of the design have benefits for reduction in labor for routine husbandry tasks, reduction in maintenance costs, and reduction of stress on aquatic animals during handling operations such as transfer, treatment, and harvest. The "exoskeleton" design of the Aquapod containment system also allows for internal structures to provide for the cultivation of flatfish species such as halibut and flounder.

Features of the Aquapod Containment System

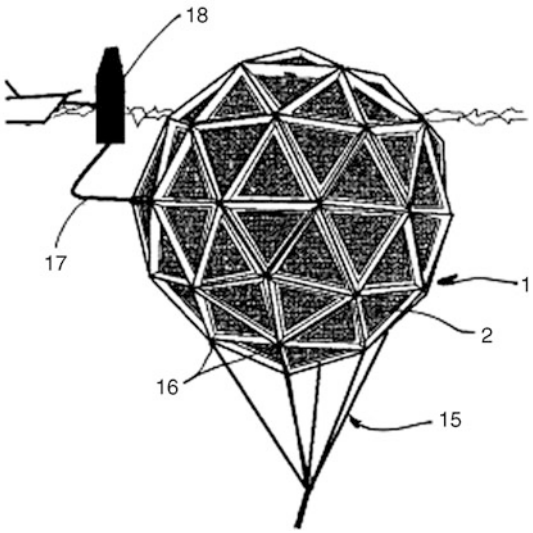
Submersibility: Submersion is the preferred, if not the only way to operate fish containment systems in the open ocean. Submergence is necessary to operate a fish pen below the destructive energy of surface waves. Submergence also allows fish to be kept at favorable temperatures below warm water thermoclines. The Aquapod can be operated partially surfaced (Fig. 2) or fully submerged (Fig. 3). Although there are other pen designs which are designed to operate submerged, the spherical Aquapod design is well suited for species such as Atlantic salmon, which require periodic access to substantial surface area in order to gulp air.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

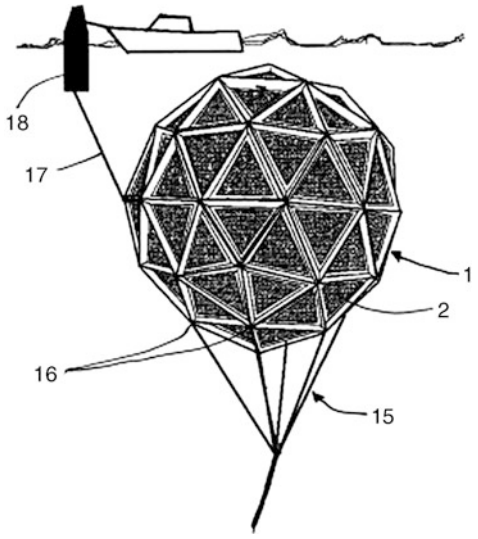
Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Aquapod Systems for Sustainable Ocean Aquaculture. Figure 1
The scale of contemporary net pens to date has become problematic especially in terms of net-handling activities, which are labor intensive, require significantly sized hydraulic equipment, and cannot be performed in rough ocean conditions. The modular nature of the Aquapod solves this problem by utilizing individual net panels which are interchangeable and scaled to be handled without difficulty in adverse conditions. These net panels are fastened together to form a secure enclosure, tailored to each customer's specific requirements



Aquapod Systems for Sustainable Ocean Aquaculture. Figure 2
The structure of the Aquapod net pen allows for many configurations of mooring attachment. Figure 2 shows an Aquapod net pen partially surfaced



Aquapod Systems for Sustainable Ocean Aquaculture. Figure 3
An Aquapod net pen submerged

Fixed volume: The Aquapod maintains its shape and volume in strong currents or undertow, reducing stress and physical damage to the fish contained within. Aquapod net pens can be constructed to any size to fit individual customer's needs, with a practical range of 8 m diameter (212 m^3) to 24 m diameter (7,000 m^3).

Containment: The structural exoskeleton of the Aquapod provides a high ratio of structural support to net area. Each triangular panel is comprised of an essentially rigid frame supporting tensioned netting. Frames can be constructed of marine grade aluminum, fiber-reinforced polymer, or even a combination of materials. The modular nature of the Aquapod containment system provides for easy inspection and inventory control as required by today's mandated containment management systems. Individual net panels are coded so they can be brought into a regular schedule of inspection, maintenance, repair, and replacement. While surfaced in suitable conditions, a single net panel or a group of net panels can be removed and replaced without compromising the integrity of the containment. No other system can offer this convenience.

Predator control and escapement: Tensioned netting has proven to be relatively predator proof in other engineered systems. The Aquapod goes one step further. Due to the modular structure and the relatively small triangular units, different types of wire mesh can be used in place of synthetic netting. This enhances predator control in areas where triggerfish, sharks, sea lions, and crocodiles have made fish farming impractical. Wire mesh also prevents escapement by chewing fish such as cod and sea bream, which readily chew through synthetic fiber netting.

Husbandry and fish health: Fish can be moved from one Aquapod containment to another without seining or pumping. Patent pending displacement technology is capable of transferring fish from one Aquapod containment pen to another without brailing or pumping, or from an Aquapod containment to a harvesting pump without seining or other stressful means of crowding the fish. While being transferred, fish can be graded and counted with automated technology. In competitive designs, these fish-handling operations are either impossible or very labor intensive and stressful on the fish.

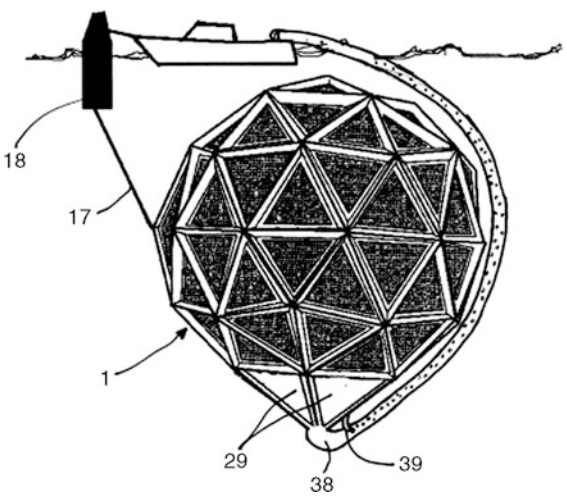
Feeding: One or more panels in each Aquapod containment is modified to receive and distribute hydraulically supplied feed from a centralized feed barge or service boat. The semirigid and modular exoskeleton of the Aquapod allows easy attachment of any number of feeding ports. Multiple feed outlets provide better distribution of feed to the fish.

Safety: Worker safety is a paramount concern when operating fish farms in open ocean conditions. Since conception, the Aquapod containment system has been designed to maximize automation of routine husbandry tasks and reduce the amount of time divers are needed in the water. Although any containment system will need some diving, the ability of the Aquapod to rotate within its mooring grid, bringing any segment of the pen to the surface or near to the surface, reduces the amount of diving, and when diving is needed, the depth of the dives will be greatly reduced. One of the most dangerous jobs on a fish farm is net changing, and the Aquapod eliminates this chore by providing a means to clean nets at the surface.

Waste management: The spherical design of the structure causes mortalities to collect at the bottom of the pen where they can be brought to the surface with a conventional airlift pump, eliminating the routine and hazardous task of mortality collection by divers (Fig. 4).

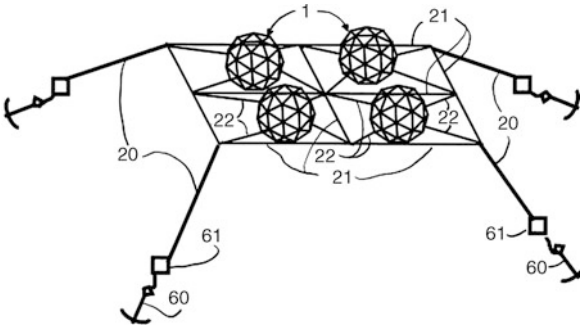
Mooring. There are many options for mooring an Aquapod net pen. Each hub of the frame is a potential mooring point, which allows enormous flexibility in mooring placement and the ability to distribute loads over a large area for safety. Any specific net panel can be strengthened to facilitate predicted maximum mooring stresses, whether the Aquapod is attached to a single-point mooring or whether it is installed in a conventional submerged grid system (Fig. 5). Another mooring option available in suitable site is a two-point mooring (Fig. 6) that facilitates Aquapod rotation.

Cost: On a capital cost per cubic meter of containment basis, the Aquapod containment system is significantly more than conventional surface pens but significantly less than the cost of currently available submersible net pens. Furthermore, when submerged, the volume used to calculate containment is accurately figured, unlike surface pens which calculate the volume in the top part of the water column, although that space is not used by the fish. Operational costs are less than



Aquapod Systems for Sustainable Ocean Aquaculture. Figure 4
A simple air lift suction pump can remove mortalities and waste from the bottom of the Aquapod net pen for removal and processing

existing systems due to the efficiency of the proprietary fish transfer technology for routine husbandry tasks. Maintenance costs are reduced by the modular nature of the net panels and the ability to orient the Aquapod so that any part of the net pen is at or near the water surface, making it easy to inspect, remove, and replace individual net panels. When optional vinyl-coated wire mesh is used for netting, net replacement is reduced and net washing is greatly facilitated.



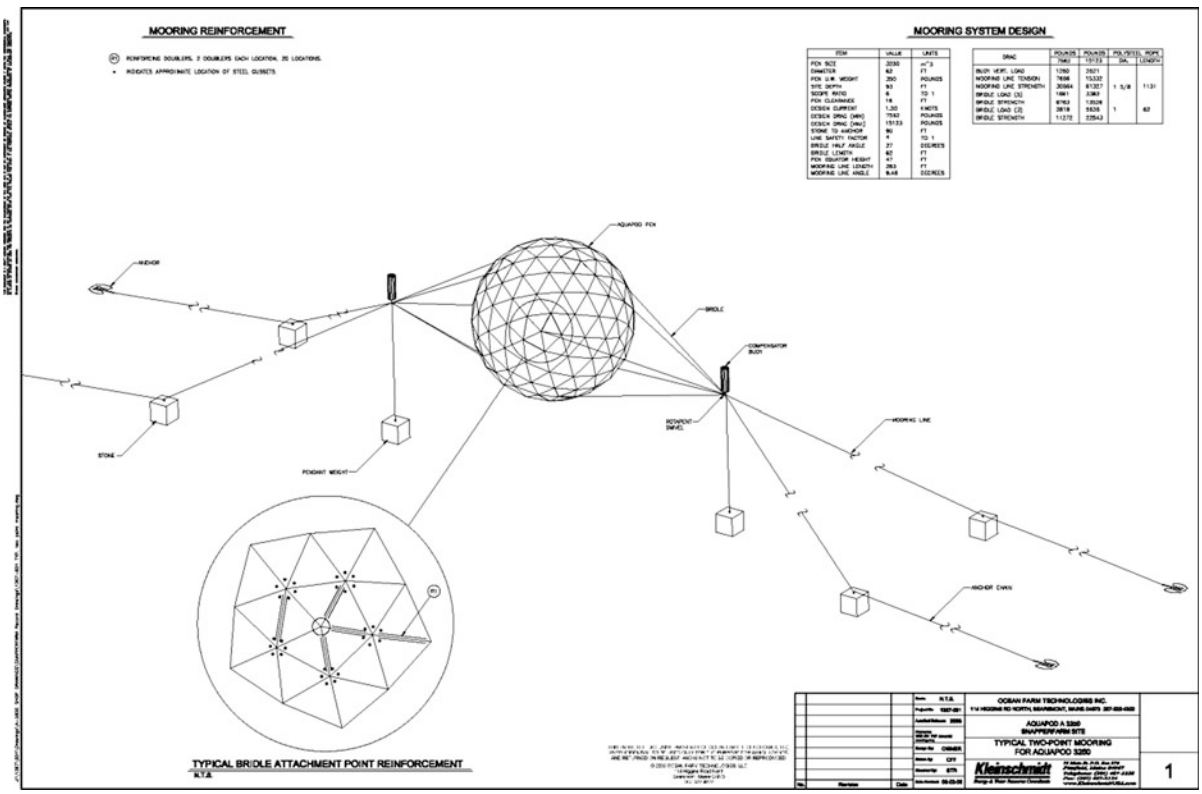
Aquapod Systems for Sustainable Ocean Aquaculture. Figure 5
Aquapod net pens can be moored in many different configurations of grids

The true cost of any system is not only the initial capital cost, but the life cycle cost and the cost of operations, including risk. The Aquapod has been designed with these factors as principal drivers.

Aquapod Technology and Engineering Description

Current State of Design and Materials

The engineering work to date on the Aquapod design has centered primarily on early 10 m (31 ft) diameter units, the larger 19 m (64 ft) diameter A3600 size, and more recent 8 m (27 ft) A212 Micropods. All these models have used recycled HDPE materials as the



Aquapod Systems for Sustainable Ocean Aquaculture. Figure 6
Single Aquapod net pens can be moored individually in a two-point mooring. With swivels at the bridle attachment points, this allows for rotation of the net pen

primary element for the struts, and plastic-coated welded wire mesh for the net fabric. Selective reinforcement around the mooring attachment points has addressed strength issues. So far these materials seem to work well and have been a basis for all Aquapod installations to date. The innovations by Ocean Farm Technologies (OFT) with testing and analysis have been steadily advancing the design, learning the relative cost and difficulty of various ideas, with the goal of refining the concept and improving the economy of the design, manufacture assembly, and installation procedures.

Mooring system components have been selected to create a two-point design allowing movement in the vertical direction and rotation, and single-point moorings. Buoyancy calculations have been done to confirm the near-neutral buoyancy of the system.

The main engineering goal is to design an economical pen that is strong enough, but not excessively heavy or expensive, by defining the real safety factor of the current design, and seeing where further structural design improvements might be made. With a large structure with so many repetitive elements, the changes in any one element are repeated hundreds of times, and thus has a real effect on cost and fabrication. Further work is also being done by OFT with more long-range improvements and concepts for different types of designs using different materials or assembly ideas.

Further data from the real size pens has been used to confirm drag forces. Loading of the pen has to date been based on a tow test performed by a University of New Hampshire boat on the 31-ft geodesic pen and has been scaled up for the 62-ft pen [1]. OFT plans to measure drag loads on existing pens as a function of current velocity and thus more accurately establish this relationship. The current drag forces used to date are on the conservative side, allowing for the effect of biofouling that increases drag.

The structural effect of wire mesh on the Aquapod is obvious but difficult to measure or model. The mesh contribution has not been credited in the analysis to date, except to provide buckling restraint for the compression member weak axes. As far as basic strength, the mesh can help reduce tension stresses, but will do nothing to reduce compressive stresses in the struts, and since these are usually of about the same magnitude over the sphere, the overall effect may not be of much importance.

Results of University of Maine Engineering Study

The use of recycled HDPE [2] plastic extrusions in this type of structural engineering application is rare and, for the designer, the available data on materials and fasteners in the material is limited. For design of the Aquapod struts, it is necessary to check the capacity of the tension and compression members. Needed for compression members are values to use for modulus of elasticity, maximum allowable stresses for compression, based on buckling, yielding, and crushing, and creep rupture of the real life struts. For design of the tension members, the modulus of elasticity, maximum and allowable tensile stresses, and fastener strengths are needed. In addition, bolts and other fasteners are used in the Aquapod and data is needed regarding end and edge distances, strengths, and yield points. Testing of the full size members and hub assemblies has been desirable to confirm the actual strength of real life members, and thus provide an indication of the real safety factor provided in the pen.

The Hybrid Structures Testing Laboratory in the Mechanical Engineering Department, at the University of Maine, was engaged by OFT and performed initial testing (1) to determine engineering data for the plastic material that is used in the Aquapod net pen system and (2) to test sub-component details of the Aquapod system. The goal has been to have design guidance to use as the limits for the members, as calculated for the pen under the various load scenarios. Testing was performed under the direction of Vincent Caccese, Ph.D., P.E., Professor of Mechanical Engineering (Caccese).

The testing consisted of a total of 62 structural tests of components as follows:

1. Material Bolt Bearing, Tensile, and Shear-out Capacity (40 tests performed)
2. Column Compression Tests (20 tests performed)
3. Mesh Assembly Buckling (2 tests performed)

The bolt tests were done first and arrived early enough to size the bolts in the Aquapod 3250. These tests were primarily to determine the bolt capacities for the tension members in the Aquapod, and to determine how many, and what size bolts would provide the required tension transfer from component to component, principally in the steel-reinforced connections

around the mooring attachment points. These tests also showed that the tensile capacity of the struts is a controlled by the holes, and tension members will fail first at the bolt holes.

The compression tests were primarily aimed at determining maximum compression loads that could be assigned to struts, which should be a safe percentage of the ultimate compression demonstrated by the full size tests, and should also be a safe amount below the maximum stress that induces the beginning of buckling.

While the sample sizes for the compression tests were small, the results indicate that the actual modulus of elasticity for design use is significantly higher than the published data provided by the plastic vendor for design of compression posts, but well under the value listed as an engineering property in their material property tables. The actual reinforcement of members with doublers could have been reduced somewhat and the current design is conservatively reinforced.

Assessment of Structural Stability of Aquapod Net Pens

The geodesic sphere that has been used for this pen is based on an icosahedron. The icosahedron is a geometric shape consisting of ten triangular faces that are further subdivided into triangular panels, in the process of creating the geodesic sphere. Each of the ten faces is subdivided into 16 triangular panels of 10 ft on a side. Each strut is a pair of extruded fiberglass-reinforced HDPE members. All panels are faced with $1'' \times 1'' \times 16''$ gage vinyl-coated galvanized steel wire mesh.

The geodesic sphere has been structurally analyzed using finite element computer modeling, for load cases due to various loading conditions that might be encountered in the life of a pen. Under each load case, an appropriate safety factor related to the likelihood of the case was used. The load cases are:

- Drag force from current at the site or when being towed. Using a total drag force that was extrapolated from the tow testing results, the total force is distributed around the pen based on locating maximum forces on the leading and trailing faces, as oriented to the current.
- Dead load of a pen hanging from a crane on its five part bridle.
- Drag forces with the pen in a current but with one and two broken bridle lines.

There have been numerous numerical simulations made of mooring line arrangements to refine the mooring system arrangement, and this has been an iterative process of examining the forces in the pen from a given loading and mooring arrangement. Through examination and rejection of a number of possible mooring schemes, OFT was able to come up with the final bridle arrangement that minimizes and localizes the maximum loading in the struts. This arrangement features five tangent bridle lines attached at the intersections of the base icosahedron faces, which, in the pen, are seen as a five panel intersection. The five panels form a pentagon. The analysis has found that locating the mooring at this position and angle will reduce the maximum strut forces and provide a superior distribution of loads within the structure.

The hub reinforcement consists of bent steel gusset replacements for the plastic gussets in each corner of the panels framing into the pentagons, in effect creating a steel ring at each of these hubs. The central hub of each pentagon receives a steel fabrication that reinforces the hub and provides an attachment point for the mooring bridle. The design for the central hub was developed for the conservative a 2-knot current loading, and a lighter design was developed for the Beta pen where the current is less.

When the member strut forces from each load case were sorted by magnitude, they were compared to critical allowable loading, assigned to the compression or tension members. When mapped to the actual positions, in all cases, the maximum strut loads, both tension and compression, are relatively concentrated in the pentagons around the mooring forces, and a few panels around them. The geodesic sphere distributes loading very quickly away from these areas and the struts forces become very low. Thus, OFT developed the recommended reinforcement of the pentagons related to the mooring attachments. This consisted of selective triangle receiving steel gussets and some additional bolting.

In addition, OFT looked at the sphere under the same drag forces but with various broken tension or

compression struts, and did not find much difference as compared to a broken bridle line. Critical strut failure, next to a mooring attachment, at most will relieve the mooring force of that line and redistribute load to the others. Failure of struts elsewhere in the sphere just redistributes around the broken strut area to adjacent struts, which have plenty of reserve. Thus, the structure appears to be very robust and redundant.

OFT has engineered on the conservative side with reinforcement requirements. The total test compressive load of 4,800 lbs on the unmeshed strut is more than the load previously calculated using the material properties provided by the vendor, and the load with the mesh included is even greater. The design has been using 3,800 lbs for the buckling strength of the short sides of the mooring panels, which is quite a bit less than either of the test results. The test data shows that the compressive buckling modulus of elasticity from the published value of the vendor is too low, since the test buckling strength is several times higher than the predicted strength. In the A3250, all members with compression loads greater than their buckling load calculated with E = 75,000 were reinforced. Since the tests show greater E, around 150,000, this reinforcement is quite conservative.

Feasibility of Scale-Up

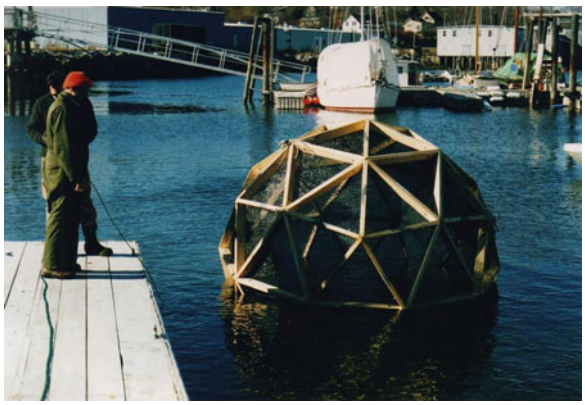
OFT performed a preliminary analysis of a 28-m diameter pen using a scaled-up version of the A3250. This is an icosahedron but uses many additional panels of the same basic size of about 10 ft to a side, and encloses 11,000 m^3. The dead load case is the most severe. This is the load seen if the sphere were hung from a crane. The drag load with one broken mooring line appears to cause a similar magnitude axial loading in the worst struts. Since the dead load is 90,500 lbs., it will exceed the drag load determined using the drag calculation. The drag at 1 m/s current (2 knots) would be around 60,000 lbs.

The most-loaded members are all associated with the mooring line attachment points as before. The pentagon at each mooring line is highly loaded, more so than the previous A3250 aquapod, and will be specifically reinforced as before. These loadings are relatively discrete areas and an inexpensive reinforcement will be devised for these pentagons, or they will be specially fabricated from a different material or configuration.

There are also high loads in the next members radiating from the pentagon on the downstream side. After the pentagon, most of the highest stressed members are in the hexagon of six panels just downstream of the pentagon. This hexagon will be treated like the pentagon and reinforced to handle the loads. The rest of the pen seems to have relatively lower loads. The extruded HDPE material with wire mesh is very capable of these loadings.

Aquapod Installations and Experience to Date

Ocean Farm Technologies has installed 26 Aquapod net pens prior to 2011. Initial prototypes were deployed in Maine and New Hampshire (Figs. 7–9). The first commercial-sized installation of a Beta model was in Culebra Puerto Rico at Snapperfarm Inc (Fig. 10). A commercial crop of cobia (*Rachycentron canadum*) was grown in this Aquapod. OFT proceeded to develop small net pens with installations in Panama (Figs. 11, 12 and 13) and Mexico 9 (Fig. 14). The first commercial sale, in 2008, was to a company in South Korea which was in transition from wild-catch fishery to farming (Figs. 15 and 16). Most recent sales have been to Mexico, where several sizes of Aquapods are deployed to grow shrimp (*p. vannemei*) (Figs. 17, 18, and 19).



Aquapod Systems for Sustainable Ocean Aquaculture. Figure 7
Belfast, Maine, USA 2004 prototype A

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 8
Bucks Harbor, Maine 2005 prototype B A400

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 10
Culebra, Puerto Rico 2006 Beta 1 A3250

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 9
Portsmouth, New Hampshire, USA 2005 prototype C A400

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 11
Puerto Lindo, Panama 2008 Beta 2 Micropods

Future Directions

Low-Volume High-Density Net Pen Culture for Open Ocean Aquaculture

The emergence of an open ocean aquaculture industry provides an opportunity to reexamine traditional practices, such as the preference for ever-larger net pens for fish containment. The industry's focus on maximizing net pen size is driven by the convention that large net pens provide more growing volume for a given investment. Because net pen costs can vary with the square of linear dimension while the volume varies by the cube, this assumption has merit. However, the assumption that larger net pens are more cost effective requires that stocking density remain unchanged throughout the size comparison. Stocking density in terms of kilograms per cubic meter is a useful fish-husbandry parameter in pond- and tank-based aquaculture where extensive research has been done and where water exchanges are predetermined. There is

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 12
Woods Hole, Massachusetts, USA 2008 Aquadome

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 13
South Korea, 2009 A3600

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 14
South Korea, 2009 3-A3600

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 15
Panama, 2010 A132 copper mesh

also a growing body of data regarding stocking density in salmon net pens; however, these studies have been done in near-shore, low-energy environments. Little or no research has been done in open ocean net pen culture comparing density optimization over a range of net pen volumes, especially small net pens.

The principle advantage of small pens is that ongrowing fish are closer to their source of clean water. By similar logic, the small net pen, as a whole, because of its shorter stream-wide dimension experiences more water exchanges in a given current. From the perspective of an individual fish in an ocean net pen, water quality depends on how many other fish are metabolizing between it and its source of clean and oxygenated water from outside the containment. At constant kg/m^3, interior fish in a large net pen experience significantly degraded water quality compared to interior fish in a small net pen.

The potential advantage of low-volume, high-density (LVHD) net pen culture is not a new concept. Schmittou pioneered the concept of LVHD in 1968, and the approach has since been applied in a project

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 16
Mexico, 2009 A3600 under tow

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 17
Sorrento, ME USA A400

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 18
Mexico, 2010 2-A3600, 1-A212

sponsored by the American Soybean Association in China [4]. Growing densities of up to 200 kg/m^3 are recorded. McMaster et al. [5] suggests stocking density ranges for Florida pompano between 35 and 100 kg/m^3. Even at close to typical salmon stocking densities, the economics of small net pens become apparent. Table 1 shows a constant crop yield per capital investment in containment as density in the smaller net pens is increased.

In addition to this economic advantage, strong, secure small net pens will alleviate some of the economic barrier to entry for offshore fish farming. Small net pens require smaller vessels and support equipment; they are easily shipped, assembled, and deployed. In the near term, OFT sees a potential market for net pen hardware export to developing world economies that are transitioning from capture fisheries to fish farming, and who want to skip the near-shore environmental problems and user conflicts by moving directly offshore. This includes third world artisanal fishermen who in global aggregate contribute to marine fish resource depletion. Also, the mooring gear required for smaller net pens is lighter and more secure; scuba diving is reduced – especially deep diving; harvesting is easier; small pens are easier to tow; and risk is diversified.

Use of Small Aquapods for Artisanal Fish Farming

According to the FAO, "Artisanal Fisheries" are traditional fisheries involving fishing households (as opposed to commercial companies), using relatively small amount of capital and energy, relatively small fishing vessels or canoes, often beach-based, making short fishing trips, close to shore, mainly for local consumption. Artisanal fisheries can be subsistence or commercial fisheries, providing for local consumption or even export. They are sometimes referred to as small-scale fisheries or day fisheries.

Numbers of artisanal fishermen are hard to obtain because the sector is informal, often outside the regulated fishery (some illegal) and part-time. In Ghana, for

Aquapod Systems for Sustainable Ocean Aquaculture. Figure 19
Mexico 2010 A212 under tow

Aquapod Systems for Sustainable Ocean Aquaculture. Table 1 The 212 m^3 net pen stocked at 51 kg/m^3 gives interior fish better access to clean water (less biomass between center of net pen and net) and an equal yield per capital cost of containment

Yield from various-sized Aquapod net pens							
Size of pen m^3	Radius (m)	Diameter (ft)	Approximate cost of aquapod (USD)	Final stocking density (kg/m^3)	Biomass of fish between center of pen and net	Yield per pen (kg)	Yield (kg) per capital cost of containment (kg/$)
7,000	11.87	77.8	$218,000	17	202	119,000	0.55
3,600	9.51	62.4	$140,000	21	200	75,600	0.54
212	4.04	26.7	$20,000	51	189	10,812	0.54

example, canoe fishermen go to sea for about 150 days a year. Half the catch is obtained in 2 months, so it is obviously a part-time endeavor. The FAO estimates that as much as 95% of the annual catch in Africa is from artisanal fisheries. In Mexico, the FAO estimates that 96.6% of the fishing fleet (108,205 boats) are *panga* (skiffs). Worldwide, the UN estimates there are 35-million people whose primary livelihood comes directly from fishing, and that artisanal fishermen outnumber large- and medium-scale fishermen by 4 or 5 to 1. This would put the number of artisanal fishermen in the range of 28 million to 30 million individuals worldwide (Table 2).

From NIOSH Second Conference on International Fishing Industry Safety and Health Overexploitation of coastal resources and decreasing catches has forced subsistence artisanal fishermen to go further offshore in search of fish in vessels designed for near-shore use. In a study done in 2000, the FAO reports an alarming increase in fishing-related fatalities as a result.

Small-scale marine aquaculture is one way to bring sustainability and consistency to this often-overlooked fishery sector. Turning artisanal fishermen into artisanal fish farmers will not be an easy task. However, small Aquapods have several advantages, beside affordability, that makes them advantageous for artisanal aquaculture:

- Size of boats and equipment can be smaller
  - The 7–10-m diameter Aquapods can be serviced with existing artisan vessels, whether motorized or not. Equipment needs are minimized and may include a small air compressor or pump.

Aquapod Systems for Sustainable Ocean Aquaculture. Table 2 Estimates of the number of fishing vessels in Pacifica [3]

Location	Locally based vessels [3]	Motorized artisanal fishing vessels [6]	Nonmotorized artisanal fishing vessels [6]
Cook Islands	10 L/L	200	120
Fiji	96 L/L; 1 P/L	1,600	400
Fed. States Micro.	34 L/L; 8 P/S	2,000	600
Kiribati	2 L/L; 1 P/S	600	5,000
Marshall Islands	54 L/L; 5 P/S	500	250
Nauru	1 L/L	100	80
Niue	100 skiffs	60	240
Palua	71 L/L; 1 P/S	700	40
PNG	40 L/L; 24 P/S	8,000	10,000
Samoa	153 L/L	80	100
Solomon Islands	8 L/L; 2 P/S; 12 P/L	1,800	5,000
Tonga	26 L/L	800	200
Tuvalu	20 skiffs	200	500
Vanatu	10 skiffs	250	500
Total	495 L/L; 40 P/S; 14 P/L	16,890	24,530

P/L pole and line vessel, *P/S* purse seiner, *L/L* longliner

- Ease of assembly and deployment – lighter mooring gear
  - Small Aquapods are easy to assemble, and can be put together by several men using hand tools on a beach and rolled into the water (or rolled out of the water for harvest). The small sectional area means a relatively low drag resistance, so mooring gear can be much lighter than traditional net pens.
- Single-point moorings are simple and inexpensive
  - An attractive option from a cost and environmental standpoint for these small pens is a single-point mooring, allowing the net pen to swing in a watch circle just as a boat moored in a harbor. Low drag forces on the anchor make this feasible.
- Less deep diving, most diving can be done by hookah
  - A net pen less than 10 m in diameter, when brought to the surface for maintenance, will allow almost all underwater activity to be performed without scuba gear. The spherical shape of the pen and the ability to rotate it allows for all portions of the net to be surfaced.
- More available deployment areas due to shallower water depth
  - Conventionally sized submersible pens require water depths of more than 30 m. The small Aquapods can be deployed in water depths of 12–25 m. This means boat trips far offshore are not necessary.
- Easier harvest – selective harvest by netting or whole pen harvest is possible.
- Diversification of risk as opposed to "all eggs in one basket."
- Ease of towing – their small size and rigidity facilitate easy repositioning, or towing of a pen to market port.
- Predator proof
  - Sharks, seals, and sea lions are a huge risk to artisanal fisheries – in some places, sharks are one of the main targets of artisanal fishermen. According to the Chamber of Commerce in

Somalia, sharks constitute 90% of the artisanal catch. Artisanal fish farmers cannot tolerate this risk to the crop and to human safety, and the shark proof Aquapod is the solution.

- Storm resistant
  - The Aquapod net pens are submersible, so they can be submerged below storm waves when a hurricane or typhoon approaches, eliminating a potentially devastating risk.

Economics of Artisanal Fish Farming with Aquapods

Catch estimates from artisanal fishermen are difficult to obtain – most are subsistence harvests, some excess is sold locally, and a little is exported. The sparse data that exists can only estimate the amount of fish that are consumed by the fisherman's family and community based on how much is sold. However, FAO estimates of annual catches range from 2.5 t per year per boat (Yemen) to 3.6 t per year per (Mexico). Considering that the smallest Aquapod ($212m^3$) could easily contain 5–10 t of fish at harvest, several of these small pens could probably produce more harvest than most artisanal fishermen ever see in a year.

Following is a spreadsheet analysis of what the economics of a small artisanal fish farm would look like. The assumptions include realistic and current pricing for commercial fish farm expenses, as low-volume pricing and sourcing of feed and fingerlings is unknown, and most likely would require some public sector support. As with all producing units, the sensitivity of market price trumps all other variables. Labor costs are assumed to be nil, as this is a family-scale operation.

The establishment of artisanal fish farms will likely be a private/public cooperative effort with governments and/or NGO's subsidizing some capital costs, providing low interest loans, organizing distribution of feed and fingerlings, and providing training to artisanal fishermen as incentives to evolve from dependence on shrinking capture fisheries to a sustainable farming economy.

Artisanal Aquapod Farm					
A212 Aquapod Net Pens (No. of Aquapod Net Pens: 4)					
Sales	$212,000		*Assumptions*		
Cost of goods sold			Biological FCR	1.7	
Feed	$124,879		Feed cost per kg	$1.65	
Fingerling	$34,980		Harvest size in kg	2.0	
Outside services	$1,000		Price of fingerlings	$1.50	ea.
Dive/Scuba expenses	$2,000		m^3 of growing vol.	848	
Workboat expenses	$3,000		Stocking final density	50	kg/m3
Fuel and oil expense	$1,500		Number of fish harvested	21,200	
Small tools	$250		Kg Harvested	42,400	
Concession	$500		Sale price	$5.00	Kg
Consumables	$750		Amortization period	7	years
Maintenance	$1,500		Aquapod A212 net pen cost	$19,000	ea.
Miscellaneous	$800		Mooring cost	$3,000	ea.
Fish health	$1,000		Total capital cost	$88,000	
Amortization of Aquapods	$12,571		Annual amortization expense	$12,571	
Cost of goods sold	$184,730				
Gross margin	$27,270				
Other overhead	$1,200				
Net Income	$26,070				

Bibliography

1. DeCew J, Page S, Turmelle CA, Irish J (2006) Interior current reduction of the Aquapod™ cage. In: OCEANS 2006, OCEANS'06MTS/IEEE, Boston
2. Caccese V (2006) Structural testing of various configurations for the aquapod net pen. Report No. C2004-018-001. Department of Mechanical Engineering, University of Maine, Maine, 38 pp
3. Gillett R (2003) Aspects of sea safety in the fisheries of pacific island countries. FAO, Rome, 12
4. Swick RA (2001) Feed based tilapia culture. Technical Bulletin AQ49-2001, American Soybean Association
5. McMaster MF, Kloth TC, Coburn JF (2003) Prospects for commercial pompano mariculture. Aquaculture America 2003
6. McCoy (1991) NIOSH Second Conference on International Fishing Industry Safety and Health 1991

Avian Specific Transgenesis

Michael J. McGrew
Avian Germ Cell Laboratory, Division of Developmental Biology, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, Midlothian, UK

Article Outline

Glossary

Founder bird (F_0) A bird into which a genetic vector was introduced. This vector may become incorporated into the chromosomes of some of the somatic and germ cells of the bird.

F_1 bird The offspring of the F_0 bird. Transgenic F_1 birds will contain the transgene incorporated into a chromosome in every cell of the animal.

Germ line chimera A genetically modified founder bird (F_0) in which the introduced biological material (i.e., DNA, virus, cells) has contributed to cells of the germ cell lineage of the host animal.

Primordial germ cells Cells in the developing embryo belonging to the germ cell lineage at a developmental stage before any sexual differentiation has occurred. These primitive germ cells may be located in the embryo in tissues other than the forming gonad.

Retrovirus A virus that carries its genome as an RNA molecule. After infecting a cell, the RNA is reverse transcribed into a DNA molecule that is inserted into the genome of the infected cell at which point it is referred to as a provirus. The provirus is passed to all daughter cells as part of the host cell's genome.

Recombinant proteins A protein produced using recombinant DNA technology. The DNA sequence encoding the protein of interest is artificially constructed using genetic engineering and the protein is produced by inserting the DNA along with DNA regulatory regions into a bacterium, a eukaryotic cell, or an animal.

Transgenic bird Any avian species in which part of its genetic component contains DNA deriving from an exogenous source, or that the genome of the bird has been altered by human intervention.

Transgenic bird lines A flock of birds deriving from a F_1 bird containing a transgenic modification at a defined genetic locus.

Definition of the Subject

Transgenesis is the process by which an *exogenous* DNA molecule is incorporated into the genome of an animal. This technology promises the possibility to investigate and manipulate the production traits of poultry, produce recombinant proteins in the eggs of genetically engineered layer lines, and directly intervene in the health and welfare of avian species. The complexity of the avian egg and the precocious development of the avian embryo in the female before oviposition (laying) have hindered endeavors in avian transgenesis. Three decades of effort have been carried out to achieve the genetic modification of the avian genome. The generation of novel methods for the modification of the avian genome has led to the current advances in the field of avian transgenesis. This entry will delineate the methods used for avian transgenesis, the current state of the art, and the potential future directions research in this field will take.

Introduction

Due to its use in meat and egg production and also as a model organism for developmental biology studies in laboratories, the chicken is the most widely used bird species for the development of transgenic technologies. There are three commonly cited incentives for the development of transgenesis in the chicken. These are the production of biopharmaceutical proteins in eggs, the generation of disease-resistant flocks along with an increase in understanding disease resistance in poultry, and the generation of useful transgenic lines for studies of developmental biology. With the recent

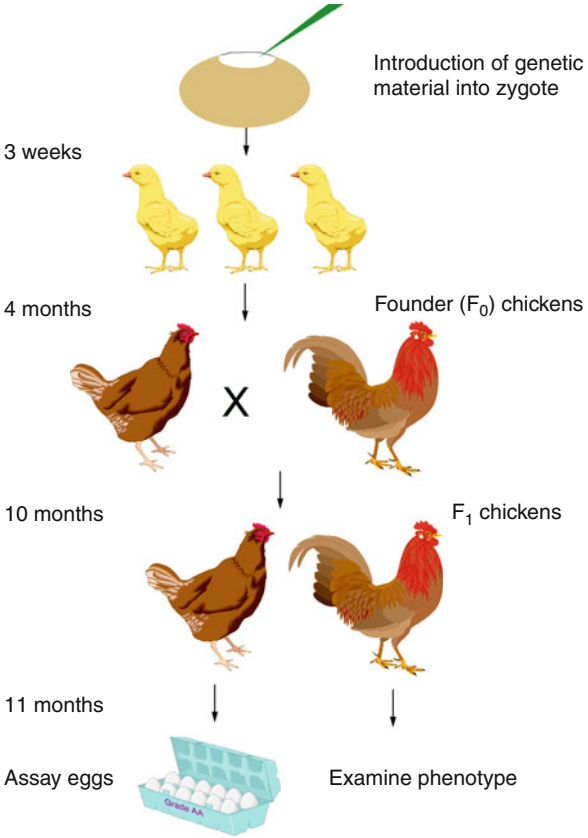
P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

development of novel avian transgenesis techniques, these goals are beginning to be achieved.

The principal rationale behind this emerging field is industry driven and is to produce a platform for the biosynthesis of biopharmaceutical proteins in chicken eggs. Animal-based bioreactors, a process sometimes referred to as animal "biopharming," were proposed as a non-cell-based method for the production of recombinant human proteins in the 1980s (reviewed in [1]). Animal-based bioreactors were inferred to be a lower cost, safe, and more efficient system in which to produce large quantities of bioactive recombinant proteins than cell-based production platforms. Efforts to produce recombinant proteins in larger animals have focused on the secretion of the target protein into the milk of the transgenic animal using the regulatory regions from a milk protein gene. Potential mammalian species commonly proposed and studied for recombinant protein production include cows, pigs, sheep, goats, lamas, and rabbits. After over two decades of investigations, the first biopharmaceutical protein produced in an animal's milk has been approved for use in humans in Europe and the United States. This product is recombinant human antithrombin (ATryn-GTC Biotherapeutics) produced and isolated from goat's milk. Profitability in the marketplace along with consumer acceptance will be the key factor in determining the success of this and similar ventures using large animal delivery systems. In addition, the regulatory approval of this recombinant protein validates the use of animal bioreactors for protein production.

The chicken has also been proposed as a potential animal-based platform for the production of recombinant human proteins. In this case, the recombinant protein is produced in the oviduct of the hen and secreted in the forming egg as it passes down the oviduct. The target protein would be targeted to the egg white of the egg using the regulatory regions from one of several proteins expressed in the albumen of chicken eggs (reviewed in [2]). The chicken has several advantages over larger animal-based bioreactors. The chicken has a shorter generation time than a larger mammal (4 months for egg production vs. 14 months for first milk production in goats), which means a more rapid time from production of the transgenic animal until production of the recombinant protein (Fig. 1). This, in turn is reflected in the second advantage, which is the lower cost of protein production in chickens. One estimate predicts that the cost of recombinant proteins in chickens will be 1/5 of that of larger mammals [3]. This is due to the reduced costs of housing, feed, and quantity of recombinant protein produced per animal. In addition, the glycosylation pattern of secreted proteins in chickens is closer to the pattern seen on human proteins than those seen on proteins secreted in several large mammals [4].



Avian Specific Transgenesis. Figure 1
Time line for production of transgenic chicken lines. The time line from the introduction of a transgenic construct into a fertilized chicken egg until the generation of F_1 offspring. The F_1 animals can be analyzed for gene expression or bred to transmit the transgene to the next generation

Several studies have reported the production of bioactive recombinant protein in chicken eggs [5–7]. The methods used to produce these transgenic animals will be discussed in the following sections. These initial

reports demonstrated that bioactive recombinant human proteins can be produced in the eggs of transgenic chickens. To date, excluding the production in influenza vaccines, no proteins produced in chicken eggs have been approved for use in humans.

A second reason for the production of transgenic chickens is the development of transgenic models for the study of the biology of the chicken and developmental biology in general. The chicken has long been a model system for the study of developmental biology due to the accessibility of the chicken embryo in the developing egg [8]. The preeminence of the chicken as the model system for embryological studies is now taken by the mouse and the zebra fish due to the lower costs associated with maintaining colonies of these animals, quicker breeding times between generations, and most importantly, the ability to investigate gene function using transgenesis. New developments in avian transgenesis have led to the development of new avian models for developmental biology [9, 10]. The generation of transgenic lines containing the green fluorescent protein (GFP) in every cell of the developing animal permits the fate mapping of living cells, the study of cells and their descendants during embryogenesis. This has facilitated the investigation of axial stem cell populations in the chicken embryo and the formation of a novel hypothesis for cellular sex identity in avian species [10, 11]. The development of more advanced methods of transgenesis will permit the generation of chickens containing genetically modified alleles, which will serve as models for investigations of early development, avian physiology, and production traits.

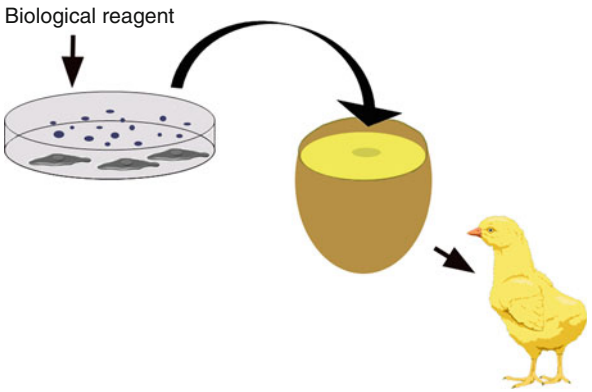
A third major reason for the generation of transgenic chickens is to use genetic modification for the increase of production traits that would lead to a decrease in environmental impact for poultry production. This could also be viewed as means of addressing the sustainability of the poultry industry with increasing demand on production and decreases in available resources. One manner in which this could be accomplished is by reducing waste production during rearing and increasing meat production for unit energy (reviewed in [12]). Several transgenic models have been developed in mammalian farm animals which exemplify the potential benefits of trait modification. A transgenic pig producing less organic phosphate waste products has been produced [13] and a similar strategy has been proposed for the chicken [14].

A comparable impact could be achieved on poultry production by reducing the losses attributable to disease. Avian transgenesis offers the potential to generate disease-resistant poultry and to investigate the genetic traits of disease resistance in poultry. It is estimated that during the avian influenza outbreak of 2004–2005, 100 million chickens were culled in Southeast Asia [15]. Using transgenic technology, the capability exists to generate a transgenic animal which will be resistant to several of the endemic diseases affecting poultry production (Avian influenza virus, Marek's disease, Newcastle disease, fowl cholera). While the production of these birds for use in meat and egg production is not currently accepted, transgenic chickens will be of use for investigating the pathogenesis of infection and the determination of viral targets of infection and pathogenesis. This in turn could generate targets for directed vaccine development. In addition, the validation of genetic traits for disease resistance also necessitates an analysis of the disease loci using transgenic technology in avian cells or the whole animal. Currently, no disease-resistant transgenic chicken models have been developed, but the production of transgenic cattle with resistance to mastitis suggests that this will be a profitable area of investigation [16]. With increasing demands on poultry production, an increase in methods of disease resistance will be needed.

Methods of Avian Transgenesis

The first efficient method developed for the introduction of exogenous DNA into mammals to create germ line chimeras was using pronuclear injection [17]. This technique entails the microinjection of DNA encoding the gene of interest (transgene) into the male pronucleus of the recently fertilized oocyte at a developmental stage previous to the first cell cleavage. Although pronuclear microinjection was primarily developed for transgenesis in the mouse, it has been exploited to generate transgenic animals in rats, sheep, cows, pigs, and rabbits [18–20]. Rates of transgenesis using pronuclear injection (transfer of the injected transgene to the genome of the offspring) in species other than mice are low. In general, only 1–5% of the offspring will have incorporated the transgene into their genome.

The use of pronuclear injection is even more problematic in the chicken. The laid chicken egg consists of a single epithelial layer of unpatterned cells termed the blastoderm consisting of 45,000–60,000 cells [21]. Injection of any biological material at this stage signifies that only a small proportion of the embryonic cells will be potentially exposed to and incorporate the transgene into their genome. The development of the chicken zygote in the hen before the egg is laid necessitates that pronuclear microinjection can only be carried out with surgical removal of the fertilized oocyte from the hen. This entails that the egg is subsequently surgical transferred to a surrogate host hen or cultured in vitro for 21 days until hatching [22]. The ovum of the avian egg is the large opaque yolk of the egg. The ovum contains a small pool of cytoplasm lying on the yolk material in which the pronuclei are located. Microinjection into the pronuclei is not possible because of the opacity of the yolk material underlying the nuclei; thus any injected biological material must be injected into the cytoplasm surrounding the pronuclei. For this reason, the rate of transgenesis for using pronuclear injection chickens is much lower than in other vertebrates and the delivery of biological material is usually performed in the laid egg (Fig. 2).



Avian Specific Transgenesis. Figure 2
Introduction of genetic material into the fertilized zygote. The genetic material to be introduced into the egg (DNA, viruses, transposons, purified proteins) is either first transfected into cells for packaging or directly introduced into the egg at an early stage of development (laid egg or fertilized oocyte). The embryo is subsequently incubated until hatching in its own shell or a host chicken shell. The hatchling will then be analyzed for the genetic modification

Injection of DNA into Early Avian Oocytes

Microinjection of DNA transgenic constructs into the early chicken oocyte has proven to be a feasible procedure for the production of transgenic chickens. As described above, the DNA construct is injected into the cytoplasm of the fertilized oocyte at an early developmental stage (Fig. 2). The egg is subsequently cultured *ex ovo* in a host shell until normal hatching occurs. Love [23] introduced a β-galactosidase reporter construct into the fertilized chicken oocyte and cultured the embryos *ex ovo* until hatching. In this report, 50% of the injected embryos contained the transgene and 14% of the hatched founder birds transmitted the transgene to the next generation. This is a similar transmission rate to that observed in mammalian species using microinjection. A comparable result was observed in which founder transgenic chickens were obtained using microinjection and a modified *ex ovo* culture system [24]. In this example, no germ line transmission to the F_1 generation was observed.

Although it is possible to produce a transgenic bird using microinjection, this avenue of research has not proven fruitful due to the cost and difficulty in obtaining and manipulating the early chicken oocyte. Instead, research has focused the use of retroviral vectors for the introduction of transgenes into the genome of the chicken at laid egg stages of development.

Replication-Competent Viral Vectors

A more efficient method to create genetically modified chickens is using retroviral vectors to introduce transgenes into the chicken genome. Retroviruses are RNA viruses that reverse transcribe the RNA genome into a DNA intermediary that is integrated into the genome of the infected cell. The integrated virus, or provirus, transcribes new copies of the viral genome that are packaged into new viral particles that bud from the infected cell and in turn infect neighboring cells. In this manner, most cells of the developing embryo will be infected along with the germ cells. Germ cells containing the integrated provirus in their genome will transmit this modification to all of the cells of the

offspring deriving from that germ cell. Using recombinant DNA technology, it is possible to remove part of the viral genome and replace it with an inserted transgene. In this manner, a line of genetically modified chickens can be produced containing a transgene of interest.

The first use of retroviruses in chicken for transgenesis used viruses that were replication-competent (i.e., infective). In this case, the virus was injected at the laid egg stage and incubated until hatching. Though initially only a small portion of the blastoderm may be infected, because the viral infection spreads during development, many more cells will contain proviral inserts. The first demonstration of the genetic modification of the chicken germ line was accomplished using a replication-competent avian retrovirus. Salter [25] injected either reticuloendothelial virus or avian leukosis virus under the blastoderm of laid eggs. They demonstrated that the integrated provirus could be found in the offspring of the injected hosts [25]. As a further demonstration of this technology, using the ALV retrovirus, 24% of founder animals contained the integrated provirus and this was transmitted to F_1 offspring with a transmission rate between 1% and 11% [26]. A replication-competent retrovirus was also used to introduce a transgene containing the bovine growth hormone under control of the mouse metallothionein promoter into founder (F_0) chicken [27]. Thus, replication-competent viruses offered an initial alternative to pronuclear injection for chicken transgenesis.

It must be noted that replication-competent viruses are limited in their usefulness in transgenesis for several reasons. First, since replicative virus can reintegrate into the genome, it is difficult to correlate a single integration site with the synthesis of recombinant protein from the integrated transgene. This is important as any regulatory approval procedure will require genetically defined transgenic animals containing a single defined transgenic insert which produces consistent levels of recombinant protein [28]. Second, insertion mutagenesis caused by the insertion of a retrovirus can disrupt an endogenous genetic locus and also lead to malignant cell growth in the host animal (reviewed in [29]). Third, and most important, a process called viral or transgene silencing is often observed in animals containing replication-competent proviral inserts. In transgene silencing, the transgene becomes silenced after it is introduced into early embryos, which is correlated with a high level of cytosine methylation throughout the proviral genome [30]. In the transgenic offspring of these founder animals, the transgene is not expressed or expression levels are severely reduced and the proviral genome is usually methylated in the long terminal repeat (LTR) region. It is believed that retroviral silencing occurs in the stem cell populations of the early embryo and leads to permanent silencing of transcription from the integrated provirus (reviewed in [31]). For these aforementioned reasons, replication-defective retroviruses have become the tool of choice for avian transgenesis.

Replication-Defective Viruses

Replication-defective retroviruses are modified retroviral vectors that are capable of infecting a host cell but lack key viral genes necessary to complete the viral life cycle and produce infective viral particles. In replication-defective retroviral vectors, a large portion of the viral genome has been removed from the virus and these genes are supplied in *trans* to allow for packaging of the defective viral particles. The envelope proteins of the viral vector can also be modified (viral pseudotyping) to permit infection of cell types and species normally outside of the retrovirus host range. The integrated provirus will contain a viral promoter in the LTR and few viral genes. It is also possible to incorporate a large transgene (8–9 kb) into the provirus in place of the truncated viral genome.

The first demonstration of germ line transgenesis in chicken used a replication-defective reticuloendothelial retroviral vector containing a neomycin reporter gene injected into the blastoderm of laid eggs [32]. Twenty-two percent of the F_0 hatchlings contained proviral integrations and the transmission rate to the F_1 generation was between 2–8%. Expression of the neomycin transgene in these birds was not demonstrated.

Since this first report, replication-defective retroviruses have been used extensively for chicken transgenesis. The most common retroviral vectors used are spleen necrosis virus, avian leukosis virus, reticuloendothelial virus mentioned previously, and Moloney murine leukemia virus (MMLV). All of these vectors have been used to generate several lines

of transgenic chickens [33–36]. Transmission rates of the integrated provirus after blastodermal injection from F_0 cockerels to F_1 offspring have been reported to be as high as 8%.

A novel development of this technology is to induce expression of the recombinant protein at defined timepoints to avoid possible transgene toxicity during development. This was achieved by using a replication-defective MMLV containing a tetracyline-regulated promoter to drive expression of human-recombinant erythropoietin protein in chicken eggs only in the presence of the inducer, doxycycline [7]. More than 90% of the F_0 chickens contained proviral integrations, and these birds transmitted the transgene to between 0.7% and 1.8% of the F_1 generation. Recombinant erythropoietin was produced in eggs after doxycycline induction.

In some replication-defective retroviral vectors, there is ongoing transcription from viral regulatory LTRs in the proviral insert. This transcription is thought to lead to a partial silencing of the adjacent transgene. An example of transgene silencing in birds was observed in transgenic quail containing a replication-defective MMLV using a LTR to drive expression of a GFP reporter construct [37]. Transgene expression was silenced in F_1 and F_2 birds. Other lines of transgenic chicken have displayed some suggestions of silencing between generations using the MMLV virus [38]. Researchers have attempted to circumvent this problem by using modified MMLV vectors containing internal promoters to drive transgene expression. This strategy appears to have worked in some transgenic birds that had no transgene silencing between generations [39]. Still, because of the perceived problems of silencing of these retroviral vectors, researchers have turned to a different class of retroviruses, the lentiviruses, for avian transgenesis.

Lentiviral Vectors

Lentiviral vectors have primarily been developed as potential viral agents for gene therapy in humans. Some of the advantages of lentiviral vectors are that the integrated provirus is preferentially incorporated into the open chromatin structure surrounding expressed genes, lentivectors will infect and integrate into the genome of post-mitotic cells, and the viral vector accepts a transgenic insert up to 9 kb in size. Moreover, there appears to be no transgene silencing upon transmission of the integrated transgene between generations [40, 41].

As an alternative to other retroviral vectors and to pronuclear microinjection, replication-defective retroviruses of the lentivirus class have proven to be very efficient for use in mammalian transgenesis [42, 43]. These vectors have been used successfully to generate transgenic cattle, pigs, rats, sheep, rabbits, and primates [44–48]. In these cases, the viral particles are injected into the perivitelline space surrounding the fertilized zygote. The zygote is reintroduced into surrogate hosts and the animals are bred and tested for germ line transmission.

The first demonstration of the use of lentiviral vectors for avian transgenesis used viral vectors containing transgenes encoding either GFP or β-galactosidase reporter genes [49]. Viral particles were injected into the blastoderm of laid eggs as described above. Efficiency of transmission of the integrated transgene from founders to the F_1 generation was between 4% and 45% and no transgene silencing was observed between generations. Since this report, lentiviral vectors have been used successfully to generate transgenic chickens containing a range of transgenes [6, 9, 10, 50, 51] and transgenic quail [52–54] and also transgenic zebra finch [55]. These vectors have also proven useful to generate biopharmaceutical proteins in eggs. Recombinant human β-interferon has been produced in the egg white of transgenic chicken [6], and recombinant human interleukin-1 receptor antagonist has also been produced in the eggs of quail [56].

While lentiviral vectors may now be the preferred method for generating transgenic birds, there will be public concerns with the use of transgenic birds containing retroviral transgenes, in particular, HIV-based lentiviral transgenes, for the production of biopharmaceutical recombinant proteins and the development of advanced production traits in avian species. It remains to be determined if consumer acceptance of these products will be forthcoming.

Transposons

Transposable elements were first identified by the seminal work by Barbara McClintock in maize [57].

Transposable elements are mobile genetic elements found in the genome of all organisms and are able to move (i.e., translocate) from one region of the genome to another. These are modules of selfish DNA that exist only to replicate their own DNA and are postulated as parasitic invaders of most genomes [58]. Transposable elements differ from viruses in that they do not spread by infection of neighboring cells; they are usually passed passively through cell division to daughter cells. Class II DNA transposons are a distinct type of transposable element that moves through the genome using a "cut and paste" method. The DNA transposon encodes an enzyme called a transposase which mediates the removal of the transposon from one chromosomal location and the insertion into a second chromosomal location. DNA transposons have been modified for the use in transgenesis in many vertebrates. This entails that the coding sequence for the transposase is deleted from the transposon and subsequently introduced into the cell with the transposon in *trans* [59]. The transposon will be incorporated into the genome of the cell but cannot "jump" to a new genomic location because it lacks the transposase gene. This is similar in approach to the packaging of replication-defective retroviruses for transgene delivery. The truncated transposon can be engineered to contain additional DNA sequences which will be inserted into the genome of the cell upon transposon integration. The size of the transgene carried by the transposon can be up to 10 kb in size [60, 61].

Transposable elements have been shown to be efficient in the generation of transgenic chickens. The mariner transposon from Drosophila *maritiana* containing an internal transposase was used to generate transgenic chickens [62]. Mariner was microinjected into the fertilized zygote and chickens were hatched and bred to generate F_1 birds. One founder bird transmitted the mariner transposon to 29% of its offspring. This result demonstrates that transposable elements can be used to modify the genome of birds although no transgene cargo was introduced in this example. So far, no reports have been made to generating a transgenic chicken line using transposons containing a transgene. However, the Tol2 and piggyBac transposons have been shown to be functional in transient transgenesis in the chicken [63, 64] and other transposable elements have been used successfully for mammalian transgenesis [59].

Use of Chicken Embryonic Stem Cells

Whist the early embryonic development of the chicken is significantly different from that of a mammal, cells of the early chicken blastoderm do contribute to somatic and germ cell lineages when transferred to host chicken embryos [65]. This indicates that the early chicken blastoderm may have the developmental equivalence of the inner cell mass of the early mouse embryo, further suggesting that embryonic cell lines derived from the chicken blastoderm may be developmentally equivalent to mouse embryonic stem cells. In support of this hypothesis, several groups were able to derive cell lines from early chicken embryos that were putatively identified as chicken embryonic stem cells because of their ability to contribute to many tissues in chimeric birds [66, 67]. The isolation of embryonic cell lines that contribute to the forming avian embryo offers the opportunity to carry out gene targeting, whereby the introduced transgene can be targeted to a precise genetic locus. Gene targeting uses the process of homologous recombination. In this process, one genomic copy of a gene can be replaced by a second copy of this gene using normal DNA repair mechanisms. The replacement gene will display homology over most of its length but can be engineered to contain a foreign piece of DNA which could encode a recombinant protein or a selection marker. Using homologous recombination, researchers can disrupt a genetic locus, introduce a gene encoding a recombinant protein, or replace one genetic allele with a second allele. Homologous recombination has been carried out in chicken cells [68]. Genetic modification of chicken embryonic stem cells also has been achieved and chimeric animals have been produced which contain extensive contribution of these cells to all germ layers of the forming embryo [69].

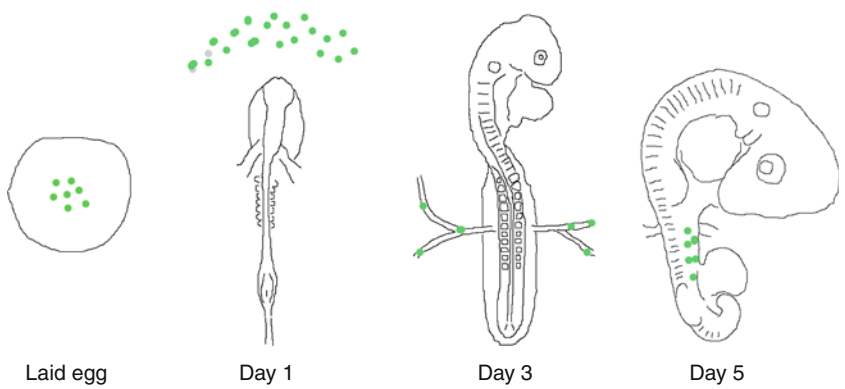
An important caveat to this work is to note here that the germ cell lineage is segregated from the somatic cell lineage very early in development (see section "Methods of Avian Transgenesis"). It is believed that for this reason cESCs do not contribute to the germ lineage in chimeras after more than 7 days of in vitro culture [67, 69]. So, although cESCs may be useful for creating somatic chimeras, these cells will not be useful for generating lines of transgenic chickens.

Recent experimental evidence suggests that it may be possible to differentiate cESCs into germ cells in vitro. These cells could then be introduced into host birds and the cells could differentiate into gametes and be transmitted to the next generation [70]. If this is achievable, it will create the possibility to carry out gene targeting in the chicken and create transgenic chicken lines using homologous recombination in chicken embryonic stem cells.

Improved Transgenesis by the Directed Targeting of the Germ Cell Lineage

Most attempts to generate transgenic chickens have resulted in inefficient transmission of the integrated transgene from F_0 to F_1 animals. This inefficiency leads to an increase in cost for production of transgenic animals due to increase in both time and breeding numbers of F_0 founder birds (reviewed in [2]). Several methods have been developed to increase the rate of transgenesis. These methods attempt to amplify the interaction of the exogenously introduced DNA or virus with the germ cell lineage of the avian embryo. The avian germ cell lineage comprises the only cells of the animal that will contribute genetic material to the next generation. Two methods developed to increase the interaction of DNA/virus with the avian germ cells are described in the next section. These methods are the purification of primordial germ cells from the developing embryo and the propagation of primordial germ cells for use as a cell-based method of transgenesis.

The germ cell lineage in birds is considered to be determined. The maternal determinants (protein, RNA) that specify the germ cell fate are thought to be deposited in the developing oocyte as it matures in the hen. During the initial segmentation of the zygote, these factors (maternal determinants) are segregated into a small number of cells of the forming embryo [71]. Descendants of these early germ cells will give rise to all cells of the germ cell lineage and are referred to as primitive or "primordial" germ cells. Moreover, since the germ cell lineage is segregated from the somatic cell lineage so precociously, chicken embryonic stem cells derived from the blastoderm will not contribute to the germ lineage when reintroduced into early embryos. The primordial germ cells are initially found in the center of the blastoderm of the laid egg [72] (Fig. 3). These cells actively migrate anteriorly in the embryo to an extraembryonic location near the future head, termed the germinal crescent. When the primitive circulatory system forms, the primordial germ cells enter the circulation and are carried to the lateral plate mesoderm adjoining the prospective gonad. During the next 2 days of development, the primordial germ cells migrate through the lateral plate mesoderm and enter the developing gonad. The germ cells subsequently differentiate into the gametes, sperm and eggs, in the



Avian Specific Transgenesis. Figure 3

Germ cell migration in the developing embryo. The primordial germ cells are initially found in the center of the developing blastoderm. From here the cells migrate anterior to the head region. The cells enter the circulation and congregate in the lateral plate adjoining the future gonad. The primordial germ cells subsequently migrate into the forming gonad

maturing gonad. In the hens, the germ cells enter meiosis by day 16 of development. In the adult cockerel, a stem cell population of germ cells remain, the spermatiogonial stem cells, which generate spermatogonia during the life of the bird.

All methods of germ line transgenesis in all species are dependent on the integration of the introduced transgene into the genome of a germ cell, which will subsequently contribute its DNA to the next generation. Any interactions between the exogenously introduced DNA/virus and non-germ-line cells of the animal will lead to a reduction of transmission of the transgene to the F_1 birds. Thus, investigations in the chicken have concentrated on increasing the interaction of the transgenic vector with the germ cells of the founder animal to increase the rate of transgenesis. The next sections detail methods to isolate avian germ cells and increase this interaction.

Germ Cell Purification

The germ cell lineage of birds can be isolated and returned to a host embryo in which these donor germ cells will migrate to the gonad and produce functional gametes [73, 74]. The intrinsic ability of donor germ cells to colonize the host germ cell lineage extends to germ cells from the germinal crescent, the circulatory blood, or post-migratory germ cells in the nascent gonad (Fig. 3). This provides the opportunity to genetically modify the donor germ cells before their introduction into host embryos. The first demonstration of this technique was carried out by Vick [75]. Primordial germ cells from the blood or the germinal crescent were incubated with replication-defective avian leukosis virus and subsequently returned to a host embryo. They observed that 3–23% of founder chickens were transgenic, and these birds transmitted the transgene to 2–4% of F_1 birds.

Several methods have been developed to purify PGCs from the developing embryo before which they are subsequently incubated with virus or transfected to introduce a transgenic vector before being reintroduced into host embryos. Primordial germ cells express the pluripotent stem cell marker, SSEA-1. Using an antibody to SSEA-1, these cells can be purified by either magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS). Both isolation techniques have been optimized for the isolation of thousands of germ cells from the blood or embryonic gonads of chicken embryos. After short periods of incubation, the isolated primordial germ cells still retain the ability to migrate to the gonad and contribute to the germ cell lineage [76]. Similarly, due to the large size of the PGC in comparison to other cells in the blood, it is possible to isolate these cells from the blood using density gradients. Density gradients have been used successfully for the purification of PGCs [77–80].

Kim [49] isolated PGCs from day 5 gonads using MACS. These cells were incubated with a lentivirus for 6 h and reinjected in host embryos. One (of 21) injected cockerels transmitted the transgene to F_1 offspring. A similar approach was used to generate transgenic quail. The rate of transmission from founder to F_1 birds was 1.9%, which was almost equivalent to what was found when the virus was injected under the blastoderm (1.6%) [54]. These results suggest that PGC isolation did not increase rates of transgenesis in comparison to blastoderm injection but further improvements on the interaction of the virus with the germ cells may augment rates of transmission.

Propagation of the Avian Germ Cell Lineage

The in vitro propagation of germ cells offers the prospect to carry out gene targeting in avian species. Both spermatogonial stem cells [81] and gonadal embryonic germ cells [82] have been cultured in vitro for shorter periods (5 and 30 days, respectively) and after culturing have contributed to the germ lineage. Moreover, the cultured gonadal embryonic germ cells generated F_1 offspring. These culture techniques, if extended, could offer the opportunity to carry out gene targeting in chickens. Recently, a breakthrough was described for the culture of primordial germ cells from the circulatory phase of development (Fig. 3). The culture of embryonic blood from day 3 chicken embryos using modified avian embryonic stem cells protocols led to the long-term expansion of primordial germ cells in vitro [83]. These cells contained telomerase activity suggesting that they may be immortal and the transmission of genetic material from both the male and female germ cells to subsequent generations was demonstrated after 110 days in culture

(0.1–86% transmission). The authors genetically modified the cultured primordial germ cells using a transgene flanked by insulator sequences to prevent silencing. Founder birds containing the modified germ cells transmitted the transgene to 1–92% of F_1 birds which accurately expressed the GFP transgene. Important caveats for this procedure were that the derivation efficiency of primordial germ cell lines was low (12%), and this culture technique has not been repeated by other research groups. It was later shown that gene targeting using homologous recombination is also possible in cultured primordial germ cells [84]. This research presents the prospect of carrying out gene targeting in avian species for the production of pharmaceutical recombinant proteins and to investigate the function of genetic pathways that may be involved in production traits by directly modifying these pathways.

Future Directions

The field of avian transgenesis has progressed rapidly in the last 5 years. Useful transgenic chicken models for use in biological studies have been developed [9, 10, 51, 85]. In the near future, it can be expected that a plethora of new transgenic models for both production traits and disease resistance to be generated. These transgenic models will generate impetus for trait selection breeding programs and rational vaccine development, first and foremost in the investigation of avian influenza.

Future uncertainties exist regarding the potential for commercialization of biopharmaceuticals proteins produced in chicken eggs and the acceptance of genetically modified chickens in general. To date, several recombinant human proteins have been produced in chicken eggs at levels up to 1 mg of protein per egg [5–7, 38, 56]. In addition, it remains to be seen if there will be consumer acceptance of genetically modified poultry products and if a functional transgenic chicken product will be brought to "market." The benefits of increased disease resistance and increased feed conversion will need to be weighed against consumer demand and acceptance. These opposing forces will have direct impact on the sustainability of the poultry industry as food demands continue to grow. The issuing of guidelines by the FDA for the regulation of genetically modified animals containing recombinant DNA should facilitate this process [28].

The development of new vectors, both viral and nonviral, for transgenesis should decrease the cost and accelerate the development of new avian transgenic models and the use of nonviral vectors may assuage public fears of transgenic modification of farm livestock. DNA transposons hold great promise as useful vectors for avian transgenesis. The next few years should bring new advances as some of the DNA transposable elements that are showing promise in mammalian transgenesis (Tol2, PiggyBac, Sleeping Beauty) are used for chicken transgenesis.

The greatest advance in avian transgenesis lies in the development of efficient gene targeting of specific genomic loci using homologous recombination. This objective may be accomplished using SSCs, gonadal embryonic germ cells, or primordial germ cells [80–83]. Gene targeting of primordial germ cells appears to be a viable technique [84] although it remains to be proven if this will be a viable method for avian transgenesis. The use of zinc finger nucleases which target specific loci in the vertebrate genome has proven to be useful for gene targeting in both zebra fish and rats [85, 86]. These proteins hold great promise for the potential manipulation of the avian genome. The generation of induced pluripotent cells (IPS cells) from somatic cells also needs to be investigated in avian species [70]. If this technique were further coupled with the conversion to germ cells, it would lay open new possibilities in avian cloning for species rescue.

Primordial germ cell culture also promises to be a viable technology for the preservation of avian germplasm (reviewed in [87]). Semen cryopreservation in avian species has proven problematic and for this reason an alternative method of germplasm cryopreservation is needed. An efficient method to preserve avian genetic resources is of importance for commercial breeders and also rare breed conservationists. The development of a robust method for avian cryopreservation would significantly enhance the biosecurity of the avian breeding industry and greatly reduce animal numbers bred for line maintenance. The use of PGCs could provide an alternative source of cellular material for cryopreservation efforts. At this stage, PGC cryopreservation offers only a slight advantage

over semen cryopreservation for the preservation of single traits. Therefore, what is needed to extend this technology is to develop improved culture conditions for the efficient expansion and cryopreservation of both male and female PGCs from different breeds of chickens. This technology will need to be coupled with an efficient methodology to reconstitute the *complete genome* of avian lines from the cryopreserved cells.

Several recent reports have described transgenic offspring from quail [52, 54] and zebra finches [55] using the genetic techniques described above. To date, no transgenic modifications have been published for turkeys and duck. Since these are also important food production animals, it can be expected that advances in transgenesis will be made in these species in the near future. These potential models will be significant to the study of avian production traits but may also be pertinent to the investigation of avian influenza in which natural disease reservoirs reside in wild duck populations. It will be difficult to foresee if the transgenic technology being developed for the chicken will be easily transferred to other poultry species beyond the use of retroviral vectors. An illustration of foreseeable problems comes from investigations of embryonic stem cells. Derivation of ES cells from mammals excluding the mouse has proven to be exceedingly difficult to achieve [88]. This may also hold true for avian transgenesis.

Bibliography

Primary Literature

1. Houdebine L-M (2009) Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infect Dis 32:2107–2121
2. Lillico SG, McGrew MJ, Sherman A, Sang HM (2005) Transgenic chickens as bioreactors for protein-based drugs. Drug Discov Today 10:3191–3196
3. Council for Agricultural Science and Technology (CAST) (2007) The role of transgenic livestock in the treatment of human disease. Issue Paper 35. CAST, Ames
4. Raju TS, Briggs JB, Borge SM, Jones AJS (2000) Species-specific variation in glycosylation of IgG: evidence for the species-specific sialyation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiology 10:477–486
5. Rapp JC, Harvey AJ, Speksnijder GL, Hu W, Ivarie R (2003) Biologically active human interferon alpha-2b produced in the egg white of transgenic hens. Transgenic Res 12:569–575
6. Lillico SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA, Sang HM (2007) Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc Natl Acad Sci USA 104:1771–1776
7. Koo BC, Kwon MS, Lee H, Kim M, Kim D, Roh JY, Park YY, Cui XS, Kim NH, Byun SJ, Kim T (2010) Tetracycline-dependent expression of the human erythropoietin gene in transgenic chickens. Transgenic Res 19(3):437–447
8. Stern CD (2005) The chick: a great model system becomes even greater. Dev Cell 8:9–17
9. Chapman SC, Lawson A, Macarthur WC, Wiese RJ, Loechel RH, Burgos-Trinidad M, Wakefield JK, Ramabhadran R, Mauch TJ, Schoenwolf GC (2005) Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. Development 132:935–940
10. McGrew MJ, Sherman A, Lillico SG, Ellard FM, Radcliffe PA, Gilhooley HJ, Mitrophanous KA, Cambray N, Wilson V, Sang H (2008) Localised axial progenitor cell populations in the avian tail bud are not committed to a posterior Hox identity. Development 135:2289–2299
11. Zhao D, McBride D, Nandi S, McQueen HA, McGrew MJ, Hocking PM, Lewis PD, Sang HM, Clinton M (2010) Somatic sex identity is cell-autonomous in the chicken. Nature 464:237–242
12. Council for Agricultural Science and Technology (CAST) (2009) Animal productivity and genetic diversity: cloned and transgenic animals. Issue Paper 43. CAST, Ames
13. Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney DJ, Plante C, Pollard DW, Fan MZ, Hayes MA, Laursen J, Hjorth JP, Hacker RR, Phillips JP, Forsberg CW (2001) Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19:741–745
14. Cho J, Choi K, Darden T, Reynolds PR, Petitte JN, Shears SB (2006) Avian multiple inositol polyphosphate phosphatase is an active phytase that can be engineered to help ameliorate the planet's "phosphate crisis". J Biotechnol 126:248–259
15. Tiensin T, Chaitaweesub P, Songserm T, Chaisingh A, Hoonsuwan W et al (2005) Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg Infect Dis 11:1664–1672
16. Wall RJ, Powell A, Paape MJ, Kerr DA, Bannermann SS, Pursel CG, Well KD, Talbot N, Hawk HW (2005) Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat Biotechnol 23:445–451
17. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 77:7380–7384
18. Hammer RE, Pursel VG, Rexroad CE, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
19. Mullins JJ, Peters J, Ganten D (1990) Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. Nature 344:541–544
20. Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R, de Boer H (1991) Generation of transgenic dairy cattle

using 'in vitro' embryo production. Biotechnology (N Y) 9:844–847
21. Kochav S, Ginsburg M, Eyal-Giladi H (1980) From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. II. Microscopic anatomy and cell population dynamics. Dev Biol 79:296–308
22. Perry MM (1988) A complete culture system for the chick embryo. Nature 331:70–72
23. Love J, Gribbin C, Mather C, Sang H (1994) Transgenic birds by DNA microinjection. Biotechnology 12:60–63
24. Naito M, Sasaki E, Ohtaki M, Sakurai M (1994) Introduction of exogenous DNA into somatic and germ cells of chickens by microinjection into the germinal disc of fertilized ova. Mol Reprod Dev 37:167–171
25. Salter DW, Smith EJ, Hughes SH, Wright SE, Fadly AM, Witter RL, Crittenden LB (1986) Gene insertion into the chicken germ line by retroviruses. Poult Sci 65:1445–1458
26. Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB (1987) Transgenic chickens: insertion of retroviral genes into the chicken germ line. Virology 157:236–240
27. Chen HY, Garber EA, Mills E, Smith J, Kopchick JJ, DiLella AG, Smith RG (1990) Vectors, promoters, and expression of genes in chick embryos. J Reprod Fertil Suppl 41:173–1782
28. U.S. Food and Drug Administration (2009) Guidance for industry #187: regulation of genetically engineered animals containing heritable recombinant DNA constructs. Final Guidance
29. Romano G, Marino IR, Pentimalli F, Adamo V, Giordano A (2009) Insertional mutagenesis and development of malignancies induced by integrating gene delivery systems: implications for the design of safer gene-based interventions in patients. Drug News Perspect 22:185–196
30. Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, Jaenisch R (1982) De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature 298:623–628
31. Pannell D, Ellis J (2001) Silencing of gene expression: implications for design of retrovirus vectors. Rev Med Virol 11: 205–217
32. Bosselman RA, Hsu R-Y, Boggs T, Hu S, Bruszewski J, Ou S, Kozar L, Martin F, Green C, Jacobsen F, Nicolson M, Schultz JA, Semon KM, Rishell W, Stewart RG (1989) Germline transmission of exogenous genes in the chicken. Science 243:533–535
33. Thoraval P, Afanassieff M, Cosset FL, Lasserre F, Verdier G, Coudert F, Dambrine G (1995) Germline transmission of exogenous genes in chickens using helper-free ecotropic avian leukosis virus-based vectors. Transgenic Res 4:369–377
34. Harvey AJ, Speksnijder G, Baugh LR, Morris JA, Ivarie R (2002) Expression of exogenous protein in the egg white of transgenic chickens. Nat Biotechnol 20:396–399
35. Mozdziak PE, Borwornpinyo S, McCoy DW, Petitte JN (2003) Development of transgenic chickens expressing bacterial beta-galactosidase. Dev Dyn 226:439–445
36. Kwon MS, Koo BC, Choi BR, Park YY, Lee YM, Suh HS, Park YS, Lee HT, Kim JH, Roh JY, Kim NH, Kim T (2008) Generation of transgenic chickens that produce bioactive human granulocyte-colony stimulating factor. Mol Reprod Dev 75:1120–1126
37. Mizuarai S, Ono K, Yamaguchi K, Nishijima K, Kamihara M, Iijima S (2001) Production of transgenic quails with high frequency of germline transmission using VSV-G pseudotyped retroviral vector. Biochem Biophys Res Commun 286:456–463
38. Kamihira M, Ono K, Esaka K, Nishijima K, Kigaku R, Komatsu H, Yamashita T, Kyogoku K, Iijima S (2005) High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. J Virol 79:10864–10874
39. Koo BC, Kwon MS, Choi BR, Kim JH, Cho SK, Sohn SH, Cho EJ, Lee HT, Chang W, Jeon I, Park JK, Park JB, Kim T (2006) Production of germline transgenic chickens expressing enhanced green fluorescent protein using a MoMLV-based retrovirus vector. FASEB J 20:2251–2260
40. Cockrell AS, Kafri T (2007) Gene delivery by lentivirus vectors. Mol Biotechnol 36:184–204
41. Wang Y, Song Y-T, Liu Q, Liu C, Wang L-L, Liu Y, Zhou X-Y, Wu J, Wei H (2010) Quantitative analysis of lentiviral transgene expression in mice over seven generations. Transgenic Res 19:775–784
42. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295:868–872
43. Pfeifer A, Ikawa M, Dayn Y, Verma IM (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci USA 99:2140–2145
44. Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL (2002) Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci USA 99:14931–14936
45. Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4:1054–1060
46. Ritchie WA, King T, Neil C, Carlisle AJ, Lillico S, McLachlan G, Whitelaw CB (2009) Transgenic sheep designed for transplantation studies. Mol Reprod Dev 76:61–64
47. Hiripi L, Negre D, Cosset FL, Kvell K, Czömpöly T, Baranyi M, Gócza E, Hoffmann O, Bender B, Bősze Z (2010) Transgenic rabbit production with simian immunodeficiency virus-derived lentiviral vector. Transgenic Res Epub:Jan 13
48. Sasaki E, Suemizu H, Shimada A, Hanazawa K, Oiwa R, Kamioka M, Tomioka I, Sotomaru Y, Hirakawa R, Eto T, Shiozawa S, Maeda T, Ito M, Ito R, Kito C, Yagihashi C, Kawai K, Miyoshi H, Tanioka Y, Tamaoki N, Habu S, Okano H, Nomura T (2009) Generation of transgenic non-human primates with germline transmission. Nature 459:523–527
49. McGrew MJ, Sherman AS, Ellard FM, Lillico SG, Gilhooley HJ, Mitrophanous KA, Kingsman AJ, Sang H (2004) Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep 5:728–733

50. Kim JN, Park TS, Park SH, Park KJ, Kim TM, Lee SK, Lim JM, Han JY (2010) Migration and proliferation of intact and genetically modified primordial germ cells and the generation of a transgenic chicken. Biol Reprod 82:257–262
51. McGrew MJ, Lillico SG, Sherman A, Taylor L, Sang H (2010) Functional conservation between rodents and chicken of regulatory sequences driving skeletal muscle gene expression in transgenic chickens. BMC Dev Biol 10:e26
52. Scott BB, Lois C (2005) Generation of tissue-specific transgenic birds with lentiviral vectors. Proc Natl Acad Sci USA 102:16443–16447
53. Poynter G, Lansford R (2008) Generating transgenic quail using lentiviruses. Methods Cell Biol 87:281–293
54. Shin SS, Kim TM, Kim SY, Kim TW, Seo HW, Lee SK, Kwon SC, Lee GS, Kim H, Lim JM, Han JY (2008) Generation of transgenic quail through germ cell-mediated germline transmission. FASEB J 22:2435–2444
55. Agate RJ, Scott BB, Haripal B, Lois C, Nottebohm F (2009) Transgenic songbirds offer an opportunity to develop a genetic model for vocal learning. Proc Natl Acad Sci USA 106:17963–17967
56. Kwon SC, Choi JW, Jang HJ, Shin SS, Lee SK, Park TS, Choi IY, Lee GS, Song G, Han JY (2010) Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. Biol Reprod 82:1057–1064
57. McClintock B (1953) Induction of instability at selected loci in maize. Genetics 38:579–599
58. Feschotte C, Pritham EJ (2007) DNA transposons and the evolution of eukaryotic genomes. Annu Rev Genet 41:331–368
59. Ivics Z, Li MA, Mátés L, Boeke JD, Nagy A, Bradley A, Zsuzsanna I (2009) Transposon-mediated genome manipulation in vertebrates. Nat Meth 6:415–422
60. Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122:473–483
61. Balciunas D, Wangensteen KJ, Wilber A, Bell J, Geurts A, Sivasubbu S, Wang X, Hackett PB, Largaespada DA, McIvor RS, Ekker SC (2006) Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. PLoS Genet 2:e169
62. Sherman A, Dawson A, Mather C, Gilhooley H, Li Y, Mitchell R, Finnegan D, Sang H (1998) Transposition of the Drosophila element mariner into the chicken germ line. Nat Biotechnol 16:1050–1053
63. Sato Y, Kasai T, Nakagawa S, Tanabe K, Watanabe T, Kawakami K, Takahashi Y (2007) Stable integration and conditional expression of electroporated transgenes in chicken embryos. Dev Biol 305:616–624
64. Lu Y, Lin C, Wang X (2009) PiggyBac transgenic strategies in the developing chicken spinal cord. Nucleic Acids Res 37:e141
65. Petitte JN, Clark ME, Liu G, Verrinder-Gibbins AM, Etches RJ (1990) Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development 108:185–189
66. Etches RJ, Clark ME, Toner A, Liu G, Gibbins AM (1996) Contributions to somatic and germline lineages of chicken blastodermal cells maintained in culture. Mol Reprod Dev 45:291–298
67. Pain B, Clark M, Nakazawa H, Sakurai M, Samarut J, Etches R (1996) Long-term in vitro culture and characterization of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122:2339–2348
68. Bezzubova O, Silbergleit A, Yamaguchi-Iwai Y, Takeda S, Buerstedde JM (1997) X-ray resistance and homologous recombination frequencies in a RAD54-/- mutant of the chicken DT40 cell line. Cell 89:185–193
69. van de Lavoir MC, Mather-Love C, Leighton P, Diamond JH, Heyer BS, Roberts R, Zhu L, Winters-Digiacinto P, Kerchner A, Gessaro T, Swanberg S, Delany ME, Etches RJ (2005) High-grade transgenic somatic chimeras from chicken embryonic stem cells. Mech Dev 123:31–41
70. Lavial F, Acloque H, Bachelard E, Nieto MA, Samarut J, Pain B (2009) Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate. Dev Biol 330:73–82
71. Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T (2000) Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development 127:2741–2750
72. Eyal-Giladi H, Ginsburg M, Farbarov A (1981) Avian primordial germ cells are of epiblastic origin. J Embryol Exp Morphol 65:139–147
73. Simkiss K, Rowlett K, Bumstead N, Freeman BM (1989) Transfer of primordial germ cell DNA between embryos. Protoplasma 151:164–166
74. Chang IK, Jeong DK, Hong YH, Park TS, Moon YK, Ohno T, Han JY (1997) Production of germline chimeric chickens by transfer of cultured primordial germ cells. Cell Biol Int 21:495–499
75. Vick L, Li Y, Simkiss K (1993) Transgenic birds from transformed primordial germ cells. Proc Biol Sci 251:179–182
76. Mozdziak PE, Wysocki R, Angerman-Stewart J, Pardue SL, Petitte JN (2006) Production of chick germline chimeras from fluorescence-activated cell-sorted gonocytes. Poult Sci 85:1764–1768
77. Tajima A, Naito M, Yasuda Y, Kuwana T (1993) Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus). Theriogenology 40:509–519
78. Ono T, Machida Y (1999) Immunomagnetic purification of viable primordial germ cells of Japanese quail (Coturnix japonica). Comp Biochem Physiol A Mol Integr Physiol 122:255–259
79. Zhao DF, Kuwana T (2003) Purification of avian circulating primordial germ cells by nycodenz density gradient centrifugation. Br Poult Sci 44:30–35
80. Park TS, Jeong DK, Kim JN, Song GW, Hong YH, Lim JM, Han JY (2003) Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos. Biol Reprod 68:1657–1662

81. Kalina J, Senigl F, Micáková A, Mucksová J, Blazková J, Yan H, Poplstein M, Hejnar J, Trefil P (2007) Retrovirus-mediated in vitro gene transfer into chicken male germ line cells. Reproduction 134:445–453
82. Park TS, Hong YH, Kwon SC, Lim JM, Han JY (2003) Birth of germline chimeras by transfer of chicken embryonic germ (EG) cells into recipient embryos. Mol Reprod Dev 65:389–395
83. van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ (2006) Germline transmission of genetically modified primordial germ cells. Nature 441:766–769
84. Leighton PA, van de Lavoir MC, Diamond JH, Xia C, Etches RJ (2008) Genetic modification of primordial germ cells by gene trapping, gene targeting, and phiC31 integrase. Mol Reprod Dev 75:1163–1175
85. Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol 26:702–708
86. Geurts AM, Cost GJ, Rémy S, Cui X, Tesson L, Usal C, Ménoret S, Jacob HJ, Anegon I, Buelow R (2010) Generation of gene-specific mutated rats using zinc-finger nucleases. Methods Mol Biol 597:211–225
87. Petitte JN (2006) Avian germplasm preservation: embryonic stem cells or primordial germ cells? Poult Sci 85:237–242
88. Muñoz M, Trigal B, Molina I, Díez C, Caamaño JN, Gómez E (2009) Constraints to progress in embryonic stem cells from domestic species. Stem Cell Rev 5:6–9

Books and Reviews

Nieuwkoop PD, Sutasurya LA (1979) Primordal germ cells in the chordates. Cambridge University Press, Cambridge

Petitte JD (2004) Isolation and maintenance of avian ES cells. In: Handbook of stem cells, vol 1, Embryonic stem cells. Elsevier, Amsterdam, pp 471–479, Chapter 44

Pfeifer A, Hofmann A (2009) Lentiviral transgenesis. Methods Mol Biol 530:391–405

Walsh G (2003) Biopharmeceuticals: biochemistry and biotechnology, 2nd edn. Wiley, Chichester

Biomass Crops for Biofuels and Bio-based Products

Elizabeth E. Hood[1], Keat (Thomas) Teoh[2], Shivakumar P. Devaiah[2], Deborah Vicuna Requesens[2]
[1]College of Agriculture and Technology, Arkansas State University, AR Biosciences Institute, Jonesboro, AR, USA
[2]Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Article Outline

Glossary

Agriculturally marginal land Land that is not arable without compromising soil stability and increasing salinity. It requires large number of inputs.

Bio-based products Products derived from biological sources as opposed to oil.

Bioenergy Energy derived from biological, renewable sources – not from petroleum.

Biofuel Fuels derived from biological sources.

Biomass Materials derived from plant or animal origin, i.e., dedicated agriculture, agricultural residues, municipal waste, and forestry.

Corn stover The remaining stalks and leaves of the corn plant after the grain has been removed.

Dedicated energy crops Crops grown only for use in the biofuels industry, e.g., switchgrass and *Miscanthus*, poplar and *Eucaly*ptus.

Herbaceous Plant materials that have green, nonwoody above-ground parts rather than lignified stems and those parts generally die at the end of the growing season.

Lignocellulose Cell wall materials from plants that contain cellulose, hemicellulose, and lignin.

Megagram (Mg) A unit of mass equal to 1,000,000 g. Also referred to as a metric ton (US)

Perennial Plants that persist for multiple years and often have woody stems.

Renewable resources Materials and products derived from plants that are generated through growth using energy from sunlight and nutrients from soil.

Sustainable Materials that can be maintained through inputs that are equal or less than harvested output.

Water use efficiency The amount of biomass produced from photosynthesis compared to the amount of water taken up by the plant. C4 plants are more efficient in photosynthetic sugar production per CO_2 taken in, thus lowering the amount of water required to obtain the CO_2 and thus the sugars.

Definition of the Subject and Its Importance

Humans currently consume at least 25% more raw materials every year than are replaced through biological growth [150]. In order to sustain quality of life and have adequate environmental resources, those resources must be balanced and renewable. Pressure on those resources has never been greater with the world population nearing seven billion people, and estimated to plateau at 10.5 billion by 2050. That number represents 35–40% more people than currently inhabit the earth.

Sustainable, renewable resources are those derived from biological sources, primarily plant biomass. The underlying principal is that the materials can be reproduced with minimal inputs using energy from the sun. Biomass is thus derived directly or indirectly from original sources that grow and reproduce biologically.

Biomass for biofuels and bio-based products can include many sources of material. In general, biomass includes any biological materials whether of plant or animal origin: agricultural harvests such as grains, agricultural residues such as stalks and leaves, perennial crops such as hay and trees, animal manures, building waste wood, municipal solid waste such as paper, and various food industry wastes.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Introduction

The heavy reliance of the western economies on fossil fuels has given rise to energy security concerns. These concerns taken together with the sustainability issue, negative environmental impacts, and the rising cost of petroleum fuel have prompted the development of viable alternatives that are sustainable, cleaner, and environmentally neutral. Biofuels, which have emerged as one of the alternatives to address these concerns, are obtained from renewable biomass which represents a key long-term component of a sustainable biofuels industry. Ethanol from corn is the first-generation biofuel produced in USA and Europe. However, corn ethanol alone will not be sufficient to address the nation's energy needs. Cellulosic ethanol from renewable resources such as forest and agricultural biomass must also be considered. The "Billion Ton Study" supported by the US Department of Energy (DOE) and US Department of Agriculture (USDA) estimated that renewable resources are available from forest and agricultural lands to produce enough cellulosic ethanol to displace 30% or more of transportation fuel needs annually [1]. This will significantly reduce the country's dependence on imported oil.

This review deals only with plant-based crops that are used as biomass sources. Crop categories discussed include agricultural residues, dedicated woody crops – poplar and *Eucalyptus*, perennial herbaceous grasses – *Miscanthus* and switchgrass, and grass species that produce soluble sugar streams – sweet sorghum and sugarcane. Although many other crop sources are generally considered as potential sources of biomass, these are the front line crops that are considered to be "near term" candidates.

Agricultural Residues for Bioenergy

Availability of Feedstock

The Billion Ton Study on biomass estimated that 388 megagrams (Mg) of agricultural postharvest residues per year could be available from agriculture for conversion to energy resources in the USA within the next 20 years [1]. The estimated crop residues were primarily from corn stover, wheat straw, rice straw, and hulls as well as other crops with lesser individual contributions. The estimated amount of ethanol that can be derived from crop residues totals 138 billion liters assuming 356 L Mg^{-1} (85 gal t^{-1}). Additionally, the study made the assumptions to support three different scenarios for productivity. The first scenario was to maintain the status quo for all resources available today, and those would be available at the same level in the future. The second scenario focused on technology changes being applied to current crops to generate higher yields. The third and most lucrative scenario assumed that the technology changes would be applied to current crops as well as new perennial crops, combined with significant land use changes. The 388 Mg estimate is based on the third scenario.

One of the major contributors to crop residue potential in the USA is corn stover, whether under current production or assuming increased crop yields [1]. Stover can be used for many bio-based products, and the use of corn stover for those products would remove it from its contribution to liquid transportation fuels. Some of these current and potential uses include pulp and paper, animal feed, composite products such as boards, and chemicals, such as furfural [2].

Impact on soil fertility: Crop residues contribute an interesting array of benefits to the soil from which grain is harvested. These benefits include lowering soil erosion, increasing moisture content, and increasing soil organic matter and total carbon. When removing crop residues from agricultural land, the loss of these benefits must be managed [3].

However, in some situations removal of residues is recommended because they cover too much of the surface and prevent warming of the soil in the spring, delaying seed germination and thus lowering yields (Table 1; [4, 5]). Moreover, too much organic matter on the surface of the soil also can increase moisture and the threat of increased fungal growth, contributing to diseases. Clearly, the issues vary with location and crop. Each situation will have a unique solution.

The most important practice that will need to be adopted for residues to be sustainably removed from crop land is no-till agriculture. Currently, only 20% of corn acreage and 15% of wheat acreage are cultivated with no-till practices [5]. Tillage increases the rate at which organic matter is decomposed and increases

Biomass Crops for Biofuels and Bio-based Products.

Table 1 Potential effects of corn stover harvest [4]

Factor	Benefits of removal	Cost of removal
Economic	Stover sale revenues ($35/t);	Yield decreases in dry years due to lower soil moisture
	Greater seed germination in colder climates	Yield decreases with increased soil loss; Poorer germination but no yield effect
Fossil fuel use	Increased EtOH production	More field passes required; Fossil fuel needed for conversion to biofuel
Micro-climate	Warmer spring temperatures	Increased evaporation, lower soil moisture
Pests and disease	Increased control for some	Decreased control of others
Carbon and nutrients	Decreased but moderated by tillage and N rate	Nutrient loss predicted greatest in Midwest
Erosion	Moderated by amount of harvest and tillage type	Increased soil loss and water run-off

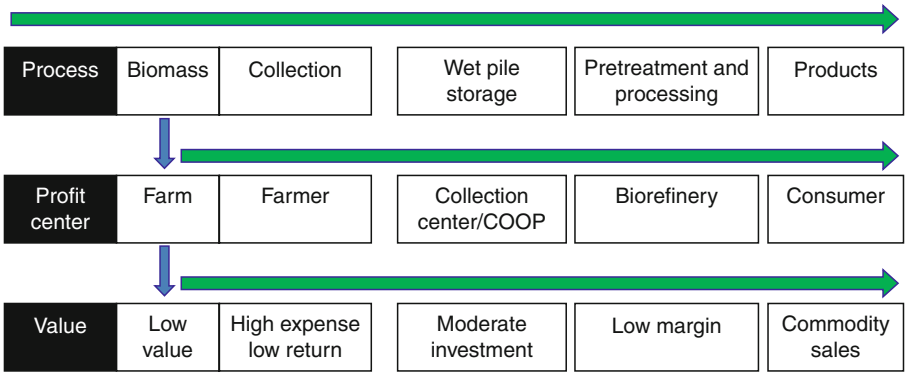
nitrogen losses due to microbial activity. No-till cultivation allows more of the remaining residue to be removed for alternative uses.

Farmer involvement: One of the major concerns with agricultural residues being used for a reliable supply of biomass is compliance of the farmer for collection and delivery of the residues. Because the residues vary by year and region, management of the residues requires consideration of many factors including maintenance of soil fertility, weather, crop yields, and economics.

The average age of US farmers is 62. This is both an advantage and a disadvantage. The collective experience of farmers is tremendous – management practices are based on their extensive knowledge. In contrast, their retirement is eminent and their replacement uncertain. Their motivation to try new crops is often high though their approach is conservative. Although most farmers may be willing to try new crops, they will approach the change cautiously, planting only a few hectares in the beginning until yield and economics are understood [5]. If the results are economically favorable, in that inputs are lower than harvested material profits, the farmers are often willing to change their practices and participate in the new system. In directed dialogs conducted in 2000–2002, farmers generally agreed that US$20 ha^{-1} pre-tax margin would generate their interest in harvesting residues, as long as grain harvesting is not hindered [5]. The value of the residues is not near to the value of the grain, thus logistics and income must make it worth the farmer's time and investment to participate in the residue harvest. The industry estimate for biomass that is delivered to biorefineries is US$30–$50 Mg^{-1} in order to produce commodity ethanol at target prices. However, in order for the farmer to reap a benefit considering the equipment, time, and field issues, the price would have to approach US$70–$100 Mg^{-1}. Clearly, policy and logistics research must address this discrepancy.

Logistics and economic issues: Small grains, mostly wheat, produce straw that can be baled and stored. However, the amount available from nonirrigated wheat is minimal after leaving USDA recommended amounts of residue on the ground to improve fertility. If the average dry land wheat straw yield is 2.7 Mg ha^{-1}, and 1.34 Mg ha^{-1} must be left on the surface to avoid erosion, then less than 2.5 Mg ha^{-1} of straw would be available for harvest [5]. Because corn stover yields about 11 Mg ha^{-1}, leaving 2.24 Mg ha^{-1} on the soil surface for organic matter and erosion control, leaves 8.8 Mg ha^{-1} for harvest. However, in each case if a cover crop is employed, the residues are not required for erosion control and can be harvested in larger amounts.

Because wheat is harvested early in the summer, the straw has low moisture content and can be immediately baled. In contrast, collecting residues as feedstocks from corn fields in particular requires altering harvesting practices. Corn is harvested in the fall and the stover moisture content is usually 30–50%, and must be left in the field to dry, although often fall rains prevent this from happening in a timely manner. In addition, if stover is harvested separately after grain harvest, field machinery must be deployed a second time and this compacts the soil more. The stover that is raked and baled also



Biomass Crops for Biofuels and Bio-based Products. Figure 1
Agricultural residue value chain. Residue collection and processing must yield value to each link in the chain for the process to be instituted. One solution to this is to have the farmer involved in the collection and storage of the residues to be sold to the biorefinery during the production year

contains rocks and soil particles which have a negative impact on biorefinery machinery. These issues suggest that one-pass harvesting is an alternative that must be considered.

A detailed description of grain and stover harvesting logistics was recently published [5]. The additional requirements for trucks, combines, and potentially one-pass harvesters, baling equipment, and additional personnel are enormous. The personnel needs are transient in that the harvest window is usually 30–50 days. However, the investment in additional equipment, whether purchased or leased is significant. Harvesting stover while harvesting corn grain requires three times the number of trucks and personnel if using a one-pass harvester. Although this equipment is not yet commercially available, several agricultural machinery manufacturers are designing these harvesters. Because most of the stover currently is either left in the field, tilled under, or used for bedding, or local markets (e.g., corn cobs), baling equipment is not adequate in most farm operations to handle a large increase in biomass harvest. One possible solution could be transporting the field materials to a collection station, possibly associated with a grain elevator operation, and the stover separated from the grain at these off-field locations. The stover then could be stored either as stacked bales or as a wet pile. Bales require protection from the elements, they are dry when baled and must remain dry, at less than 20% moisture, to keep them from deteriorating. In addition, the height of the stacks is limited by the weight and heat that is generated, thus requiring large amounts of field space. However, if the biomass is in compacted, anaerobic, wet piles at greater than 60% moisture, fungal growth is inhibited and the biomass is very stable [5]. The pile size is only limited by the height of a pump head that continuously recycles the water from a base collection reservoir. This configuration has the added advantage of washing contaminants out of the biomass that were collected from the field. These "haystacks" are large and much higher than bale stacks and thus have far less of a footprint on the ground, minimizing field requirements. Wet storage is a proven industry logistic in that the nonwood fiber pulpers moved to this method of feedstock storage several decades ago.

Recommendations: The production of ethanol from corn stover as opposed to grain has significantly more greenhouse gas reduction potential, 79% versus 25%, when burning E85 (85% ethanol with 15% gasoline) on a per km basis [4, 6]. This fact alone motivates development of logistics and infrastructure solutions to develop the industry for all concerned. Nevertheless, county by county plans will be required to sustainably harvest agricultural residues from any crop, based on climate, tillage, and residue type. Farmer networks should be included as part of the value chain to encourage participation (Fig. 1). Small biomass facilities,

including portable facilities, could be a positive development for on-farm production of ethanol from excess residues without investment in large biorefinery infrastructure.

Perennial and Annual Herbaceous Biomass Crops

Sugar Crops

Introduction Sugar cane and sweet sorghum which produce sugary syrup in their stems are members of the Panicoid subfamily of the *Poaceae* family [7]. They share the physiologically distinctive and highly productive C4 photosynthetic pathway. The C4 pathway is fundamentally more efficient than the C3 classic Calvin cycle alone and C4 plants are able to convert up to 2% of incident solar energy into biomass [8]. Compared to C3 plants, C4 plants lose less water as they can photosynthesize with stomata nearly closed, thus reducing water loss to the environment and increasing water use efficiency. In addition, plants using the C4 photosynthetic pathway are better equipped to handle high temperatures, drought, and nitrogen limitations than closely related C3 plants [9, 10].

Sugar from sugar-producing plants can be used for direct fermentation into ethanol. Among three major sugar-producing plants, sugar cane and sweet sorghum are adapted to warm temperate to tropical areas, whereas sugar beet is grown only in temperate areas. Sugar cane is the major crop in the Brazilian national ethanol program which produces 15.9 billion liters of ethanol a year [11]. Sweet sorghum is considered to be one of the promising feed stocks for the production of first-generation ethanol. Studies are being conducted to produce ethanol from sweet sorghum sugary syrup in the USA, India, and China [12–14]. Most ethanol production using sugar beet takes place in Europe; however, using sugar beet to produce ethanol could potentially increase soil erosion and lower net energy balance [15]. All the three sugar-producing plants are a good source for first-generation ethanol production. Compared to sugar beet, both sugar cane and sweet sorghum produce higher biomass and additionally are a good source of lignocellulosic biomass which can also be used for second-generation ethanol production.

Sugar Cane Crystallized sugar from sugar cane was reported in India 5,000 years ago [16]. Sugar cane is a tall perennial grass of the genus *Saccharum*, native to warm temperate to tropical regions of Asia. The plant grows in clumps, and has solid, jointed, fibrous stalks that are rich in sugar. Sword-shaped leaves, similar to those of the corn plant, fold in a sheath around the stem. Mature canes may be 3–6 m tall and 2.5–7.5 cm in diameter (Fig. 2a). All sugar cane species interbreed, and the major commercial cultivars are complex hybrids. Different species likely originated in different locations with *S. barberi* originating in India and *S. edule* and *S. officinarum* coming from New Guinea. Sugar cane is grown in over 110 countries with an estimated total production of 1,627 million Mg in 2009, (FAOSTAT, http://faostat.fao.org/site/339/default.aspx) more than six times the output of sugar beet. Brazil is the world's largest producer of sugar cane, producing about one-third of the world's crop, followed by India (FAOSTAT).

Cultivation: Sugar cane cultivation requires a tropical or temperate climate, with a minimum of 60 cm of annual moisture. In prime growing regions of India, Peru, Brazil, Bolivia, Colombia, Australia, Ecuador, Cuba, Philippines, El Salvador, and Hawaii, sugar cane can produce 20 kg m^{-2} biomass exposed to the sun. Although sugar cane species produce seeds, sugar cane propagation is through stem cuttings of immature canes 8–12 months old, called "setts." The setts are best if taken from the upper third of the cane because the buds are younger and less likely to dry out. Each sett must contain at least one bud. The setts can be planted at a 45° angle or laid horizontally in a furrow. It takes 12,500–20,000 setts to plant 1 ha. The setts are lightly covered with soil until they sprout (10–14 days) and then the sides of the furrow are turned inward [17, 18]. In the USA and Australia, billet planting is common. Billets harvested from a mechanical harvester are planted by a machine which opens and recloses the ground.

Sugar cane is a perennial crop which usually produces harvests for about 3–6 years before being replanted. The first crop is called the "plant crop" and takes 9–24 months to mature, depending on location [18, 19]. The cane is cut close to the ground because the lower stem has the highest sugar content and low cuts aid in ratooning, the emergence of new crops from the stems and trash (leaves and tops) left behind. Ratoon crops take about 1 year to mature.

Biomass Crops for Biofuels and Bio-based Products. Figure 2
(**a**) Sugar cane. (**b**) Sweet Sorghum

As many as four or more ratoon crops may be produced before replanting is necessary, mostly due to the slow decline in yields.

The complete sugar cane crop cycle is variable, depending on local climate, varieties, and cultural practices. In Brazil, usually it is a 6-year cycle, in which five cuts, four ratoon cultivation treatments, and one field reforming are performed. Generally, the first harvest is made 12 or 18 months after planting. The following ratoon cane harvests are made once a year, during 4 consecutive years [20].

Sugar cane is harvested by hand and mechanically. Hand harvesting accounts for more than half of the production, and is dominant in the developing world. Mechanical harvesting uses a sugar cane combine, a harvesting machine that can harvest 100 Mg each hour, but machine-harvested cane must rapidly arrive at the processing facility. Once cut, sugar cane begins to lose its sugar content, and damage to the cane during mechanical harvesting accelerates this decline. Some sugar cane varieties are known to be capable of fixing atmospheric nitrogen in association with a bacterium, *Acetobacter diazotrophicus*. Unlike legumes and other nitrogen fixing plants which form root nodules in the soil in association with bacteria, *A. diazotrophicus* lives within the intercellular spaces of the sugar cane's stem [21].

Breeding: The goal of cane breeding is to produce an economic yield of sugar that can be sustained over several ratoons. Breeding and selection of cane are not simple processes since viable seeds are seldom produced. Sugar canes are highly polyploid, wind-pollinated outbreeders. They are clonally propagated, highly heterozygous, and intolerant to inbreeding. New varieties are sought from the first-generation progeny of crosses between clones. Five species are of interest to cane breeders. *S. officinarum* ($2n = 80$) has good sugar quality and low fiber, although it is susceptible to most

of the main diseases, except gumming disease and smut. *S. spontaneum* ($2n$ = 40–128) is a source of resistance to many diseases, including "Sereh," mosaic, gumming, red rot, and downy mildew. *S. barberi* ($2n$ = 82–124) are considered the most important breeding canes and are immune to gumming and mosaic and resistant to downy mildew, but susceptible to smut and red rot. *S. sinense* ($2n$ = 82–124) is difficult to breed, but has given rise to some useful breeding lines. *S. robustum* ($2n$ = 60–194) has been used to some extent in breeding lines [22].

Yields: The annual global production of dry cut sugar cane (sugar content: 55% dry basis) is about 328 million Mg. Asia is the primary production region, which produces 44% of the total. South America stands second with a total production of 110 Tg of sugar cane (34%). The annual yield of dry sugar cane ranges from 14 to 22 Mg ha^{-1} with an average of 17 Mg ha^{-1}. Brazil is the largest single producer of sugar cane with about 27% of global production and a yield of 18 Mg ha^{-1}. The highest yield occurs in Peru, which produces more than 32 Mg ha^{-1} of dry sugar cane [23].

Diseases and pests: Many diseases and pests affect sugar cane. Bacterial diseases include gumming disease caused by *Xanthomonas vasculorum* (Cobb) Dows. Yellowish stripes occur at the leaf tips, leaf blisters occur, and the vascular bundles exude a yellowish gum when cut. *X. albilineans* (Ashby) Dows causes yellow stripes to occur on the leaf blade, many side-shoots are produced, and the vascular bundles of the stalk are red [24]. Fungal diseases such as red rot (*Colletotrichum falcatum* Went), root rot (*Pythium graminicolum* Subr.), pineapple disease (*Thielaviopsis parodoxa* (de Seynes) C. Moreau), downy mildew (*Sclerospora sacchari* Miy), and smut (*Ustilago scitaminea* Syd.) can also cause damage. Red rot causes the setts to be seriously damaged at low temperatures. Root rot was responsible for the failure of "Otaheite" (a noble cane) in Mauritius in 1846. Downy mildew is currently only found in the western Pacific and was responsible for severe losses in Queensland until rigorous controls were initiated. Smut causes black whiplike organs to emerge from the center of the leaf-roll and affects crops in southeastern Asia and South Africa [18]. Mosaic is a viral disease, whose vectors include *Aphis maidis* Fitch. It was first documented in Java in 1892 and causes severe stunting in some cases. Other viral diseases include ratoon stunting, chlorotic streak, Fiji disease, and Sereh disease. The most destructive insects of sugar cane are stem-borers, the larval stage of several genera of moths. The larvae burrow into the stem and on emergence cause weakened stems and loss of sucrose. Biological control and use of transgenic *Bt* sugarcane are the most efficient control for these insects [18].

Commercial and industrial use: Sugar cane has many industrial uses and is one of the most widely used and cheapest sources of domestic products, including table sugar, molasses, and ethanol. Cane juice contains 10–20% sucrose and about 1 Mg ha^{-1} of raw sugar can be extracted from 8 to 9 Mg ha^{-1} of cane. Molasses is a by-product of the manufacturing of cane sugar. It is residual syrup from which no more crystalline sucrose can be obtained by simple techniques. Approximately 2.7% of cane can be extracted as molasses, which can be used as an animal feed as it has high carbohydrate contents. Molasses along with cane juice and other by-products can be fermented to produce an alcoholic distillate, rum, or similar liquors. Ethanol is also produced from molasses, which can be used in vinegar, cosmetics and pharmaceuticals, cleaning preparations and solvents, and coatings [16]. One of the important uses of ethanol is as a transportation fuel (see below). Still other products from molasses are butanol (a solvent), lactic acid (a solvent), citric acid (mostly for foods and beverages), glycerol, and yeast media [25].

Sugarcane as a bioenergy crop: Sugar cane being a C4 plant is one of the most efficient photosynthesizers in the plant kingdom [26]. Sugar cane's high concentration of sugar, which is readily available to microorganisms, makes it uniquely suitable for ethanol production. As a producer of sugar for fermentation bio-products, sugar cane is found to have advantages in relation to fossil-energy input, emissions of greenhouse gases, and possibly acidification, when compared with corn and sugar beet [15]. Another useful by-product of sugar production is bagasse, the fibrous residue left after the juices are extracted from the cane. It is the main source of fuel in sugar factories. It can also be used in paper, cardboard, fiber board, and wall board [27]. It is quite possible that further uses of sugar cane will be developed in the future, but even now it can be seen that sugar cane is a very important and useful plant crop worldwide. Uses of sugar cane in different

Biomass Crops for Biofuels and Bio-based Products. Table 2 Uses of sugar cane [23]

	Feed (%)	Seed (%)	Waste (%)	Food manufacture (%)	Food (%)	Other uses (%)
Africa	0.14	2.02	2.12	89.43	4.44	1.85
Asia	3.14	4.68	1.13	86.19	4.57	0.30
Europe	0.18	0.00	0.00	87.90	0.00	11.92
North America	0.00	5.37	0.00	94.62	0.00	0.00
Central America	1.80	0.25	1.06	95.40	0.05	1.45
Oceania	0.00	0.00	0.00	99.99	0.01	0.00
South America	0.98	0.00	0.68	97.83	0.27	0.24
World	1.91	2.35	0.97	91.88	2.40	0.48

parts of the world are shown in Table 2. The resulting energy and greenhouse gas benefits of sugar-cane-derived products have been shown previously [28]. For sugar cane, the main co-product is surplus energy from bagasse. The most successful story of using sugar cane as a bioenergy feed stock is in Brazil. Brazil is considered to have the world's first sustainable biofuels economy and is the biofuels industry leader. Its sugar cane ethanol program is considered a model for other countries and is the most successful alternative fuel program to date. Brazil's 40-year-old ethanol fuel program is based on the most efficient agricultural technology for sugar cane cultivation in the world. It uses modern equipment and cheap sugar cane as feedstock. The residual cane-waste (bagasse) is used to produce heat and power, which results in a very competitive price and a high-energy balance (output energy/input energy). This energy balance varies from 8.3 for average conditions to 10.2 for best practice production. Brazil is recognized as the world's second-largest producer of ethanol (DOE-EIA, 2007, http://www.eia.doe.gov/emeu/cabs/Brazil/full.html). Brazil and the USA lead the world in global ethanol production, accounting for nearly 70% of the world's production. Brazil produces approximately 37% of the world's total ethanol and 48% of the world's ethanol uses as fuel. They began promoting the production of crops for ethanol in the mid-1970s after the first global energy crisis. In 2008, 454,000 barrels per day (bbl/day) of ethanol were produced, up from 365,000 in 2007. Because ethanol production continues to grow faster than domestic demand, Brazil has sought to increase ethanol exports, becoming the largest ethanol exporter in the world, holding over 90% of the global export market. According to industry sources, Brazil's ethanol exports reached 86,000 bbl/day in 2008, with 13,000 bbl/day going to the USA (DOE-EIA, 2007).

Sugar cane bagasse is the residue obtained after crushing of sugar cane during sugar production and consists of cellulose 43.6%, hemicellulose 33.8%, lignin 18.1%, ash 2.3%, and wax 0.8% on a dry weight basis [29]. About 180–280 kg ha^{-1} of sugar cane bagasse could be produced after squeezing [30]. The second-generation process revolves around accessing the large amounts of cellulosic material blocked within the lignin-based shell and creating ethanol from it [31]. Currently the leftover bagasse is burned to co-generate power. In the future, the process should be similar, but instead of burning all of the bagasse, the material will be sorted so that the cellulose and hemicellulose are processed further into cellulosic ethanol with only the remaining high-lignin content materials being used as an alternative energy source. The move toward utilizing sugar cane bagasse for the production of ethanol instead of power generation is a question of economics. The current process of burning the bagasse for energy is not as energy efficient as using it to produce ethanol. Theoretically, 1 Mg of sugar cane bagasse can produce up to 300 L of ethanol. In reality the yield depends on a number of parameters including efficiency of the process. Currently, 6,000–7,000 L of ethanol is produced from 1 ha of sugar cane-not including the bagasse. When bagasse can be utilized for ethanol production, the output could double to 12,000–15,000 L ha^{-1} [31].

Sweet Sorghum Sweet sorghum is a C4 crop in the grass family belonging to the genus *Sorghum,* which also includes grain and fiber sorghum and is characterized by high photosynthetic efficiency. Sweet sorghum is of interest as a dedicated agricultural energy crop because of its drought tolerance, relatively low input requirements and ability to produce high yields under a wide range of environmental conditions [12, 32]. These traits make sweet sorghum a potentially important feedstock for bioenergy production, mainly in regions where conditions are not favorable for growing starch-rich crops such as maize. The great advantage of sweet sorghum is that it can become dormant under adverse conditions and can resume growth after relatively severe drought which has implications for crop management. Sweet sorghum is typically an annual, but some cultivars are perennial. Like other sorghum types, sweet sorghum probably originated from East Africa and spread to other African regions, Southern Asia, Europe, Australia, and the USA. Plants grow in clumps and height of stalks ranges from 0.8 to 4 m (Fig. 2b). The thickness of stalks also varies, ranging between 1.25 cm and 3.75 cm. Prop roots regularly grow from the lower nodes. Seeds are produced by self-pollination from a panicle that emerges at the top of the plant and contains both the male and female inflorescences. Seeds are small, round, and may be white, yellow, brown, or red in color. Each panicle can produce up to 4,000 starch-containing grains. Although native to the tropics, sweet sorghum is well adapted to temperate climates. Like its close relative sugar cane (*Saccharum* spp.), sweet sorghum has been selected to accumulate high levels of edible sugars in the stem. Sweet sorghums are tall and produce high biomass in addition to sugar. In all varieties, the primary carbohydrate is sucrose, with variable amounts of reducing sugars and starch [33].

Cultivation: Sweet sorghum is very drought resistant and shows good adaptability to poor soil types including saline soils. It has a very short vegetation period and thus is ideal for double cropping, either three crops of sweet sorghum or with an alternative crop [13]. Propagation is accomplished through seeds. It is easily grown in areas that are too dry for maize. Seeds are typically sown in widely spaced rows (75–100 cm) manually or using a corn planter in spring after the rainy season and as soon as the soil temperature remains above 15–18°C. The ideal seeding rate for most sweet sorghum varieties is 3–4 seeds per foot of row with a final stand of 2–3 plants per foot of row. If plant populations are too high, the canes will be spindly and contain less juice than an equal tonnage of larger diameter canes. Seed germination takes place within 24 h in warm and moist soils, and the time to maturity lies between 90 and 120 days. The plant grows to a height of from about 1.2 to above 4 m, depending on the varieties and growing conditions. Even though sweet sorghum is predominately self-pollinating, hybrids and crosses can be produced using male-sterile maternal parents. Sugar content in the juice increases with maturity, and is low prior to seed development. Controlling nitrogen fertilizer and its application time promotes sucrose content and growth rate in sweet sorghum. Application of adequate amounts of K fertilizer increases yield responses more than high levels of nitrogen fertilizer alone [34]. Currently, the only commercially viable harvest method for sweet sorghum is removing the entire crop with a forage harvester and transporting it to a mill/pretreatment/ethanol facility. Bennett and Anex [12] indicate that fermentable carbohydrates can be produced at less expense from sweet sorghum than from corn grain. Further results on costs associated with off-farm transportation, storage, or capital costs associated with milling and energy recovery equipment reevaluates sweet sorghum as a biocommodity feedstock [12].

Breeding: Roughly 4,000 cultivars of sweet sorghum are distributed throughout the world [35] providing a diverse genetic base from which to develop regionally specific, highly productive cultivars. In addition to producing large amounts of sugar-rich biomass, hybrids can be developed from crosses between grain-type seed parents and sweet-type pollen parents [36]. The product of these crosses typically increases biomass yields and sugar content when compared to the original grain-type seed parents. Such hybrids can co-produce grain at levels approaching the yields of the grain-type seed parent [37]. The co-produced, protein-rich grain can be consumed as food, animal feed, or converted to bioproducts like ethanol [13, 36]. Proper variety selection will play a large role in the success of sweet sorghum production for ethanol. The ideal variety for a particular location should produce high yields with minimal inputs, have a high percentage of high quality

Biomass Crops for Biofuels and Bio-based Products. Table 3 Uses of sweet sorghum [23]

	Feed (%)	Seed (%)	Waste (%)	Food manufacture (%)	Food (%)	Other uses (%)
Africa	6.90	2.01	13.02	5.21	72.76	0.11
Asia	32.29	2.21	4.94	0.00	60.52	0.04
Europe	98.76	0.53	0.71	0.00	0.00	0.00
North America	86.80	0.30	0.00	0.00	3.03	0.00
Central America	94.85	0.38	2.19	0.00	2.58	0.00
Oceania	97.71	0.39	0.04	0.11	1.75	0.00
South America	95.09	0.69	4.21	0.00	0.00	0.00
World	49.10	1.39	6.11	3.20	40.15	0.05

and easily extractable juice, be disease and insect tolerant, and tolerate both drought and wet conditions.

Yields: Sweet sorghum yields vary considerably depending on the varieties/hybrids that are used, the location (soil, water, climate, pests and diseases), inputs, and production practices. The annual global production of dry sorghum is about 53 Tg with the an average yield of 1.2 Mg ha^{-1}. The USA is the largest producer of sweet sorghum (23%) at a yield of 3.7 Mg ha^{-1}. The highest yield occurs in Israel and Jordan, which produce more than 10 Mg of dry sorghum per hectare [23]. When considering sweet sorghum for ethanol production, the most important yield components are biomass yield, juice yield, and sugar production per hectare. The concentration of soluble sugars in sorghum ranges widely depending upon variety. Biomass yields of sweet sorghums are also variable ranging from 20 to 120 Mg ha^{-1} and juice content from 65% to 80% of biomass. The combined sugar content of the juice varies between 9% and 15%. Sugar yields vary from 4 to 17 Mg ha^{-1}. The bagasse (crushed stalks) and leaves make up the remainder of the wet biomass. The bagasse represents approximately two-thirds of the dry matter and leaves represent the remaining portion. Fermentation of the sugar in the juice yields 600–900 L ha^{-1} of ethanol [38].

Diseases and pests: Disease and insect problems may also limit yield potential, suggesting that further research in this area is essential. Leaf diseases are the most troublesome for forage producers. These are anthracnose caused by *Colletotrichum graminicola* (which can be overcome by using resistant varieties) and leaf blight caused by *Helminthosporium turcicum*. Charcoal rot (*Macrophomina phaseoli*) causes plants to lodge badly. Grain may be affected by covered smut (*Sphacelotheca sorghi*) in which the seed is replaced by a sac of spores; fungicidal seed treatment before planting prevents this latter malady (FAO, 2009, http://www.fao.org/ag/agp/agpc/doc/gbase/data/pf000319.htm). From a forage point of view, grasshoppers appear to be the worst pest, and feral pigs can damage the crop in some locations. Grain pests include the sorghum midge, *Contarinia sorghicola*, whose larvae feed on the developing seeds. Bird damage is also important with the weaver bird, *Quelea quelea*, causing major losses in Africa. Damage can be prevented by using awned varieties of sorghum, giving some hope of reducing losses. The high tannin content of sweet sorghum seed is another deterrent, and early harvesting for silage avoids the major problems.

Commercial and industrial use: Worldwide, the major uses of sweet sorghum are animal feed (49%) and human food (40%; Table 3). In Africa and Asia, over 60% of sweet sorghum is used for human food. In the other regions, most sorghum is used for animal feed. There is no use of sorghum for human food in Europe and South America. Although the juice, grain, and bagasse from sorghum provide many opportunities, most applications around the world are for syrup and forage. An average yield of 1,900 L ha^{-1} of syrup can be achieved, although yields of 800–1,200 L ha^{-1} can result if weather conditions are poor. In forage applications, chickens can be fed with seed heads and ruminant livestock can use the grains, leaves, and

stalks. The organic by-product from sweet sorghum syrup processing is often fed to livestock, left on the field, or composted. Sweet sorghum bagasse is used currently to manufacture chemical pulp. The quality of the pulp obtained is excellent for the paper industry. Sweet sorghum can be considered as a major raw material for the paper industry [39].

Sweet sorghum as a bioenergy crop: Sweet sorghum is attractive for bioethanol production because of its high fermentable sugar content and very high yield of green biomass (20–30 dry Mg ha^{-1}), its low requirement for fertilizer, high water use efficiency, and short growth period; and, it is well adapted to varied climate and soil conditions. These advantageous agricultural characteristics make sweet sorghum a promising substitute feedstock for fuel ethanol production in the southern USA [32]. Based on a recent economic analysis, sweet sorghum is considered to be one of the most drought-resistant crops and has higher biomass yield and lower production costs than many other plants [40]. Sweet sorghum can produce fermentable sugars (sucrose, glucose, and fructose) in its juice, starch in its grain, and lignocellulose, which can be used in both current starch-based ethanol plants and future cellulosic ethanol plants. Of the 20–30 dry Mg ha^{-1} of biomass, approximately 40–45% is fermentable sugars and starch, equivalent to more than 500 bushels ha^{-1} of corn yield. If all fermentable sugars in sweet sorghum are converted to ethanol, potential ethanol yield could be 5,500–6,100 L ha^{-1}. However, normal pressing can recover only ≈50% of the total sugars in the sorghum stalk [41]. Increasing the juice yield or making proper use of remaining sugars in the bagasse is crucial for realizing the high ethanol yield of sweet sorghum and is of important economical value.

Similar to sugar cane bagasse, sweet sorghum bagasse is the residue obtained after crushing of sweet sorghum cane for sugar production and consists on average of cellulose 34%, hemicelluloses 25%, lignin 18%, and ash 4% on a dry weight basis [42]. Sweet sorghum bagasse was found to be a remarkable raw material for the paper industry, yielding high-quality pulp [39]. The most promising future utilization of bagasse is cellulose-based ethanol production, while the residual solids (mainly lignin) can be burned to provide heat and power. Hydrolysis of the cellulose and hemicellulose fractions can be catalyzed by acids or cellulolytic enzymes. Enzymatic processing needs a pretreatment step to increase the susceptibility of the cellulose, which can be degraded by cellulolytic enzymes to glucose.

Miscanthus and Switchgrass – Dedicated Perennial Energy Crops

Introduction *Miscanthus* and switchgrass are two perennial herbaceous crops with attributes generally considered as ideal for biomass crops, making them well suited as dedicated energy crops for biofuel production (Table 4). They are members of the grass family, *Graminae* (*Poaceae*). The perennial herbaceous crops have long-lived roots that may establish beneficial interactions with root symbionts, facilitating acquisition of mineral nutrients from the soil which may result in lesser amounts of fertilizer needed [44]. This can translate into cost saving on fertilizer and

Biomass Crops for Biofuels and Bio-based Products.

Table 4 Attributes of an "ideal" biomass crop [43]

The "ideal" biomass crop?	Corn	Short-rotation coppice[a]	Perennial grass
C4 photosynthesis	⋆		⋆
Long canopy duration		⋆	⋆
Recycles nutrients to roots			⋆
Clean burning			⋆
Low input		⋆	⋆
Sterile (noninvasive)	N/A	⋆	*M. giganteus*
Winter standing		⋆	⋆
Easily removed	⋆		⋆
High water use efficiency			⋆
No known pests or disease			*M. giganteus*
Uses existing farm equipment	⋆		⋆

[a]Coppice is a grove of densely growing small trees pruned to encourage growth

minimizing water pollution from leachates and run-off. A very important physiological characteristic of the perennial herbaceous crops is the recycling of nutrients within the plants. Nutrients are moved from roots to growing shoots at the beginning of each growing season and from the senescing shoots to the roots at the end of the growing season [45]. This recycling characteristic gives the perennial herbaceous crops many advantages over other plants and at the same time reduces the amount of fertilizer needed. In spring, when mineral nutrients are translocated from the roots to the shoots, the perennial grasses get a rapid start in forming a photosynthetically active canopy leading to biomass accumulation when many annuals are still seedlings. Conversely, in fall, mineral nutrients from the senescing shoots are transferred to the roots where they are stored over the winter. This has the positive effect of lowering the ash content of the shoots, improving their overall energy content and quality for biofuel production and at the same time improving the viability of the roots during the cold winter. Another advantage of the perennial grasses is the low input cost in establishing the crops. Once the perennial grasses are established, they can be harvested annually without replanting and no tillage is needed for the next 15–20 years. This leads to a substantial saving in labor cost and at the same time reduces soil erosion and nutrient loss. *Miscanthus* and switchgrass are C4 plants [46]. A higher solar energy conversion rate should lead to a higher total biomass yield per unit of land area. Perennial grasses are also known to exhibit increased soil-carbon levels by sequestering portions of atmospheric carbon in the soil in root biomass and in root turnover creating soil organic matter. The perennial herbaceous crops therefore, represent significant soil carbon sinks [47]. The perennial grasses are also known to be better adapted than conventional crops to different types of soils which means they can also be grown on marginal land not used for food crops.

Miscanthus *Origin and distribution*: *Miscanthus* is native in many parts of Asia such as China, Japan, and the Pacific Islands, and can grow up to 4 m tall. *Miscanthus* was first introduced into Denmark from Japan in 1935 as an ornamental plant and is now cultivated throughout Europe and North America primarily for energy production with other end uses being explored. It is estimated that there are about 14 species of *Miscanthus* [48] and the most common species investigated as a biofuel in Europe and North America is *Miscanthus* × *giganteus*, a naturally occurring sterile triploid hybrid that has its origin in Japan [49]. The genetic origin of *M.* × *giganteus* is uncertain but some evidence suggests that this hybrid is a result of a cross between *M. sinensis* (diploid) and *M. sacchariflorus* (tetraploid) [50]. In general, *M. sinensis* types are well adapted for cooler climates, whereas *M. sacchariflorus* can provide genetic resources for warmer regions [51].

Growing conditions: Extensive studies and evaluation of *Miscanthus* as a biomass resource for biofuel and bio-based products have been going on in Europe for the last 2 decades under the umbrella of the *Miscanthus* Productivity Network [52]. These studies have provided much of the currently available information on *Miscanthus* as a renewable feedstock for bioenergy. In recent years, similar studies have also been conducted in the Midwest of North America but on a smaller scale. The studies in Europe were conducted in 15 cities from southern Italy (37° N latitude) to Denmark (56° N latitude) [53]. Although *Miscanthus* prefers the mild temperatures and high water availability of its natural habitats in the tropics and subtropics, the successful establishment of *M.* × *giganteus* in Europe and North America suggests that it is relatively tolerant of temperate temperatures and low water availability. In North America, *M.* × *giganteus* has been established successfully in the Midwest from latitude 38° N to 48° N covering Ohio, Michigan, Indiana, and Illinois, and expanding into other areas in the south like Georgia and Florida and as far north as Quebec, Canada. *M.* × *giganteus* has been demonstrated to be more tolerant of low temperatures than most C4 perennials [54, 55]. Nevertheless, low temperature still limits the growth of *Miscanthus*. It is estimated that *Miscanthus* requires a minimum of 500 mm of annual rainfall and where the annual rainfall is typically below that amount, irrigation is necessary for substantial growth and biomass yields [53]. *M.* × *giganteus* can adapt to a wide range of soil, from sands to high organic matter soils. It is also tolerant to a wide range of pH, the optimum being between pH 5.5 and 7.5 [56]. Generally, areas with higher rainfall and

soils with high water-holding capacity will favor production of *Miscanthus*. Limited soil water availability during the growing season will decrease production.

M. × *giganteus* being a sterile hybrid is established from rhizomes. The rhizome pieces, approximately 200 mm in length, are planted directly into the soil at a depth of 200 mm [56]. Fresh rhizomes are generally used because rhizomes stored for a period of time are likely to dry out and exhibit less vigor. Planting takes place after the risk of the latest spring frost is over, typically between March and May. The annual fertilizer requirement is very low. Results from field trials in Europe and from reviews of literature showed that nitrogen has only a modest influence on the yields of *M.* × *giganteus* [43]. It is estimated that the annual requirement of nitrogen is 50–70 kg ha^{-1}, phosphorus is 5–10 kg ha^{-1} and potassium is 70–100 kg ha^{-1} [56, 57].

Harvesting is carried out after the crop has senesced with moisture content preferably at its lowest and with nutrients translocated into the rhizomes. Harvesting is timed to achieve a balance between attaining lowest moisture content and minimizing biomass losses caused by adverse winter conditions. Crops grown in cooler climates are typically harvested in early spring when moisture content is lowest. Crops grown in warmer climates reach their maturity earlier and can be harvested in late autumn or delayed until early spring. However, delaying harvest until early spring will result in yield reduction by as much as 30–50% due to winter losses of dead and decaying leaves and upper stem parts. But such loses are tolerated because the lower moisture content (20%) improves the fuel quality, permits ease of handling, and requires little drying.

Yield: Miscanthus takes about 2–3 years to establish and so the first-year crop does not yield sufficient biomass to merit harvesting. The crop is normally harvested from the second year onwards but yields will continue to improve after year two until they level off. Ceiling yields can be reached in 2 years under good growing conditions but may take up to 5 years at some locations. The ceiling yields are attained more quickly in warmer climates and those total yields are higher than in cooler climates. The winter yields reported for Europe following the third growing season varied with location and ranged between 6.4 and 23.6 Mg ha^{-1} from northern to southern Europe [53]. Data on *Miscanthus* productivity in North America are still lacking but mathematical productivity modeling [58] based on the European studies projected yields of *Miscanthus* in the Midwest to far exceed those of switchgrass [43]. A recent report comparing productivity of *Miscanthus* and a locally adapted switchgrass cultivar (Cave-in-Rock) grown side by side in field trials in Illinois seems to support the projection [59]. The reported average winter yields of dry matter for the 3 years following the third growing season were 19, 30.3, and 31.4 Mg ha^{-1} in the northern, central, and southern plains, respectively [59]. The average yield per year across the three sites is approximately 27.2 Mg ha^{-1}. This yield is significantly higher than the yields reported in Europe and almost three times higher than for switchgrass cv. Cave-in-Rock, grown side-by-side with *Miscanthus* in the same field trials (Table 5). A yield of 27.2 Mg ha^{-1} would achieve the projected target of 342 million Mg on 12.6 million hectares, half the area estimated in the billion-ton-study projections [1]. No disease has been reported to date for *Miscanthus* but the crop has been known to be susceptible to *Fusarium* blight and Barley Yellow Dwarf Luteovirus [52]. Weed control is essential during the long establishment phase. Fields are typically sprayed with herbicide before planting followed by at least two sprayings a year for the first 2–3 years.

Uses: Miscanthus is grown primarily to be used in the bioenergy industry. It is being tested in Europe as solid fuel for combustion in farm-heating plants as well as for co-combustion with coal, straw, and wood to generate power [53]. Its potential lies in the abundance of raw cellulose biomass that can be converted to cellulosic ethanol. Nonenergy applications for *Miscanthus* include material for thatching [60], paper pulp production [61], and as a bio-composite in construction/building materials such as panel board and building block [62] and as a substitute for the plastics or light metals in the core of light natural sandwich material (LNS). LNS materials are light building materials used for plane and molded structural parts with high form stability at low weight, used for a broad range of applications. Such substitutions are used in carrying cases for musical instruments and lab tops, rotor blades of wind power stations, small boats, and parts of yachts [63].

Biomass Crops for Biofuels and Bio-based Products. Table 5 Comparison of mean harvestable dry matter, Mg ha^{-1} ($\pm$ 1 SE), at the time of peak biomass production (Hmax), and after complete plant senescence (H1) from *Miscanthus* (M) and switchgrass (S) grown at three locations in the Midwest USA during 2004–2006 (n = 4) [59]

		North		Central		South		State average	
Year	Harvest time	M	S	M	S	M	S	M	S
2004	Hmax	38.1 (5.7)	*	60.8 (3.9)	26.0 (3.1)	48.5 (1.8)	*	48.3 (3.5)	26.0 (3.1)
	H1	13.7 (1.6)	*	25.1 (2.5)	12.8 (1.2)	37.3 (3.0)	*	25.4 (3.2)	12.8 (1.2)
2005	Hmax	25.6 (1.1)	7.8 (0.6)	40.7 (2.3)	11.5 (1.8)	40.4 (4.1)	7.8 (0.6)	33.3 (2.6)	7.9 (0.8)
	H1	13.7 (1.6)	7.8 (0.6)	31.1 (3.2)	10.6 (1.3)	27.3 (5.7)	7.8 (0.6)	25.8 (3.8)	7.9 (0.8)
2006	Hmax	29.9 (3.3)	8.4 (0.9)	44.1 (2.6)	22.0 (5.2)	51.3 (2.6)	9.6 (2.9)	39.0 (4.6)	15.6 (2.6)
	H1	29.9 (3.3)	7.7 (1.0)	44.1 (2.6)	15.6 (2.6)	39.2 (2.9)	9.1 (2.6)	377 (2.4)	15.6 (2.6)
3-year	Hmax	31.2 (3.7)	8.1 (0.5)	45.5 (3.9)	19.8 (2.6)	42.3 (3.6)	8.7 (1.8)	**38.2 (2.3)**	**12.5 (1.8)**
average	H1	20.9 (2.4)	7.8 (0.6)	33.4 (2.8)	13.0 (1.1)	34.6 (2.6)	6.7 (1.1)	**29.6 (1.8)**	**29.6 (1.8)**

*Data is not available for these points. Bold values highlight geographic and temporal averages of both parameters in both species

Future directions: One of the drawbacks of using *Miscanthus* as a biomass crop is its narrow genetic base. However, efforts have been undertaken to broaden the genetic base of *Miscanthus* and maximize the productivity and adaptive range of the crop through traditional breeding as well as modern genetic engineering by the European *Miscanthus* Improvement (EMI) project (www.biomatnet.org/secure/Fair/F659.htm) and research institutes across North America. Even with an unimproved *Miscanthus* crop, the yields achieved so far look very promising. Therefore, yield would be expected to increase dramatically and the cost of production to fall with breeding improvement efforts. A potential hybrid of *M. sinensis* that combines winter hardiness with high biomass potential has been identified through the effort of the EMI project. This hybrid will result in improved crop quality through delayed harvest without significant loss in yield [53]. The high cost of establishment is another major concern associated with cultivating *Miscanthus* as a biomass crop. This concern can be addressed with improvement in farming equipment and farming practices that will help to reduce loss of harvested material, time, and labor cost. The development of new machinery to process rhizomes (that includes lifting, cleaning, splitting, sorting and boxing) and carry out precision rhizome planting has made commercial rhizome multiplication farming and commercial production of *Miscanthus* feasible. Improvement in the crop together with improvement in farming technology and processing of this biomass crop into fuel will undoubtedly make *Miscanthus* a very attractive crop to the biofuel industries.

Switchgrass *Origin and distribution:* Switchgrass (*Panicum virgatum*) is a warm-season grass indigenous to the Central and North American prairie with its northern limit of adaptation at about 51° N [64]. In the USA its adaptive range stretches from the eastern seaboard to as far west as the east side of the Rocky Mountains and from the Texas Coastal Plain to as far north as Hudson Bay [65]. This wide geographic distribution can be seen as the manifestation of the great genotypic and consequently phenotypic variation seen within the species. In addition to its broad adaptation, switchgrass exhibits great adaptability to diverse edaphic conditions. Switchgrass can reach the height of 0.5–3 m or more in wetter areas of the country. Switchgrass has been extensively studied and planted in North America. Historically, switchgrass grown in the USA has been used as forage but over the last 2 decades it has been investigated intensively for its potential as an energy crop. In 1991 switchgrass was identified by

Biomass Crops for Biofuels and Bio-based Products.

Table 6 Characteristics of Upland and Lowland switchgrass ecotypes [67]

Upland ecotypes	Lowland ecotypes
Developed on higher "mesic" (moderately moist) sites	Developed in lower lying, "hydric" (considerably moist) sites, more sensitive to moisture stress
Adapted to mid- to northern latitudes	Adapted to lower latitudes
Predominantly octaploid ($2n = 7x = 72$)	Tetraploid ($2n = 4x = 36$)
Longer root length and internodes	Bunch form, larger root diameter
Shoots: originate from more active rhizomes and basal nodes of previous year culms	Shoots: originate from buds on rhizomes
Shorter and fine stems	Taller, coarser, thick stems, long, wide bluish-green leaves with long ligules, large panicles

the US Department of Energy for development as a model herbaceous energy crop because of its ability to tolerate a wide range of environmental conditions and at the same time offer high biomass yield [66].

Ecotypes: A fair amount of genetic variability exists in the switchgrass populations found across North America. The environment exerts a selective pressure on the population's diverse genotypes. The interaction between environment and genotype has over time created different ecotypes as well as variations within an ecotype (reproductive phenology, cold, heat, and drought tolerance) resulting in varieties with specific genetic and morphological characteristics that provide a good "fit" to a particular region. Through natural selection, two genetically and phenotypically distinct types of switchgrass have emerged, the lowland and the upland varieties (Table 6) [68]. The lowland varieties are vigorous, tall, thick-stemmed and generally found in wetter and more southern habitats. The upland forms are typically shorter and fine-stemmed and mainly found in drier mid- and northern latitudes. The ecotypical differences are generally related to local soil and climatic characteristics, with eastern and southern varieties adapted to higher moisture conditions, and western and northern varieties adapted to drier conditions. The phenotypic and ecotypic variation between the lowland and the upland ecotypes can be explained by their cytotypic diversity. Lowland switchgrass ecotypes are tetraploid, with a base chromosome number of 9 (diploid number 18), leading to 36 chromosomes ($2n = 4x = 36$). Upland ecotypes with the exception of the cultivar Summer, which is tetraploid, are octoploid ($2n = 8x = 72$) and less frequently, hexaploid ($2n = 6x = 54$) [69]. Breeding programs aimed at improving the forage yield have developed cultivars that adapt to specific locales ensuring the success of switchgrass in a large variety of conditions, from arid sites in the shortgrass prairie to brackish marshes and open woods [70].

Growing conditions: Switchgrass will grow in a wide range of soil types from fine to coarse textured soils but prefers soils of finer texture. Loamy and sandy soils will allow the roots and crown to spread more easily than do denser clay soils. Sandy soils dry out quickly potentially limiting the establishment success and biomass yield. Switchgrass can grow in acidic soil with pH as low as 4.3 [71] but optimum growth occurs at a pH between 5.0 and 8.0 [72]. Lowland switchgrass fares better in heavier and wetter soils while the upland types favor drier soils. Within the species, upland types are generally considered more drought tolerant [73]. Switchgrass grows best in association with site-adapted mycorrhizal fungi. The presence of mycorrhizae is thought to enhance phosphorus uptake by the plants [74, 75]. Therefore, mycorrhizae play a key role in moderating switchgrass growth in phosphorus-limited environments as well as reducing fertilizer inputs for biofuels production. Warm soil temperature and moisture are important in the successful establishment of switchgrass. Once established, switchgrass can survive extreme periods of drought. Under nonirrigation conditions switchgrass grows best in areas with greater than 500 mm of average annual rainfall.

Switchgrass seed germination is temperature and pH sensitive [72]. The optimum temperature for germination and growth are cultivar-dependent. Some cultivars such as the southern uplands germinate at about 11°C [76] while other cultivars may require as

high as 35°C to germinate [77]. Planting is typically done in spring from mid-March to late May. Dormant seeds require a breaking step for them to germinate. Dormancy-breaking can be achieved by stratification. Planting dormant seeds into a cool soil (10–20°C) and allowing them to stratify in situ has been shown to increase germination [77] but this may lead to weed control problems. The cool season weeds can germinate first and choke out the switchgrass seedlings when the soil warms [78]. Weed control is very important in the early stages of establishment. Unfortunately, there are very few herbicide options available to control the weeds after switchgrass emergence. The herbicides almost universally used for some time as pre- or post-emergent herbicides for switchgrass and other warm-season grasses are triazines [79–82]. However, the types of herbicides and the rates used will best be determined by considering the weed species to be controlled and switchgrass' tolerance to the chemical.

Most research on switchgrass fertility has focused on its use as forage and higher nitrogen applications can ensure high yields and better quality feed. Nonetheless, some researchers have considered nitrogen fertilizer recommendations for switchgrass to be higher than necessary for biomass production. Switchgrass has a remarkable ability to extract nitrogen from unfertilized soils and is inherently thrifty in its use of phosphorus and potassium and often shows little or no response to additions [83, 84]. Mineral cycling within the plant and the presence of mycorrhizae in the soil play an important role in nitrogen and phosphorus nutrition of switchgrass. Switchgrass as a biomass crop in general can be grown without fertilizer or limited addition of fertilizer and still maintain productivity [67]. The nitrogen, phosphorus, and potassium recommendations should ideally be specific to a site or soil.

Harvesting switchgrass for biomass as opposed to forage should preferably be delayed until shoots have essentially all senesced and died which may not be until November or December. This will allow nutrients to be recycled from shoots to below-ground parts at the end of the growing season [85]. Harvesting once per year in late fall or early winter is recommended to maintain the highest sustainable biomass yields in the long term [67]. Harvesting late in the fall or early winter allows moisture content of the crops to drop to 15% or less which will facilitate quick baling and ease of transport as well as improving the quality of the biomass feedstock. For co-firing in coal plants the moisture content should preferably be around 12% to 13% [86]. Harvesting can be carried out with conventional haying equipment.

Yield: Switchgrass becomes fully productive only upon the third year after planting. Unlike other crops where yield data have been available for many years, data for switchgrass as a biomass crop are rather limited and are based mainly on small-plot research. A search across the literature provides switchgrass yield estimates that vary considerably, from less than 0.9 Mg ha^{-1} to almost 36.3 Mg ha^{-1}. Nonetheless, the most frequently observed yield class across all ecotypes, cultivars, soils, and management practices is between 9 and 10.9 Mg ha^{-1}. This great variability in yields is explained by the wide range of ecotypes as well as the strong interactions between genotypes and the environment. Gunderson et al. [70] observed higher yields on average within the lowland cultivars, 11.9 Mg ha^{-1} versus 8.4 Mg ha^{-1} in upland cultivars. Among the lowland varieties Alamo and Kanlow give the highest yield while Cave-in-Rock gives the highest yield for the upland varieties [87].

With newer varieties of switchgrass, yields in excess of 18.2 Mg ha^{-1} have been reported for test plots. Sanderson et al. [88] have reported yields of 13.6–18.2 Mg ha^{-1} in field trials in Texas and Thomason et al. [89] have reported yields in excess of 27.2 Mg ha^{-1} from field work in central Oklahoma. These yields are relatively high and site-specific. In a larger scale study, Parrish and Fike [67] reported average biomass yield in a 10-year study of 12.9 Mg ha^{-1}. Schmer et al. [90] reported on-farm yields ranging from 4.7 to 10.1 Mg ha^{-1} for field trials in the USA. According to the US Agricultural Research Service, growers can expect yields from 15.4 to 36.2 Mg ha^{-1} in the southeast, 10.9–13.6 Mg ha^{-1} in the western Corn Belt, and 2.3–9.1 Mg ha^{-1} in the northern plains [91].

Uses: Switchgrass was originally used as forage, either grazed [92, 93] or for making hay [94, 95]. The use of switchgrass has since been expanded to include nonforage purposes such as for bioenergy, soil stabilization and erosion control in critical areas like strip-mine soils, sand dunes, and dike, and soil improvement

in areas degraded by overcropping. "Alamo" switchgrass has also been tested for its ability to remediate soils contaminated with cesium-137 and strontium-90, two radionuclides released during nuclear testing, nuclear reactor accidents, and weapons production. The level of cesium and strontium removed over a 5 months period was reported at 36% and 44%, respectively [96].

Benefits: Benefits associated with switchgrass are numerous. Planting switchgrass, even as a monoculture, is expected to enhance prairie biodiversity by providing forage, habitat, cover, and nesting areas for a diverse prairie wildlife that includes mammals, birds, amphibians, reptiles, invertebrates, and insects. Hohenstein and Wright [97] estimated a 95% reduction in soil erosion rates and a 90% reduction in pesticide use for herbaceous energy crops such as switchgrass relative to annual row crops like corn and soybean. It is also suitable for short windbreak plantings in truck farm fields [98] and it has been shown to improve water and soil quality by reducing carbon emissions through carbon sequestration [99]. Switchgrass can be easily integrated into existing farming operations because conventional equipment for seeding, crop management, and harvesting can be used [100].

Future directions: A significant amount of knowledge of the biology and agronomy of switchgrass has accumulated over the years through research on switchgrass as forage and recently as a biomass feedstock. There is now an understanding of the adaptation of existing cultivars and the development of new cultivars with improved yield and adaptation ability for different agro-ecoregions. However, there are still constraints that limit the use of switchgrass as a bioenergy crop. Problems with seed dormancy, improper or nonuniform planting depth, lack of weed control options at establishment, and variable weather and soil conditions precluded the development of reliable and economic establishment methods [101]. A better understanding of how nitrogen is used and recycled in switchgrass under different growing conditions will help to develop fertilizer guidance and nutrient management specific to sites and soils [102]. Current research on the genetics, breeding, and molecular biology of switchgrass will result in new switchgrass cultivars with improved yield, greater establishment ability, and altered cell-wall properties for more efficient conversion.

Woody Biomass Crops

Introduction

Biomass, especially woody biomass and energy crops, already contribute substantially to cover energy demands around the world. Dedicated woody crops for biofuels have the potential to be an important energy source and will contribute to the substitution for fossil fuel energy [103]. In fact, the US Department of Energy has a vision to replace 30% of the liquid petroleum fuel for transportation with biofuels and similarly, the European Union Directive 2003/30/EC has targeted 5.75% of all petrol and diesel transport fuels to be derived from biomass by December 2010 [104]. Clearly, a multifaceted approach that includes both agricultural crops and dedicated woody crops will be necessary to attain these goals.

The principal challenge for biomass production is to develop and grow crops with improved physical and chemical traits while increasing biomass yields. Woody perennial plants like trees and shrubs grown for biofuels have the potential to play a central role in providing a renewable source of biomass for conversion to fuels while also providing a wide array of conservation benefits and ecological services, such as favorable habitat for wildlife, clean surface and ground water, conservation of soil and species diversity, that will exceed those associated with conventional annual crops [105].

Poplar

Origin and distribution: Hybrid poplars (*Populus* spp.) are among the fastest-growing trees in North America and among the oldest types of dicotyledonous plants. The genus *Populus* consists of close to 35 species of deciduous flowering plants in the family *Salicaceae*, native to most of the Northern Hemisphere. It includes the cottonwoods, poplars, and aspens, all of which are sometimes termed poplars. The hybrids themselves represent crosses among various cottonwood species [106].

Cottonwood (*Populus deltoides*) was first introduced by early French explorers in North America,

which crossed naturally with Black Poplar (*Populus nigra*), creating what is referred to as *Populus* × *euramericana* hybrids (http://www.ag.ndsu.edu). They were first cultivated around fields as windbreaks and were selected for fast-growing characteristics. In 1912, hand-pollinated poplar hybrids were produced in Britain. The shortage of timber after World War II spurred an increase in hybrid poplar plantations in Europe. Even though some of these European varieties were reintroduced to North America in the early 1920s, it was not until the 1970s that commercial planting of hybrid poplar started in the USA. Since then, a national consortium involving government researchers from several agencies, universities, and the private sector has been working on improving hybrid poplar. Research in this area is targeting reduction of production costs by targeting pest and disease resistance, increasing yields and improving management systems. Also, studies are being performed to establish the environmental impact of producing hybrid poplar [107] (http://bioenergy.ornl.gov/main.aspx).

Physiology: Poplars are deciduous trees with alternate leaves, furnished with the appendages known as "stipules." Their flowers are dioecious; the calyx and corolla are replaced by simple scales. The female flower consists of a solitary one-chambered ovary, containing many ovules. Flowers are borne in long, sessile (attached directly to the stem) or pedunculated (growing from a stalk) aments, which are produced from buds formed in the axils of the leaves of the previous year. Leaves are green to dark green and measure 5–12 cm. The fruit is a two- to four-valved capsule, ripening before the full development of the leaf. The seed is light brown and surrounded by a tuft of long, soft, white hairs. Poplars can grow from anywhere between 15 to 50 m, with trunks of up to 2.5 m in diameter They grow upright, with spreading branches and their root system is shallow and wide-spreading, equal or greater than the height of the tree (Fig. 3). *Populus* species are somewhat susceptible to insects and diseases. Common diseases may include *Melampsora* leaf rust, *Septoria* leaf spot and canker, *Cytospora* canker, wetwood, and stem decay. Common insect pests include poplar borer, aphids, poplar bud gall mite, poplar vagabond aphid, and poplar leaf beetles (http://www.2020site.org/trees/poplar.html).

Biomass Crops for Biofuels and Bio-based Products. Figure 3
Hybrid poplar grove

Culture: Hybrid poplar is a short-rotation woody crop (SRWC). SRWC's are species that usually are planted and harvested in less than 15 years [108]. Hybrid poplar stands are typically planted at 750–1,700 trees ha^{-1} and allowed to grow for 6–12 years before harvest. Hybrid poplars are capable of resprouting from their rootstocks after harvest but it is recommended to reestablish the cultures to reduce the potential of diseases and also to exploit the new improved hybrid varieties. For the production of hybrid poplars, clay loams or sandy-loam, slightly alkaline (pH 5 to 7.5) and medium-textured soils are recommended. They require a moist site and will not tolerate drought on upland sites. The use of herbicides or manual weed removal must be used in the first 3 years, but this is no longer necessary when the canopy closes, creating its own weed control. Furthermore, fertilizer applications are only necessary if the nitrogen level in leaves falls below 3% on a dry weight basis (http://www.ag.ndsu.edu, [107]).

Biomass Crops for Biofuels and Bio-based Products.

Table 7 Hybrid poplar yields in regions of the USA [109]

Region	Hybrid poplar yields	
	Average Mg ha^{-1} per year	Range Mg ha^{-1} per year
Lake States	9.9	7.9–11.8
Corn Belt	10.4	8.4–11.7
Southeast	10.1	8.6–11.7
Appalachia	8.0	9.0–11.7
North Plains	8.6	7.3–9.7
South Plains	8.4	7.3–9.0
Northeast	9.0	7.7–10.0
Pacific Northwest	12.9	12.4–13.5

Yield: Hybrid poplars, when grown under short-rotation silviculture, can produce between 8 and 22 Mg ha^{-1} per year and achieve a height of 20 m in as little as 6 years. Average yields in the USA and range of yields by geographic region on hectares currently planted are shown in Table 7 [109].

Hybrid poplar growth and biomass yield could vary greatly depending on the site, the soil properties and weather conditions, as well as the species genotype. The above-ground biomass yield of 4-year-old hybrid poplar stands in Europe (in short-rotation coppice culture) and north central USA averaged between 2 and 11 Mg ha^{-1} per year. In comparison, a recent study at the University of Saskatchewan reported growth and yield data in western Canada of 4-year-old Walker hybrid poplar stands from five locations to average from 0.17 to 0.19 Mg ha^{-1} per year [110]. It has been suggested that once the crop is established, the combination of precipitation and temperature and its influence on soil moisture becomes the most limiting factor for maximizing biomass production [111].

Advantages: Many advantages can be linked to growing hybrid poplars, from its positive effect on the environment to the fact that they are expected to be grown on agricultural cropland using standard production methods, thus reducing the need for new technology. Environmental benefits are linked to hybrid poplar's perennial nature. Chemical and fertilizer applications are considerably lower and these trees can intercept run-off nutrients to rivers and wetlands close by. Also, wind and water erosion over the life of the rotation is inferior to the erosion caused by annual crops and there is a clear ecological benefit of year-round trees for birds and habitat for small mammals. Their buds provide a source of food for birds, and their twigs and young branches make good forage for wildlife (http://www.ag.ndsu.edu, [107]).

The use of SRWCs for bioenergy production could substantially decrease the overall use of fossil fuels for energy. It would reduce CO_2 emissions into the atmosphere, increase soil organic carbon sequestration, and improve soil erosion control. Additionally, growing woody species like poplar on waste disposal sites and agriculturally marginal lands would definitely benefit rural communities [110].

Uses: Hybrid poplars are grown on plantations mainly for pulpwood used in the manufacture of paper. This wood is also sold as inexpensive hardwood timber, used for pallets and cheap plywood [106]. Wood and wood-derived fuels are a primary energy source in developing countries' domestic households as well as industrial facilities. From a medical point of view, *Populus* species can be a source of salicin, used for fevers and headaches. (http://www.ag.ndsu.edu, [107, 112]). More importantly, they are well suited for the production of bioenergy (e.g., heat, power, transportation fuels) and other bio-based products (e.g., organic chemicals, adhesives).

Poplar as a source of biofuels: Bioethanol is traditionally produced by converting either starch from grains such as corn, or sugar from sugarcane into ethanol. A second technology is based on the hydrolysis of cellulose or lignocelluloses into sugars, followed by fermentation to produce fuel ethanol [113]. The cellulose comes from woody parts of plants or trees, such as hybrid poplar and other short rotation woody crops. The main objective in the production of ethanol from cellulose is to obtain a maximum biomass output with a minimum input. Developing new poplar varieties could expand the biofuel production without incurring the type of environmental problems that intensive agriculture can generate [114]. Major improvements have been achieved through breeding and genetic selection.

Poplars are the first tree for which the entire genome has been sequenced. Its 45,000 genes are

being investigated to find ways to improve this tree, from genes that regulate its root system, improving water and nitrogen absorption, to cell wall modification for more extractable cellulose [115, 116]. New varieties focusing on more efficient roots not only would result in higher tolerance to drought but also in bigger trees and thus more biomass for the biofuels industry [115]. Alternatively, modification of cell wall polymers, such as lignin, which interfere with the enzymes needed to degrade cellulose, can benefit the extraction of cellulose from poplar trees for the production of ethanol [116].

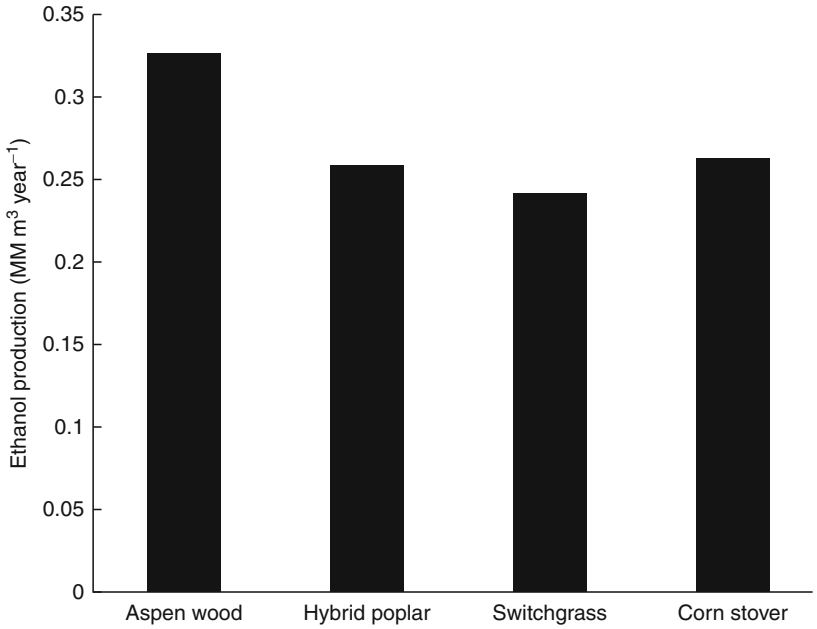
To increase vigor and yield of poplar species, new crosses are being performed and examples of studies on new varieties are easy to find. Among these, a cross is found between *Populus trichocarpa* and *Populus deltoides* which has achieved a hybrid with leaves about four times as large as the leaves of either parent at a similar age, expanding their photosynthetic surface area. Other projects are looking at the biochemical indicators for drought tolerance in these hybrids, by growing them with or without irrigation and characterizing biochemical changes. All efforts are focused on fast-growing highly adapted poplar clones [106].

Up to now, traditional row crops like corn have been utilized for ethanol production. Several corn-to-ethanol plants are commercially in use around the world and it is known that they offer an alternative green energy source while minimizing greenhouse gas (GHG) emissions [117]. Nonetheless, poplar trees might have several additional benefits. In fact, corn ethanol reduces GHG emissions by about 13%, while cellulosic ethanol could greatly reduce GHG by 88% [118]. Even though wood from hybrid poplar can produce roughly the same ethanol production per unit biomass (Fig. 4), hybrid poplar generates 40% more excess electrical energy than corn. In addition, fast-growing cellulosic energy crops such as hybrid poplar can be grown on a variety of land types, without having to compete with cropland for food and feed needs, as it is necessary for crops such as corn [117]. Poplar plantations do not require intensive inputs and are not harvested every year. There is a wide range of varieties and they could be grown in an equally wide range of climates, from subtropics in Florida to subalpine areas in Alaska. It should not be forgotten that the machinery necessary for poplar growth already exists and the infrastructure to handle the trees is already available [116].

The use of dedicated energy crops, such as hybrid poplars will not only address energy security issues, but will also inevitably be beneficial for the environment while addressing global climate change and economic development.

Eucalyptus

Origin and distribution: Eucalyptus is indigenous to Australia, Indonesia, and Papua New Guinea. There are more than 700 species of *Eucalyptus*, almost all (except for two) are native to Australia [119]. Species of *Eucalyptus*, prized globally for excellence in paper and energy production, are cultivated throughout the tropics and subtropics in more than 90 countries worldwide representing 8% of all planted forests (FAO, ftp://ftp.fao.org/docrep/fao/008/A0400E/A0400400.pdf). A few cold-tolerant species and their hybrids grow in the temperate regions of Europe, New Zealand, and South and North America. The global land area under *Eucalyptus* cultivation is estimated at over 20 million hectares and Brazil has emerged as the major global producer and exporter of *Eucalyptus* wood with approximately 4.2 million hectares on plantations followed by India and China with ~3.5 million hectares and 2.9 million hectares, respectively [120]. *Eucalyptus* was first introduced to California as an ornamental plant in 1853. Soon after, it was widely planted throughout the state because it was fast growing and a renewable source of timber and fuel [121]. Its popularity was later replaced with the criticism that *Eucalyptus* forests compete with native plants and do not support native animals. Eucalypt forests in some parts of California were eventually removed [122]. The fuel crisis in the 1970s brought back *Eucalyptus* species as prime candidates for woody biomass cultivated on experimental farms mainly in central California. Two other places in the USA where *Eucalyptus* short-rotation research projects are conducted are located in Hawaii and Florida [123, 124]. Other regions in the USA considered highly suitable for growing *Eucalyptus* are in Texas, Louisiana, and Georgia. Only four *Eucalyptus* species, *E. grandis (EG), E. urophylla (EU), E. camaldulensis, and E. globulus*, and their hybrids



Biomass Crops for Biofuels and Bio-based Products. Figure 4
Comparison of ethanol production utilizing aspen, hybrid poplar, switchgrass, or corn stover

account for nearly 80% of the eucalypt plantations worldwide with *E. grandis* being the most widely used species in tropical and subtropical areas while *E. globulus* is the premier species for temperate zone plantations [125]. *E. grandis* is also used as a parental species in hybrid breeding and is rated worldwide as one of the fastest growing species and has the widest adaptability of all *Eucalyptus* species [125]. The greatest area of plantations of *EG* and its hybrids with other species are in Brazil and several other Central and South American countries.

Importance: *Eucalyptus* species are fast-growing woody perennials with many uses making them economically important trees. Eucalypts are commonly cultivated for the paper and pulp industries because of their high fiber yields and use as fuelwoods. In many poor countries in Africa [126], South America [127], and Asia [128] eucalypts are grown as a cash crop. *Eucalyptus* woods are used as mine props, poles, firewood, and charcoal [119]. The nonwood products derived from eucalypts include essential oils, honey made by bees from its flowers, and tannin [129]. The potential of *Eucalyptus* as a biomass feedstock for cellulosic ethanol has drawn attention to this species of woody perennial. The US Department of Energy (DOE) identified *Eucalyptus* as one of the crops with the potential to contribute to the biomass needed for biofuel to reduce the nation's dependence on imported fossil fuels [1]. *Eucalyptus* has many attributes and advantages that make it suitable as a dedicated energy crop. As a perennial, *Eucalyptus* can be cultivated under the coppice system. The cut stumps resprout to provide another crop. The retained roots with stored carbohydrates and access to soil water and nutrients help sustain rapid regrowth rates [130]. The tree withdraws the mineral nutrients into its roots at the end of a growing season, thus reducing the amount of fertilizer needed and thereby reducing the fertilizer costs and minimizing water pollution caused by run-off. The fact that harvesting only once every few years over a period of 15–20 years reduces the environmental impact created by disturbances at harvesting and planting (soil erosion and nutrient loss) and saves on the cost of establishment (site preparation, seedling, and planting costs). *Eucalyptus* is one of the fastest growing hardwoods which grows well even on environmentally harsh lands (infertile soils, arid lands) [131]. It is a low input crop requiring minimum fertilizer and

precipitation. *Eucalyptus* is also the world's most widely planted hardwood species that has multiple end uses, including feedstock for traditional forest products and energy products such as cellulosic ethanol and power generation through direct firing, co-firing or wood pellets in short rotation. In regions of the USA where *Eucalyptus* will most likely be cultivated, infrastructure already exists for planting, harvesting, handling, and processing wood for pulp that could also support the production, processing, and distribution of *Eucalyptus* crops. Last but not least, *Eucalyptus* species have been grown in the USA for many decades and have not demonstrated any invasive characteristics.

Growing conditions: Eucalypts are hardy trees and the majority of *Eucalyptus* species will grow in a range of soils from pH 4.5 to 8.5. They do not require fertile soils and many species naturally occur in shallow, low fertility soils. When grown on deeper more fertile soils they generally grow faster, bigger, and more luxuriant. Some species do require good drainage while others will grow on heavy clay and even on boggy, partially waterlogged sites. Several species grow well on high pH soils, and most species adapt to higher pH soils, although usually with reduced growth rates. *Eucalyptus* will tolerate an annual rainfall of 500–5,000 mm and an annual temperature of 12.3–27.9°C (Table 8). *Eucalyptus* is considered one of the most adaptable plant genuses, and the trees are remarkably hardy in all kinds of weather conditions. Once established the mature trees adapt to the local growing conditions and become very tolerant to drought and prolonged dry periods. However, the young trees of most *Eucalyptus* species do not tolerate frost very well and will succumb to extreme fluctuation of temperatures [132]. Fertilization and nutritional studies of *Eucalyptus spp.* in Australia, Brazil, and Hawaii pointed to a general need for supplementary nitrogen, and sometimes phosphorus, especially at planting and soon thereafter [133, 134]. Fertilizer (N-P-K) is generally applied at planting and again about 6 months later. Subsequently, only nitrogen is needed on most sites. The total amount required depends on the nitrogen status of the topsoil and may range from 224–673 kg ha^{-1} in four to eight applications (depending on the site quality and rotation length) [123]. The application of nitrogen may be eliminated or reduced with the practice of intercropping with nitrogen-fixing species such as *Leucena* or *Albizia*. Such practices were studied in Hawaii [135], Brazil [136], and India [137] and have shown that it is possible to maintain both the yields and nitrogen status of the soils without using nitrogen fertilizers, or with much reduced fertilizer inputs. *Eucalyptus* can be planted at any time of the year in tropical and subtropical areas but spring time is recommended for colder regions. Young *Eucalyptus* trees do not compete well with weeds; therefore, weed control in the early stages of establishment is very important in the management of *Eucalyptus* cultivation. Weed control is carried out before planting the trees and during the first 2 years of establishment. Pre-emergence and post-emergence herbicides found safe and efficacious for *Eucalyptus* are listed in a report by Elmore [138]. The projected rotation age for bioenergy production is 5 years under optimum growing conditions and a year or two longer under less favorable conditions [125]. However, new and high-yielding varieties have the potential to reduce rotation length to 4 years or less [139].

Yield: The yield of *Eucalyptus* biomass varies widely from species to species and also with provenances (place of seed origin), sites, and management systems around the world. Small plot studies conducted at the University of Massey, New Zealand, comparing the yields of 19 *Eucalyptus* species grown under similar conditions and managed under a coppice regime highlighted the differences in survival rates, the number of shoots per tree, the shoot/tree sizes and ultimately the yields between different species. The yields obtained vary from 4 to 20 Mg ha^{-1} per year and only six species: *E.brookerana*, *E. botryoides*, *E. botryoides* × *saligna*, *E. ovata*, *E. elata,* and *E. oblique* had yields averaging 16 Mg ha^{-1} per year [140]. The yield evaluation of *Eucalyptus* species in small plot trials in Hawaii emphasized the suitability of sites and species (Table 9). *E. grandis* and *E. urophylla* are relatively highly productive at a higher elevation with moderate to high precipitation but not at a lower elevation. *E. camaldulnesis* performed poorly at low elevation even with irrigation. *E. urophylla* has the highest annual yield with ∼35 Mg ha^{-1} per year at the moderately upland site [141]. The importance of provenance selection for different sites was brought out in the data for *E. camaldulensis* planted in Afaka, Nigeria. Five-year-old trees had a mean annual increment (MAI) that ranged from

Biomass Crops for Biofuels and Bio-based Products. Table 8 Growth requirements of *Eucalyptus* species in USA

Growth requirements	*E. grandis*	*E. saligna*	*E.* camaldulensis	*E. globulus*
Adapted to coarse textured soils	Yes	Yes	Yes	Yes
Adapted to fine textured soils	Yes	No	Yes	Yes
Adapted to medium Textured soils	Yes	Yes	Yes	Yes
Drought tolerance	Low	Medium	Medium	Low
Fertility requirement	Medium	Low	Low	Medium
Frost free days, minimum	340	340	180	240
Moisture USE	Medium	Medium	High	High
pH, minimum	4.0	4.0	5.0	5.0
pH, maximum	6.0	6.0	8.5	6.8
Planting density per acre, minimum	170	170	170	170
Planting density per acre, maximum	1,200	1,200	1,200	1,200
Precipitation, minimum, (mm)	1,016	1,524	508	553.4
Precipitation, maximum, (mm)	1,778	5,080	2,540	1,524
Temperature, minimum (°C)	2–2.2	−6.1°C	−8.3°C	−8.3°C
Coppice potential	Yes	Yes	Yes	Yes
Resprout ability	Yes	Yes	Yes	Yes

(Source: http://www.plants.usda.gov)

17.3 to 5.1 Mg ha^{-1} per year depending on where in Australia the seed had come from. This demonstrates a possible threefold increase in yield achieved simply by selecting a seed source location [131].

A well-managed *Eucalyptus* plantation will improve the performance of the crop and will maintain a relatively high level of yield throughout the coppice regime. *Eucalyptus* cultivated with irrigation and fertilizer gives a higher yield over *Eucalyptus* grown without these additions. This was demonstrated in a pilot experiment in Brazil under the Brazil *Eucalyptus* Potential Productivity Project (BEPP). The yields of *Eucalyptus* cultivated with no fertilization were 28% lower than yields achieved with fertilization, and the response to irrigation was found to be far larger than that of fertilization. The growth increase from irrigation ranged from a low of 7% at the cooler, higher elevation site to 67% at the driest site [142]. The stand uniformity also has an influence on the yield. Stands with uniform structure (trees in plots planted in a single day) showed 13% greater growth than stands with higher heterogeneity of tree sizes (owing to staggered planting). The yields reported 2 decades ago in field trials in California, Florida, and Hawaii were typically 20–24 Mg ha^{-1} per year [124, 141]. Over the last 10–20 years of intensive research, improved silviculture (site preparation, fertilization and weed control) and superior clones have improved yields, exceeding 37 Mg ha^{-1} per year with the potential to reach ~66 Mg ha^{-1} per year [124]. In Brazil, commonly planted *Eucalyptus* hybrids such as *E. grandis* × *E. urophylla* routinely yield ~24–30 Mg ha^{-1} per year [142]. Large gains in yield can be achieved by the careful selection of species, sites, provenances, and management system to suit the local conditions.

Economics: Growing biomass and producing bioenergy require a substantial financial investment. Hard numbers on the economics of growing *Eucalyptus* for bioenergy on a commercial scale are unavailable. However, the cost projection from small plot trials in Hawaii for producing and delivering biomass feedstock

Biomass Crops for Biofuels and Bio-based Products. Table 9 *Eucalyptus* biomass yields at five Hawaiian sites after 5 years [141]

Site/Island	Tree species	Elevation (m)	Annual rainfall (mm)	Average temperature (°C)	Agronomic conditions	Planted density (trees/ha)	Annual yield (Mg ha^{-1} per year)
Mountain View, Hawaii	*E. grandis*	296.3	4,623	21.1	Wet, upland	405	20.16
	E. saligna					405	11.2
	E. urophylla					405	20.16
Honokaa, Hawaii	*E. grandis*	232.3	2,057	21.1	Moderately dry, upland	405	29.12
	E. saligna					405	20.60
	E. urophylla					405	31.80
Puunene, Maui	*E. grandis*	7.6	483	25.0	Dry, irrigated, lowland	485	16.57
Kilohana, Kauai	*E. grandis*	256.6	3,023	20.6	Wet, intermediate elevation	405	16.35
	E. urophylla					405	17.47
Hoolehua, Molokai	*E. calmaldulensis*	76.2	711	22.8	Dry, irrigated, intermediate elevation	405	9.85

to the central facility, excluding storage, processing, and biomass conversion ranges from US$30 to US $100 per dry Mg [143]. The estimated cost of producing and delivering *Eucalyptus* in the southern USA ranges from US$50 to US$60 per dry Mg, based on a yield of 28–16.8 Mg ha^{-1} and on a delivery range within 48 Km of the processing site [144]. Factors that contribute to the cost of production are establishment, maintenance, harvesting, transportation, rotation length, and productivity. The cost can be greatly reduced by growing highly productive species, having higher tree stand density and a shorter rotation length. The economic feasibility of growing *Eucalyptus* for biomass can be improved by increasing productivity through improved germplasm, an efficient management system, and improvements in biotechnology. Introducing traits such as improved growth, stress tolerance, reduced lignin composition for easier processing of the biomass, and improved wood quality through increasing cellulose composition will increase productivity and add value to the crop.

Conclusions and Future Directions

Agricultural residues: Residues can contribute as much as 428 million dry Mg per year to biomass harvests for conversion to transportation fuels. Nevertheless, it is clear that although there is a positive energy balance from residue utilization, many factors other than ethanol output have an impact on the use of these residues. Maintenance of soil fertility is paramount to the sustainability of this biomass source. Compensation for the farmer is highly important as well, with potential of their participation in farmer cooperatives (co-op's) to capture part of the value chain and ensure their continued involvement. Additionally, harvest and storage logistics must be explored and resolved. Handling large quantities of loosely packed biomass, transporting it over long distances and storing it in bales or wet stacks are only a few of the logistics issues. Harvesting activities must be coordinated so that the process of grain collection and storage is not slowed with resultant loss in their valuable revenues. However, the potential of

this biomass source is important enough for the investments necessary to make these residues a feasible feedstock.

Sweet sorghum and sugar cane: Sugar cane and sweet sorghum are members of same family and share physiological characteristics. Their use as bioenergy crops is almost identical. For the last 40 years, sugar cane has been used as a bioenergy crop in Brazil, which is a successful example of a country that has reduced its gasoline usage by producing bioenergy. Sweet sorghum is considered to be one of the most drought-resistant agricultural crops as it has the ability of remaining dormant during the drought period. de Vries et al. [145] analyzed different biofuel production systems in geographical regions where they are currently important for ethanol. Among the six "first generation" biomass crops, e.g., maize (USA), wheat (Northwest Europe), sugar beet (Northwest Europe), cassava (Thailand), sweet sorghum (China), and sugar cane (Brazil), biofuel production from sugar cane and sweet sorghum delivers substantially more energy per unit energy spent than from the other crops. These two C4 sugar-producing plants appeared to be most sustainable and most efficient in the use of land, water, nitrogen, and energy resources, while pesticide applications were relatively low in relation to the net energy produced. An agronomic and yield comparison of sugar cane and sweet sorghum is presented in Table 10.

Both sugar cane and sweet sorghum are excellent feed stock for first-generation bioethanol production. However, in order to reduce the usage of fossil fuel and attain the goal of sustainability, utilization of lignocellulosic biomass for the production of cellulosic ethanol plays a vital role. Lignocellulosic-rich bagasse from sugar cane and sweet sorghum has the most positive net energy balance among the common feedstocks potentially used for bioethanol production. Sugar cane and sweet sorghum have comparable energy balance, with 8.3 and 8 units of energy produced for every unit of energy invested in their cultivation and production, respectively, especially when compared with 1.8 units for corn grain. On the other hand, only 0.8 unit of energy is produced in fossil fuel production for every unit invested [147]. Currently eight times more energy is produced from sugar cane than what is used in its creation. When bagasse is included in the equation, it is estimated that the number may increase to as much as 16 times. Bagasse is the feedstock with the most positive energy balance available in the near term. Sugar cane bagasse shows significant potential for making the biomass-based ethanol an economically viable solution.

Biomass Crops for Biofuels and Bio-based Products.

Table 10 Comparison of sugar cane and sweet sorghum [146]

	Sugar cane	Sweet sorghum
Crop duration	About 7 months	About 4 months
Growing season	Only one season	One season in temperate and two or three seasons in tropical area
Soil requirement	Grows well in drain soil	All types of drained soil
Water management	36,000 m^3 h^{-1}	12,000 m^3 h^{-1}
Crop management	Requires good management	Little fertilizer required; less pest and disease complex; easy management
Yield (ha^{-1})	70–80 Mg	54–69 Mg
Sugar content on weight basis	10–12%	7–12%.
Sugar yield	7–8 Mg ha^{-1}	6–8 Mg ha^{-1}
Ethanol production directly from juice	3,000–5,000 L ha^{-1}	3,000 L ha^{-1}
Harvesting	Mechanical harvested	Very simple; both manual and through mechanical harvested

Switchgrass and Miscanthus: Perennial grasses are nonfood crops which can yield high-quality materials for both energy and fiber production. Switchgrass and *Miscanthus* are immense biomass producers and show great potential as bioenergy crops. They can be grown over a wide range of conditions and with minimal agricultural inputs. Switchgrass has deep roots that makes it less susceptible to drought stress and enables

it to adapt better in areas where the water precipitation is lower than that required by *M.* × *giganteus*. An analysis of published yields indicates *M.* × *giganteus* produces more biomass per unit area and per unit input than switchgrass but the yields of *M.* × *giganteus* are more strongly influenced by water, while those of switchgrass are more strongly controlled by nitrogen [43]. Therefore, in locations with ample rainfall but with concern over nitrogen contamination of water supplies, it may be better to grow *M.* × *giganteus* while conversely, in arid areas without contamination concerns, greater yields may be obtained growing switchgrass as long as adequate nitrogen fertilization is provided.

Woody crops: Woody crops such as Poplar and *Eucalyptus* represent a critical component of the bioenergy future. They have the potential to be a significant part of the bioenergy solution in the USA and in many other parts of the world. In the USA, perennial woody crops are expected to account for part of the 377 million dry Mg of the 1.37 billion dry Mg total biomass resource potential. The projected yield for the long-term feasibility of renewable energy production defined by DOE is 20 Mg ha^{-1} per year [1]. The projected yield is not only achievable but can be exceeded with improved silviculture, the use of superior clones, and genetic improvement. Investments in research and technology are needed to improve productivity and bring down the cost of production. Recent advances in genetically modified *Eucalyptus* include a freeze-tolerant variety and a low-lignin and high-cellulose variety. The freeze-tolerant variety was developed using a highly productive tropical hybrid, *E. grandis* × *E. urophylla* [139]. A field test of the freeze-tolerant variety demonstrated its ability to maintain its productivity even at temperature as low as −9°C. This has made possible commercial plantings of tropical *Eucalyptus* species in the Southeastern USA where cold winter temperatures would normally restrict such cultivation. The low-lignin and high-cellulose variety with 18% less lignin and 4.5% more cellulose developed by a team of Taiwanese and US scientists (China Post, 2007, http://www.chinapost.com.tw/taiwan/2007/09/14/122524/Gene-modified-eucalyptus.htm) would add value to the crop by improving efficiency in the pretreatment step utilized in fermentation systems for biofuels production from lignocellulosic raw materials. The authors estimated that an output of 0.9 million Mg of this new variety could generate extra revenues of about US$36 million every year. Advances in research and technology in growing *Eucalyptus* in Brazil over the last 40 years have paid off significantly. Productivity of *Eucalyptus* biomass has increased twofold to fivefold depending on the site quality [148, 149]. Improved new varieties of poplar trees are currently being obtained through genetic engineering and breeding. Varieties with larger leaves, improved root system, and modified lignin are associated not only with higher biomass yields and better net energy balance but they would definitely have a lower environmental impact [115, 116].

Growing *Eucalyptus* and hybrid poplar for biomass is sustainable and will be economically feasible with improved varieties and technology. The use of woody crops for bioenergy offers many benefits with no anticipated long-term environmental impact and may be part of a long-term solution to the nation's energy security.

In order to ensure feedstock availability whether from dedicated crops or from residues, changes in farm and energy policies will need to be made, making sure they are connected to incentivize this new industry.

Bibliography

1. Perlack R et al (2005) Biomass as feedstock for a bioenergy and bioproducts industry: The technical feasibility of a billion-ton annual supply
2. Kadam K, McMillan J (2003) Availability of corn stover as a sustainable feedstock fo bioethanol production. Bioresour Technol 88:17–55
3. Green S (2011) Sustainability: soil. In: Hood E, Nelson P, Powell R (eds) Plant biomass conversion. Wiley-Blackwell, Ames, IA
4. Andrews S (2006) Crop residue removal for biomass energy production: effects on soils and recommendations. USDA – National Resource Conservation Service, Washington
5. Hettenhaus J (2011) Agricultural crop residues for biomass conversion. In: Hood E, Nelson P, Powell R (eds) Plant biomass conversion. Wiley-Blackwell, Ames, IA
6. Wang M, Saricks C, Santini D (1999) Effects of fuels ethanol use on fuel-cycle energy and greenhous gas emissions. Center for Transportation Research, Argonne National Laboratory Argonne, IL, USA
7. Lawrence C, Walbot V (2007) Translational genomics for bioenergy production from fuelstock grasses: maize as the model species. Plant Cell 19:2091–2094

8. Osborne C, Beerling D (2006) Nature's green revolution: the remarkable evolutionary rise of C$ plants. Philos Trans R Soc B Biol Sci 361:173–194
9. Makino A et al (2003) Differences between maize and rice in N-use efficiency for photosynthesis and protein allocation. Plant Cell Physiol 44:952–956
10. Raines C (2006) Transgenic approaches to manipulate the environmental responses of the C3 carbon fixation cycle. Plant Cell Environ 29:331–339
11. de Oliveira M, Vaughan B (2005) Ethanol as fuel: energy, carbon dioxide balances, and ecological footprint. Bioscience 55(7):593–602
12. Bennett AS, Anex RP (2009) Production, transportation and milling costs of sweet sorghum as a feedstock for centralized bioethanol production in the upper Midwest. Bioresour Technol 100(4):1595–1607
13. Reddy BVS, Kumar AA, Ramesh S (2007) Sweet sorghum: a water saving bio-energy crop. In: Linkage between energy and water management for agriculture in developing countries, Hyderabad
14. Gnansounou E, Dauriat A, Wyman CE (2005) Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China. Bioresour Technol 96(9):985–1002
15. Renouf M, Wegener M, Nielsen L (2008) An environmental life cycle assessment comparing Australian sugarcane with US corn and UK sugar beet as producers of sugars for fermentation. Biomass Bioenergy 32:1144–1155
16. Sharpe P (1998) Sugar cane: past and present. Southern Illinois University, Carbondale
17. McIlroy R (1963) An introduction to tropical cash crops. Ibadan University Press, Ibadan, p 163
18. Purseglove J (1979) Tropical crops: monocotyledons. Longman, London, p 607
19. Goldemberg J, Coelho S, Guardabassi P (2008) The sustainability of ethanol production from sugar cane. Energy Policy 36:2086–2097
20. Macedo IC, Seabra JEA, Silva JEAR (2008) Green house gases emissions in the production and use of ethanol from sugarcane in Brazil: the 2005/2006 averages and a prediction for 2020. Biomass Bioenergy 32(7):582–595
21. Dong z et al (1994) A nitrogen-fixing endophyte of sugarcane stems (a new role for the apoplast). Plant Physiol 105: 1139–1147
22. Wrigley G (1982) Tropical agriculture: the development of production, Fourthth edn. Longman, New York, p 496
23. Kim S, Dale B (2004) Global potential bioethanol production from waste crops and crop residues. Biomass Bioenergy 26(4):361–375
24. Destefano S et al (2004) Differentiation of 'Xanthomonas' species pathogenic to sugarcane by PCR-RFLP analysis. Eur J Plant Pathol 109:283–288
25. Laopaiboon P et al (2010) Acid hydrolysis of sugarcane bagasse for lactic acid production. Bioresour Technol 101:1036–1043
26. Reijnders L, Huijbregts M (2009) Transport biofuels: a seed to wheel perspective. Springer, London
27. Banerjee R, Pandey A (2002) Bioindustrial application of sugarcane bagasse: a technology perspective. Int Sugar J 1:3–7
28. Beeharry RP (2001) Carbon balance of sugarcane bioenergy systems. Biomass Bioenergy 20(5):361–370
29. Sun J et al (2004) Isolation and characterization of cellulose from sugarcane bagasse. Polym Degrad Stab 84: 331–339
30. Pessoa J, de Manchilha I, Sato S (1997) Evaluation of sugarcane hemicellulose hydroyzate for cultivation of yeasts and filamentous fungi. J Indian Microbiol Biotechnol 18:360–363
31. Halling P, Simms-Borre P (2008) Overview of lignocellulosic feedstock conversion into ethanol – focus on sugarcane bagasse. Int Sugar J 110:191–194
32. Rooney WL et al (2007) Designing sorghum as a dedicated bioenergy feedstock. Biofuels Bioproducts Biorefining (Biofpr) 1(2):147–157
33. Billa E et al (1997) Structure and composition of sweet sorghum stalk components. Ind Crops Prod 6(3–4):297–302
34. Almodares A, Mostafafi D (2006) Effects of planting date and time of nitrogen application of yield and sugar content of sweet sorghum. J Environ Biol 27:601–605
35. Grassi G, Tondi G, Helm P (2004) Small-sized commercial bioenergy technologies as an instument of rural development, in biomass and agriculture: sustainability, markets and policies. OECD Publication Service, Paris, pp 277–287
36. Hunter EL, Anderson IC (1997) Sweet sorghum. Hortic Rev 21:73–104
37. Miller FR, McBee GG (1993) Genetics and management of physiological systems of sorghum for biomass production. Biomass Bioenergy 5(1):41–49
38. Vermerris W et al (2009) Production of biofuel crops in florida: Sweet sorghum. Institute of Food and Agricultural Sciences, University of Florida, Gainesville
39. Belaychi L, Delmas M (1997) Sweet sorghum bagasse: a raw material for the production of chemical paper pulp. Effect of depithing. Ind Crops Prod 6:229–232
40. Corredor D et al (2009) Evaluation and characterization of forage sorghum as feedstock for fermentable sugar production. Appl Biochem Biotechnol 158(1):164–179
41. Bryan W, Monroe G, Caussariel P (1985) Solid-phase fermentation and juice expression systems for sweet sorghum. Trans ASAE 28(1):268–274
42. Sipos B et al (2008) Sweet sorghum as feedstock for ethanol production: enzymatic hydrolysis of steam-pretreated bagasse. Appl Biochem Biotechnol 153:151–162
43. Heaton E, Voigt T, Long SP (2004) A quantitative review comparing the yields of two candidate C4 perennial biomass crops in relation to nitrogen, temperature and water. Biomass Bioenergy 27(1):21–30
44. Finlay R (2004) Mycorrhizal fungi and thier multifunctional roles. Mycologist 18:91–96
45. Beaty E, Engel J, Powell J (1978) Tiller development and growth in switchgrass. J Range Manage 31:361–365

46. Monteith JL (1978) Reassessment of maximum growth rates for C3 and C4 crops. Exp Agric 14(01):1–5
47. Ney R, Schnoor J (2002) Incremental life cycle analysis: using uncertainty analysis to frame greenhouse gas balances from bioenergy systems for emission trading. Biomass Bioenergy 22:257–269
48. Hodkinson T, Renoize S, Chase M (1997) Systematics of *Miscanthus*. Aspects of Applied Biology 49:189–198
49. Greef JM, Deuter M (1993) Syntaxonomy of Miscanthus × giganteus, vol 67. Blackwell, Berlin
50. Linde-Laursen I (1993) Cytogenetic analysis of Miscanthus 'Giganteus', an interspecific hybrid. Hereditas 119(3): 297–300
51. Surlock J (1999) Miscanthus – a review of the European experience with a novel energy crop. Oak Ridge National Laboratory, Oak Ridge
52. Walsh M, McCarthy S (1998) Miscanthus handbook. In: Tenth European bioenergy conference, Wurzburg, 1998. CARMEN Publishers
53. Clifton-Brown J, Long SP, Jorgensen U (2001) Miscanthus productivity. In: Jones MB, Walsh M (eds) Miscanthus for energy and fibre. James & James, London
54. Naidu SL et al (2003) Cold tolerance of C4 photosynthesis in Miscanthus × giganteus: adaptation in amounts and sequence of C4 photosynthetic enzymes. Plant Physiol 132(3):1688–1697
55. Naidu SL, Long SP (2004) Potential mechanisms of low-temperature tolerance of C4 photosynthesis in Miscanthus × giganteus: an in vivo analysis. Planta 220(1): 145–155
56. Christian DG, Haase E (2001) Agronomy of Miscanthus. In: Jones MB, Walsh M (eds) Miscanthus for energy and fibre. James & James, London, pp 21–45
57. Lewandowski I et al (2000) Miscanthus: European experience with a novel energy crop. Biomass Bioenergy 19(4):209–227
58. Clifton-Brown J et al (2002) Modelled Biomass production potential of Miscanthus and actual harvestable yield as influenced by harvest time. In: 12th European Biomass conference and exhibition, Amsterdam
59. Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of Miscanthus. Glob Change Biol 14(9):2000–2014
60. Wikberg S (1990) The genus Miscanthus: a summary of available literature. Project Agro-fibre. University of Lund, Sweden
61. Booth E, Batchelor S, Walker K (1996) An evaluation of the potential of fiber crops in Scotland. In: Ninth international conference on Jojoba and its uses and third international conference on new industrial crops and products, Catamarca, 1996
62. Harvey J, Hutchens M (1994) Progress in commercial development of Miscanthus in England. In: Biomass for energy, environment, agriculture and industry – Proceedings of the eighth EC conference, Elsevier Science, Oxford
63. Visser P et al (2001) Utilization of Miscanthus. In: Jones M, Walsh M (eds) Miscanthus for energy and fibre. James & James, London, p 218
64. Jefferson P et al (2002) Performance or American native grass cultivars in the Canadaian prairie provinces. Native Plants J 3:24–33
65. Hitchcock AS, US Department of Agriculture (1935) Manual of the grasses of the United States. Government Printing Office, Washington
66. McLaughlin S (1992) New switchgrass biofuels reserach program for the Southeast. In: Proceedings of the annual automotive technology development contractors' coordination meeting, Dearborn, 1992
67. Parrish DJ, Fike JH (2004) The biology and agronomy of switchgrass for biofuels. Crit Rev Plant Sci 24:423–459
68. Porter CJ (1966) An analysis of varitation between upland and lowland switchgrass (Panicum Virgatum L) in central Oklahoma. Ecology 47:980–992
69. Hopkins A et al (1996) Chromosome number and nuclear DNA content of several switchgrass populations. Crop Sci 36: 1193–1195
70. Gunderson CA et al (2008) Exploring potential U.S. switchgrass production for lignocellulosic ethanol. Oak Ridge National Laboratoy, Oak Ridge
71. Jung G, Shaffer J, Stout W (1988) Switchgrass and bluestem responses to amendment on strongly acid soil. Agron J 80:669–676
72. Hanson J, Johnson H (2005) Germination of switchgrass under various temperature and pH regimes. Seed Technol 27(2): 203–210
73. Stroup J et al (2003) Comparison of growth and performance in upland and lowland switchgrass types to water and nitrogen stress. Bioresour Technol 86:65–72
74. Brejda J et al (1993) Dependence of 3 Nebraska sandhills warm-season grasses in vesicular-arbuscular mycorrhizae. J Range Manage 46:14–20
75. Johnson N (1998) Responses of Salsola kali and Panicum virgatum to mycorrhizal fungi, phosphorus and soil organic matter: implication for reclamation. J Appl Ecol 35:86–94
76. Hsu F, Nelson C (1986) Planting date effects on seedling development of perennial warm-season forage grasses II. Seedling Growth. Agron J 78:38–42
77. Hsu F, Nelson C, Matches A (1985) Temperature effects on germination of perrenial warm-season forage grasses. Crop Sci 25:215–220
78. Vogel K (2004) Switchgrass. In: Moser LE, Sollenberger L, Burson B (eds) Warm-season grasses. ASA-CSSA-SSSA, Madison
79. Cossar R, Baldwin B (2004) Establishment of switchgrass with sorghum-sudangrass. In: Third Eastern native grass symposium, Omnipress, Chapel Hill, 2004
80. Zarnstorff M (1993) Physioecological effects of cultural and pesticide management on no-till establishment of switchgrass and seed dormancy. Dissertation Abstract International. Science and Engineering

81. McKenna J, Wolf D, Lentner M (1991) No-till warm-season grass establishment as affected by atrazine and carbofuran. Agron J 83:311–316
82. Wolf D et al (1989) No-till establishment of perennial, warm-season grasses for biomass production. Biomass 20:209–217
83. Brejda J (2000) Fertilization of native warm-seaon grasses. In: Anderson B, Moore K (eds) Native warm-season grasses: research trends and issues. Crop Science Society of America, Madison, pp 177–200
84. Hall KE, George JR, Riedl RR (1982) Herbage dry matter yields of switchgrass, big bluestem, and indiangrass with N fertilization. Agron J 74(1):47–51
85. Clark F (1977) Internal cycling of 15nitrogen in shortgrass praire. Ecology 58:1322–1333
86. Tin X et al (2006) Switchgrass as an alternate feedstock for power generation: integrated environmental, energy, and economic life-cycle analysis. Clean Technol Environ Policy 8(4):233–249
87. McLaughlin S et al (1999) Developing switchgrass as a bioenergy crop. In: Janick J (ed) Perspectives on new crops and new uses. ASHS Press, Alexandria
88. Sanderson MA et al (1996) Switchgrass as a sustainable bioenergy crop. Bioresour Technol 56(1):83–93
89. Thomason W et al (2004) Switchgrass response to harvest frequency and time and rate of applied nitrogen. J Plant Nutr 27:28
90. Schmer MR et al (2008) Net energy of cellulosic ethanol from switchgrass. Proc Natl Acad Sci 105(2):464–469
91. Comis D (2006) Switching to switchgrass makes sense. In: Agricultural research, USDA-ARS
92. Anderson B (2000) Use of warm-season grasses by grazing livestock. In: Anderson B, Moore K (eds) Native warm-season grasses: research trends and issues. Crop Science Society of America, Madison, pp 147–158
93. Moore K et al (2004) Sequential grazing of cool- and warm-season pastures. Agron J 96:1103–1111
94. McLaughlin SB, Adams Kszos L (2005) Development of switchgrass (Panicum virgatum) as a bioenergy feedstock in the United States. Biomass Bioenergy 28(6):515–535
95. Sanderson MA, Read JC, Reed RL (1999) Harvest management of switchgrass for biomass feedstock and forage production. Agron J 91(1):5–10
96. Entry JA, Watrud LS (1998) Potential remediation of 137Cs and90 Sr contaminated soil by accumulation in alamo switchgrass. Water Air Soil Pollut 104(3):339–352
97. Hohenstein W, Wright LL (1994) Biomass energy production in the United States: an overview. Biomass Bioenergy 6(3):161–173
98. Fike JH et al (2006) Long-term yield potential of switchgrass-for-biofuel systems. Biomass Bioenergy 30(3):198–206
99. Duffy M, Nanhou V (2002) Costs of producing switchgrass for biomass in Southern Iowa. In: Janick J, Whipkey A (eds) Trends in new crops and New Uses. ASHS Press, Alexandria
100. Vogel KP et al (2002) Switchgrass biomass production in the midwest USA: harvest and nitrogen management. Agron J 94(3):413–420
101. Vogel K et al (2006) Switchgrass for biomass: farm-scale production practices affect feedstocck costs and quanitities in the Northern Great Plains. In: Eighth symposium on biotechnology for fuels and chemicals, program abstracts, Nashville, 2006
102. Sanderson M et al (2004) Alternative uses of warm-season grasses. In: Moser L et al (eds) Warm-season (C4) grasses. ASA, CSSA and SSSA, Madison, pp 389–416
103. Parikka M (2004) Global biomass fuel resources. Biomass Bioenergy 27(6):613–620
104. Ragauskas AJ et al (2006) The path forward for biofuels and biomaterials. Science 311(5760):484–489
105. Ruark G et al (2006) Perennial crops for bio-fuels and conservation. USDA Forest Service/UNL Faculty Publications, 2006, p. 18
106. Stettler R et al (1996) Biology of populus and its implications for management and conservation. NRC Researc Press, Ottawa
107. Tuskan G. Popular poplars. trees for many purposes. Available from: http://bioenergy.ornl.gov/main.aspx. Accessed November 28, 2010
108. Demchik M et al (2002) Hybrid poplars as an alternative crop. Natural Resources Special Report
109. Walsh M et al (2003) Bioenergy crop production in the United States. Potential quantities, land use changes, and economic impacts on the agricultural sector. Environ Resour Econ 24:313–333
110. Amicheva BY, Johnston M, Rees KCJV (2010) Hybrid poplar growth in bioenergy production systems: biomass prediction with a simple process-based model (3PG). Biomass Bioenergy 34(5):687–702
111. Labrecque M, Teodorescu TI (2003) High biomass yield achieved by Salix clones in SRIC following two 3-year coppice rotations on abandoned farmland in southern Quebec, Canada. Biomass Bioenergy 25(2):135–146
112. Lattimore B et al (2009) Environmental factors in woodfuel production: opportunities, risks, and criteria and indicators for sustainable practices. Biomass Bioenergy 33(10): 1321–1342
113. Perez-Verdin G et al (2003) Woody biomass availability for bioethanol conversion in Mississippi. Biomass Bioenergy 33: 492–503
114. Pellis A, Laureysens I, Ceulemans R (2004) Growth and production of a short rotation coppice culture of poplar I. Clonal differences in leaf characteristics in relation to biomass production. Biomass Bioenergy 27(1):9–19
115. Goodrich M (2009) From the roots up, developing trees for sustainable biofuel. In: Michigan Tech News, 2009
116. Reidy S (2006) Purdue University Researchers Study poplar trees as feedstock for cellulose ethanol. BioFuels Journal. http://www.biofuelsjournal.com/articles/Purdue_University_Researchers_Study_Poplar_Trees_as_Feedstock_for_Cellulose_Ethanol-37114.html. Accessed November 28, 2010

117. Huang H et al (2009) Effect of biomass species and plant size on cellulosic ethanol: a comparative process and economic analysis. Biomass Bioenergy 33(2):234–246
118. Farrell A, Plevin R et al (2006) Ethanol can contribute to energy and environmental goals. Science 311(5760): 506–508
119. Davidson J (1993) Ecological aspects of Eucalyptus plantations. in regional expert consultation on eucalyptus. RAPA publication 1995
120. Iglesias T, Wilstermann D (2009) Global Eucalyptus Map (v 1.2). In: GIT forestry consulting's eucalyptologics: information resources on Eucalyptus cultivation worlwide, 2009
121. Groenendaal G (1983) History of Eucalyptus in California. In: Proceedings of a workshop on Eucalyptus, Sacramento, 1983. Pacific Southwest Forest and Range Experiment Station, Berkeley
122. Henter H (2005) Tree wars: the secret life of Eucalyptus. In Alumni. 2005, University of California, San Diego
123. Whitesell C et al (1992) Short rotation management of eucalyptus: guidelines for plantations in Hawaii. USDA Forest Service, Pacific Southwest Research Station, Albany
124. Stricker J et al (2000) Short rotation woody crops for Florida. University of Florida, Gainesville
125. Rockwood D et al (2008) Energy product options for Eucalyptus species grown as short rotation woody crops. Int J Mol Sci 9:1361–1378
126. Whitesell C, DeBell D, Schubert T (1988) Six-year growth of eucalyptus
127. Luzar J (2007) The political ecology of a "forest transition": Eucalyptus forestry in the Southern Peruvian. Ethnobot Res Appl 5:85–93
128. Pousajja R (1996) Eucalyptus plantations in Thailand. In: Kashio M, White K (eds) Reports submitted to the regional consultation on eucalyptus
129. Song Y (1992) Utilization of Eucalyptus in China. Appita 45(6):382–383
130. Steinbeck K, McAlpine R, May J (1972) Short rotation culture of sycamore: a status report. J Forest 70(4): 210–213
131. Skolmen R (1983) Growth and yield of some Eucalyptus of interest in California. In: Proceedings of a workshop on eucalyptus in California, Sacramento, 1983. USDA Forest Service, Pacific Southwest Forest and Range Experiment Station, Berkeley
132. Lull G (1908) A handbook for Eucalyptus planters, State Board of Forestry (ed), US Dept of Agriculture, Forest Service
133. Cremer K, Cromer R, Florence R (1978) Stand establishment. In: Hillis W, Brown A (eds) Eucalyptus for wood production. Commonwealth Scientific and Industrial Research Organization, Aulmlia, pp 81–135
134. Knudsen D, Yahner E, Carrea H (1970) Fertilizing Eucalyptus on Brazilian savannah soils. Commonw Forest Rev 49(1): 30–40
135. DeBell D, Whitesell C, Schubert T (1989) Using N_2-fixing Albizia to increase growth of Eucalyptus plantations in Hawaii. Forest Sci 25:64–75
136. De Jesus R, Dias G, Cardoso E (1992) Eucalyptus/leucaena mixture experiment – growth and yields. Biomass and Bioenergy, IPEF, 39:41–46
137. Shailaja R, Ravindranath N, Jagadish K (1991) Experiments in mixed species forestry. In: Annual symposium of bioenergy society, New Delhi, 1991
138. Elmore C (1983) Vegetation management in Eucalyptus. In: Proceedings of a workshop on Eucalyptus in California, Sacramento, 1983. Pacific Southwest Forest and Range Experiment Station, Berkeley
139. Arbogen (2010) Purpose grown trees as an economical and sustainable biomass feedstock. Available from: http://www1.eere.energy.gov/biomass/biomass2010/pdfs/biomass2010_track2_s2_wells.pdf
140. Sims R et al (2008) 1st to 2nd generation biofuel technologies: an overview of current industry and RD&D activites. International Energy Agency, Paris
141. Kinoshita C, Zhou J (1999) Siting evaluation for biomass-ethanol production in Hawaii. National Renewable Energy Laboratory, Golden
142. Stape J et al (2010) The Brazil Eucalyptus potential productivity project: influence of water, nutrients and stand uniformity on wood production. For Ecol Manage 259:1684–1694
143. Hubbard H, Kinoshita C (1993) Investigation of biomass-for-energy production on Molokai. Hawaii Naturall Energy Institute, Honolulu
144. Gonzalez R, Wright J, Saloni D (2010) The business of growing eucalyptus for biomass, Biomass Magazine. http://biomassmagazine.com/articles/3620/the-business-of-growing-eucalyptus-for-biomass. Accessed November 29, 2010
145. de Vries S et al (2010) Resource use efficiency and environmental performance of nine major biofuel crops, processed by first-generation conversion techniques. Biomass Bioenergy 34(5):588–601
146. Almodares A, Hadi M (2009) Production of bioethanol from sweet sorghum: a review. Afr J Agric Res 4:772–780
147. Koppen S, Reinhardt G, Gartner S (2009) Assessment of energy and greenhouse gas inventories of sweet sorghum for first and second generation bioethanol. Food and Agriculture Organization of the United Nations, Rome
148. Santana R, Barros N, Comeford N (2000) Above-ground biomass, nutrient content, and nutrient use efficiency of Eucalyptus plantations growing in different sites in Brazil. NZ J Forest Sci 30:225–236
149. Stape J, Goncalves J, Goncalves A (2001) Relationship btween nursery practices and field performances for Eucalyptus plantations in Brazil: a historical overview and its increasing importance. New Forest 22:19–41
150. Raven P (2009) Biodiversity: What to Do About It in an Age of Rapid Extinction. Presented at the International Plant Molecular Biology Symposium, St. Louis, MO

Biotechnology and Nutritional Improvement of Crops

Gemma Farre[1], Sonia Gomez-Galera[1], Shaista Naqvi[1], Chao Bai[1], Georgina Sanahuja[1], Dawei Yuan[1], Uxue Zorrilla[1], Laura Tutusaus Codony[1], Eduard Rojas[1], Marc Fibla[1], Richard M. Twyman[2], Teresa Capell[1], Paul Christou[3,4], Changfu Zhu[1]
[1]Department of Vegetal Production and Forestry Science, ETSEA, University of Lleida, Lleida, Spain
[2]Department of Biological Sciences, University of Warwick, Coventry, UK
[3]Department de Produccio Vegetal i Ciencia Forestal, Universitat de Lleida/ICREA, Lleida, Spain
[4]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Article Outline

Glossary

Antinutrients Substances that interfere with the absorption of nutrients.

Bioavailability The amount of a nutrient in food that can be absorbed and utilized.

Biofortification Any fortification strategy that improves nutritional content at source (before harvest).

Conventional breeding Any process used to create new plant varieties without recombinant DNA technology (e.g., introgression, mutagenesis, and hybridization).

Gene silencing Prevention of gene expression, usually through epigenetic means such as antisense RNA, RNA interference, or de novo DNA methylation.

Genotype The total genetic constitution of an organism.

Inbred Progeny produced as a result of breeding between genetically similar parents.

Introgression The introduction of a new allele from one species into the gene pool of another by repeated backcrossing of an interspecific hybrid with one of its parents.

Locus The position of a gene or other genetic marker on a chromosome.

Macronutrient A nutrient required at levels exceeding 100 mg/day (includes carbohydrates, fats, proteins, water, and fiber as well as certain minerals).

Malnutrition The situation resulting from a nutrient imbalance in the diet, usually referring to a lack of one or more nutrients but equally applicable to nutrient excess.

Micronutrient A nutrient required in minute amounts (typically less than 10 mg/day); includes vitamins and many minerals.

Mineral An inorganic nutrient, usually represented by a soluble ion.

Phenotype The sum of observable characteristics of an organism.

Phytate (phytic acid) A phosphorus-containing compound in the outer husks of cereal grains that, in addition to limiting the bioavailability of phosphorous itself, binds to other minerals and inhibits their absorption.

Promoter The DNA sequence upstream of a gene that regulates transcription.

Quality protein maize (QPM) A variety of maize (corn) developed by CIMMYT that contains 70–100% higher levels of lysine and tryptophan in the grain compared to normal varieties.

Transgene A gene from one species that has been incorporated into the genome of another organism using recombinant DNA technology.

Transgenic plant A plant that carries integrated exogenous DNA in the nuclear genome.

Vitamin An organic micronutrient.

Definition of the Subject and Its Importance

Food insecurity is one of the most important social issues faced today, with nearly one billion people

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

enduring chronic hunger and an additional two billion people suffering from nutrient deficiencies, mostly in the developing world. Strategies to address food insecurity must ultimately address underlying problems such as poverty and poor governance/infrastructure, but the improvement of agricultural productivity in the developing world is an important goal, and biotechnology is one of a raft of measures being considered to achieve it. Genetically engineered plants provide one route to sustainable higher yields, which will increase the quantity of food available. However, genetic engineering can also increase the nutritional quality of crops, and this is the definition elaborated in this article. In particular, the focus is on biotechnology-based methods to increase the availability of essential nutrients, which are often limiting in human diets and lead to specific deficiency diseases.

Introduction

Food security is taken for granted in the industrialized world, where stable political and social structures ensure that everyone has access to enough safe and nutritious food. In contrast, almost one billion people are chronically undernourished in the developing world, regularly consuming less than 2,000 calories per day [1]. There are also a further two billion people who, despite having access to an adequate source of calories, nevertheless lack essential nutrients; perhaps surprisingly, a significant number of these people are citizens of industrialized countries. This means that up to half the world's population at any one time may suffer from malnutrition [1].

The underlying causes of food insecurity in the developing world are complex but poverty is one of the main factors, reflecting the fact that more than one billion people live on less than US $1 per day and another two billion are only marginally better off. The world's poorest people tend to be rural farmers in developing countries, depending entirely on subsistence agriculture, whereas less than 1% of the population in the industrialized world are farmers and they tend to farm for profit. Because of limited purchasing power, the poorest farmers cannot irrigate their crops or buy fertilizers, herbicides, and pesticides. This leads to soil exhaustion, falling yields and quality, and the crops become susceptible to pests, diseases, and natural disasters such as drought. In the industrialized world, malnutrition can reflect poverty on the fringes of society, but is also an educational/lifestyle issue that needs to be approached differently [1].

Any long-term strategy to address food insecurity in the developing world must tackle the underlying problem of poverty by providing rural employment–based income through increased agricultural productivity. Given projected increases in the world's population, the higher cost of oil, falling reserves of fresh water, and greater urbanization, it will certainly be necessary to increase the quality, quantity, and diversity of major food crops. A variety of strategies have been proposed, including the efficient use of organic and inorganic fertilizers, irrigation strategies, soil and water conservation, pest and disease management, and the production of improved plant varieties with higher yields or novel products. Biotechnology provides a range of tools that can be used to improve agriculture in the developing world by lifting yields or increasing food quality, and the latter aspect of biotechnology is considered in this article – the use of biotechnology for nutritional improvement [2, 3].

Overview of Essential Nutrition in Humans

Nutrients and Their Roles in Human Health

Nutrients are chemical substances in food that are necessary for humans to have healthy and active lives. Humans require at least 49 defined nutrients to meet their metabolic needs (Table 1), some highly specific and some represented by families of related molecules. Inadequate consumption of even one of these nutrients will result in adverse metabolic effects leading to poor health, impaired development in children, and (if this is a population-wide problem) an economic impact on society. Table 2 lists the daily guideline amounts in adults for some of these nutrients, as reported by the Food and Agricultural Organization (FAO) of the United Nations and the World Health Organization (WHO) [4]. Table 3 shows some common dietary sources of different nutrients. Generally, nutrients are divided into two broad categories: macronutrients (needed in gram or hundred milligram quantities per day, mainly for energy, structural components of body tissues, and bulk composition of body fluids) and

Biotechnology and Nutritional Improvement of Crops. Table 1 The 49 nutrients essential for sustaining human life [37]

Water and energy	Protein (amino acids)	Lipids-fat (fatty acids)	Macro-elements	Micro-elements	Vitamins
Water	Isoleucine	Linoleic acid	Na	Fe	A
Carbohydrates	Leucine	α-Linolenic acid	K	Zn	D
	Lysine		Ca	Cu	E
	Methionine		Mg	Mn	K
	Phenylalanine		S	I	C (ascorbic acid)
	Threonine		P	F	B1 (thiamine)
	Tryptophan		Cl	B	B2 (riboflavin)
	Valine			Se	B3 (Niacin)
				Mo	B5 (pantothenic acid)
				Ni	B6
				Cr	B9 (folate)
				V	Biotin
				Si	B12 (cobalamin)
				As	Choline
				Sn	
				Co	

Source: [37]

micronutrients (needed in microgram or milligram quantities per day, for many diverse functions). Macronutrients include carbohydrates, fats, proteins, certain mineral ions, water, and fiber, whereas micronutrients include a range of inorganic ions (minerals) and organic molecules (vitamins) [4].

Macronutrients

Carbohydrates and fats consist only of carbon, hydrogen, and oxygen. They are primarily used to derive energy, although they also contribute to some structures in the body, such as the carbohydrates heparan sulfate, chrondoitin sulfate, and hyaluronic acid in the extracellular matrix, and various lipid components of cell membranes. Carbohydrates range from simple monosaccharides (e.g., glucose, fructose, and galactose) to complex polysaccharides (e.g., starch, glycogen, the matrix carbohydrates listed above, and cellulose, the predominant component of plant cell walls). All polysaccharides are polymers of monosaccharide units. Therefore, carbohydrates that can be digested by humans are broken down into monosaccharides and metabolized for energy, used as substrates to synthesize other molecules, or reassembled into energy-storing or structural polymers. Many carbohydrates cannot be broken down in the human digestive system because the corresponding enzymes are not present (e.g., cellulose, inulin) and these are collectively known as dietary fiber. Insoluble fibers such as cellulose help the digestive system function properly (preventing constipation and diarrhea), but there are also many soluble forms of fiber such as inulin which have positive roles in digestion, slowing the movement of food through the gut and facilitating nutrient absorption. Both insoluble and soluble fibers are thought to help prevent colon cancer and cardiovascular disease. Although carbohydrates as a general class of

Biotechnology and Nutritional Improvement of Crops. Table 2 Recommended nutrient intakes for males and females between the ages of 25 and 50 years [37]

Nutrient	Assessment	Male	Female
Energy (kcal)	AEA[a]	2,900	2,200
Protein (g)	AEA	63	50
Vitamin A (mg retinol equivalent)	RDA[b]	1,000	800
Vitamin D (mg)	RDA	5	5
Vitamin E (mg α-tocopherol equivalent)	RDA	10	8
Vitamin K (mg)	RDA	80	65
Riboflavin (mg)	RDA	1.7	1.3
Niacin (mg niacin equivalent)	RDA	19	15
Thiamine (mg)	RDA	1.5	1.1
Pantothenic acid (mg day^{-1})	ESADDI[c]	4–7	4–7
Vitamin B6 (mg)	RDA	2	1.6
Vitamin B12 (mg)	RDA	2	2
Biotin (mg day^{-1})	ESADDI	30–100	30–100
Folate (mg)	RDA	200	180
Vitamin C (mg)	RDA	90	60
Ca (mg)	RDA	800	800
P (mg)	RDA	800	800
Mg (mg)	RDA	350	280
Na (mg)	MR[d]	500	500
K (mg)	MR	2,000	2,000
Cl (mg)	MR	750	750
Fe (mg)	RDA	10	15
Zn (mg)	RDA	15	12
Cu (mg)	ESADDIC	1.5–3	1.5–3
Se (mg)	RDA	70	55
I (mg)	RDA	150	150
Mn (mg)	ESADDI	2 ± 5	2 ± 5
Mo (mg)	ESADDI	75 ± 250	75 ± 250
Cr (mg)	ESADDI	50 ± 200	50 ± 200
F (mg)	ESADDI	1.5 ± 4	1.5 ± 4

Source: [37]

[a]AEA, average energy allowance

[b]RDA, recommended dietary allowances

[c]ESADDI, estimated safe and adequate daily dietary intakes

[d]MR, minimum requirement

Biotechnology and Nutritional Improvement of Crops. Table 3 Sources of some essential nutrients [4, 5]

Component	Examples of sources
Carbohydrates	Cereal grains and potato
Insoluble fiber	Whole grain barley and vegetable peels
Soluble fiber	Fruits, vegetables, and legumes
Essential fatty acids	Fish, flax seed oils, soybeans, pumpkin seeds, sunflower seeds, walnuts, most vegetables, nuts, seeds, and marine oils
Dietary protein	Tofu and other soy-products, eggs, grains, legumes, and dairy products such as milk and cheese, meat, fish
Vitamin A	Carrots, yams, pumpkins, yellow or orange fruits, fish, eggs, and tuna
Vitamin B1	Whole grains, rice bran, lean meats, legumes, wheat germ, oranges, poultry, fish, and enriched pastas
Vitamin B2	Fortified grains and cereals, leafy green vegetables, poultry, fish, yogurt, milk, and cheese
Vitamin B3	Fortified breads and cereals, brewer's yeast, broccoli, carrots, cheese, dandelion greens, eggs, fish, milk, peanuts, potatoes, tomatoes, tuna, beef liver, and chicken breast
Vitamin B6	Whole grain breads and cereals, fish, chicken, and bananas
Vitamin B9	Pinto beans, navy beans, green leafy vegetables, beef, brown rice, bran, cheese, lamb, liver, milk, mushrooms, oranges, pork, and tuna
Vitamin B12	Ham, clams, cooked oysters, king crab, herring, salmon, tuna, lean beef, liver, and low fat dairy products
Vitamin C	Citrus fruits, strawberries, broccoli, melons, peppers, collards, dandelion greens, onions, radishes, and watercress
Vitamin D	Sun exposure, sardines, salmon, fortified milk, fortified cereals, herring, liver, tuna, margarine, and cod liver oil
Vitamin E	Whole grains, wheat germ, nuts, spinach, and sunflower seeds
Vitamin K	Dark-green leafy vegetables and the skins of fruit and vegetables
Calcium	Dairy products and green leafy vegetables
Magnesium	Fish, dairy products, nuts, soybeans, and cocoa
Phosphorus	Milk, cheese, meats, fish, and eggs
Sulfur	Meats, fish, dairy products, eggs, and garlic
Potassium	Legumes, whole grains, and bananas
Sodium	Table salt (sodium chloride, the main source), milk, and spinach
Chlorine	Table salt and unprocessed foods
Iron	Red meat, and leafy vegetables (especially spinach)
Zinc	Wheat germ, pine nuts, sesame seeds, sunflower seeds, and beefsteak
Manganese	Avocados, berries, nuts, egg yolks, whole grains, green leafy vegetables, and legumes
Iodine	Seafood, dairy products, and iodized salt (table salt)
Fluorine	Drinking water, seafood, teas, and toothpaste
Selenium	Nuts, lamb's kidney, mushrooms, and sunflower seeds
Molybdenum	Legumes, dark green leafy vegetables, and grains
Nickel	Chocolate, nuts, fruits, and vegetables
Chromium	Whole grain breads, brown rice, cheese, and lean meats

Biotechnology and Nutritional Improvement of Crops. Table 3 (Continued)

Component	Examples of sources
Vanadium	Seafood, mushrooms, olives, whole grain breads, carrots, and vegetable oils
Cobalt	Vitamin B12, red meat, fish, eggs, cheese, and milk
Silicon	Whole grain breads and cereals, beets, bell peppers, and legumes
Copper	Beans, almonds, broccoli, garlic, soybeans, peas, and seafood

Source: Revised and updated from [4, 5]

compounds are essential nutrients, no specific carbohydrate is essential because the human body can synthesize all the carbohydrates it needs from simple sugars.

In contrast, fats in the diet include both essential and nonessential molecules. The majority of dietary fat is in the form of triacylglycerides (fatty acid esters of glycerol) and the fatty acids can be interconverted or converted into more specialized phospholipids and sphingolipids that have numerous roles in the body, such as cell membrane components and signaling molecules. Other lipids are derived from cholesterol, which is synthesized in the liver. There are several essential fatty acids as well as fat-soluble vitamins that the body cannot synthesize de novo, and these are discussed in detail below.

Proteins are the third major class of macronutrients and these contain nitrogen in addition to carbon, oxygen, and hydrogen. Many proteins also contain sulfur (see below). Fibrous proteins form the structural and mechanical components of tissues (e.g., muscles owe their contractile ability to the proteins actin and myosin, hair and nails are comprised predominantly of keratin, and skin contains large amounts of collagen and elastin). In contrast, most globular proteins are enzymes, the biological catalysts that ensure chemical reactions can take place at the body's ambient temperature. Other proteins fulfill a range of biological functions – signaling molecules and receptors, transmembrane conduits such as ion channels, components of the immune system, effectors of blood clotting, transport and storage of other molecules (e.g., hemoglobin, oxygen, ferritin, and iron), and the control of gene expression. Proteins are linear polypeptides derived from a panel of 20 "standard" amino acids specified by the genetic code (as well as two specialized variants encoded through unique mechanisms), plus a range of posttranslational forms that are generated by chemical modification after protein synthesis. Many of the standard amino acids can be synthesized de novo by humans but some cannot, and these are known as essential amino acids (see below). Proteins are described as "complete" in the sense of nutritional completeness if they provide a source of all the essential amino acids but this designation refers to the sum of all proteins in a meal, not an individual protein molecule.

Essential Amino Acids

Of the 20 standard amino acids, eight (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) (Table 1) are described as essential because the human body cannot synthesize sufficient amounts de novo so they must be obtained from food [5]. A diet that contains adequate amounts of essential amino acids is particularly important during pregnancy, lactation, early development, and following injury. Most plants are deficient in at least one essential amino acid but a balanced diet provides adequate quantities of all. For example, cereal storage proteins are generally deficient in lysine and threonine whereas legumes lack the sulfur-containing amino acids methionine and cysteine. A diet solely comprising one of these protein sources will therefore be deficient for one or more essential amino acids, but it is possible to combine two incomplete protein sources (e.g., rice and beans) to make a complete protein meal, and such characteristic combinations are the basis of distinct cultural cooking traditions. Food from animals, such as meat, eggs, fish, milk, and cheese, provide all the essential amino acids.

Essential Fatty Acids

Fats are more correctly described as triglycerides (three fatty acids attached to one glycerol backbone), and most fatty acids are nonessential in that the body can synthesize them by interconverting other fatty acids. The exceptions are the omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), so named because the first double-bond is found at the third (or sixth) carbon from the terminal CH_3 group. These cannot be synthesized de novo from saturated fats, nor interconverted, and must be obtained from the diet [6]. There are several essential fatty acids in each category, but the body can synthesize any omega-3 fatty acid given a source of one of them (ditto for the omega-6 fatty acids) and therefore only the group is defined as essential. The simplest source of omega-3 is α-linolenic acid and the simplest source of omega-6 is linoleic acid. Good sources of both these molecules include fish, shellfish, seeds, nuts, and leafy vegetables.

Macrominerals

Minerals are inorganic molecules, and those required at levels exceeding 100–200 mg/day (calcium, chlorine, magnesium, phosphorus, potassium, sodium, and sulfur; see Table 2) are known as macrominerals [4]. Their function is generally structural and/or electrolytic/osmotic, although they may also perform additional specialized roles. For example, calcium is an important electrolyte in muscles and in the digestive system, but is also a structural component of bones and teeth, and furthermore acts as a signaling molecule and as a trigger for the transmission of nerve impulses. Sodium, potassium, and chlorine are key electrolytes that maintain osmotic balance in cells. Magnesium is an important buffer and stabilizer for organic phosphates including nucleic acids, and is absolutely required for the activity of adenosine triphosphate (ATP). It is also part of the active site of many enzymes. Phosphorus is the key energy currency in cells (as part of ATP). It is also an important structural component of nucleic acids and some proteins, the basis of a key regulatory mechanism in signal transduction pathways, a component of membrane lipids, and (along with calcium) forms the mineral structure of bones and teeth. Finally, sulfur is found in two essential amino acids (methionine and cysteine) as well as two posttranslational variants with central roles in core metabolism (homocysteine and taurine). The ability of cysteine residues to form disulfide bridges is critical to the structural integrity of many proteins. Sulfur is also found in coenzyme A, which is needed for carbohydrate and fatty acid metabolism, as well as providing the thiol group in many other reactions.

Micronutrients

Micronutrients are required in small quantities (generally less than 100 mg/day) and are divided into organic molecules (vitamins) and inorganic molecules (microminerals). Vitamins are organic substances that (a) are not carbohydrates, amino acids, or fats, and (b) cannot be manufactured by the body in sufficient amounts in all circumstances and must therefore be obtained from the diet. There are 13 human vitamins, although some are represented by a small family of interconvertible molecules rather than a single substance. Most are required because humans lack the metabolic capability to produce them, although vitamin D is an exception because it is produced in the skin during exposure to UVB irradiation albeit not always in sufficient quantities to make dietary sources unnecessary. Vitamins are classified as either water soluble or fat soluble. In humans, there are four fat-soluble vitamins (A, D, E, and K), which are transported throughout the body in fat globules and stored in the liver and other fatty tissues, and nine water-soluble vitamins (eight B vitamins and vitamin C) that are not stored in the body and must be replaced every day.

Minerals that are required at levels below 100–200 mg/day are known as microminerals or trace minerals (Table 2) and these are required in minute amounts usually because they are required for the catalytic activity of a small number of specific enzymes. However, certain microminerals have more significant roles [4]. For example, iron can act as both an electron donor and acceptor, and in this context forms the functional core of the heme complex, which is found in the oxygen-binding molecules hemoglobin and myoglobin, and in the catalytic center of cytochromes (enzymes that carry out redox reactions). Iron is therefore required for oxygen transport in the body and for energy metabolism, also contributing to the catalytic activity of a range of nonheme enzymes such as

ribonuclease reductase. Zinc also has multiple functions – it is an essential component of hundreds of enzymes (e.g., carboxypeptidase, liver alcohol dehydrogenase, and carbonic anhydrase) as well as transcription factors (zinc fingers and related molecules) and signaling proteins. The roles of other microminerals are more specialized and are discussed below.

Facultative Nutrients

The eight essential amino acids are needed by everyone. In addition, cysteine, tyrosine, histidine, and arginine are essential for infants and children because they are required in large amounts for growth and development, and that demand cannot be satisfied without a dietary source [5]. For the same reason, children have greater requirements for the essential fatty acids than adults, although these are not facultative because they are also essential nutrients in adults despite being required in lower quantities. The amino acids arginine, cysteine, glycine, glutamine, histidine, proline, serine, and tyrosine are also considered conditionally essential, meaning they are not normally required in the diet, but must be supplied to specific populations that do not synthesize them in adequate amounts [7].

It is sometimes more convenient to define the essentialness of amino acids on a group basis in the same way as the essential fatty acids, since interconversion is possible within a group but no member of that group can be synthesized de novo. This applies to the sulfur-containing amino acids methionine, homocysteine, and cysteine, and to the aromatic amino acids phenylalanine and tyrosine. Likewise, arginine, ornithine, and citrulline, which are interconvertible within the urea cycle, can be considered as a group for nutritional purposes.

Nutrient Deficiencies

The lack of essential nutrients causes widespread malnutrition, a hidden problem in many communities because its effects are often subclinical or affect physical and cognitive development in a cumulative manner. Micronutrient deficiencies in particular have been referred to as "hidden hunger"; such deficiencies occur on a population-wide basis when the diet lacks diversity or is overly dependent on a single staple food [4], but in individual cases may reflect a genetic abnormality that prevents nutrient absorption or metabolism. Some important nutrient deficiencies and disorders are discussed below.

Macronutrient Deficiencies and Disorders

A lack of carbohydrates in the diet leads to a calorie deficit and results in hunger, then eventually catabolysis and atrophy as the body uses its own tissues as a source of energy to maintain essential functions. These are the early signs of starvation, and can result in permanent organ damage and ultimately death. Besides this general process, there are several specific disorders of carbohydrate metabolism such as galactosemia and glycogen storage diseases, which reflect an inability to break down or synthesize particular carbohydrates. As in the case for carbohydrates, a general deficiency in fats results in a calorie deficit, which if prolonged results in the symptoms of starvation. Deficiencies for essential fatty acids occur rarely, and mostly in infants. The symptoms include scaly dermatitis, alopecia, hair loss, thrombocytopenia, and stunted growth. The symptoms can be reversed by supplying adequate quantities of omega-3 and omega-6 fatty acids in the diet.

The essential amino acids are components of many proteins, so the symptoms associated with deficiency are often quite general. In adulthood, amino acid deficiencies may result in tiredness, inability to concentrate, irritability, bloodshot eyes, and in the longer term stunted growth, hair loss, anemia, connective tissue defects, inefficient wound healing, and reproductive problems. Acute amino acid deficiencies in childhood are more severe and result in a disease called kwashiorkor which involves swelling of the feet and abdomen, anorexia, ulcerating dermatoses, loss of hair, nails, and teeth, an enlarged fatty liver, and irritable behavior. It is possible that the symptoms of kwashiorkor may also be caused in part by concomitant micronutrient deficiencies and in some cases by poisoning. The disease also affects the immune system, often rendering children incapable of producing antibodies against vaccines.

As well as deficiency diseases, there are also several disorders of amino acid metabolism that need special dietary provisions. These include phenylketonuria (PKU), in which the enzyme phenylalanine hydroxylase is missing so phenylalanine cannot be converted into

tyrosine, and maple syrup urine disease (MSUD), in which the body is unable to use the amino acids isoleucine, leucine, and valine.

Vitamin A Deficiency

Vitamin A in its reduced form (retinal) is required for the production of rhodopsin in the eyes, and helps to maintain epithelial and immune cells (making it necessary for a healthy immune system). In its acidic form (retinoic acid), it is a morphogen in development. Although many foods are said to be good sources of vitamin A, it should be noted that these generally do not contain retinal itself but derivatives that can be converted into retinol and then into either retinal or retinoic acid. Meat and dairy sources of vitamin A primarily contain an esterified form called retinyl palmitate, whereas plants produce pro-vitamin A carotenoids such as β-carotene that are cleaved to produce retinol. These are abundant in a wide variety of dark green, yellow, and orange fruits; and vegetables such as oranges, broccoli, spinach, carrots, squash, sweet potatoes, and pumpkins [8].

Vitamin A deficiency (VAD) causes night blindness, i.e., the deterioration of light sensitive cells (rods) essential for vision in low light intensity and it can also damage the cornea resulting in a total form of blindness called xerophthalmia. The lack of vitamin A has a particularly severe effect on the immune system leaving individuals susceptible to infections [4]. More than four million children worldwide exhibit signs of severe VAD, including 250,000–500,000 per year that become partially or totally blind [9]. During pregnancy, women have a higher demand for vitamin A, and VAD in pregnancy causes nearly 600,000 deaths every year [9].

Vitamin B Group Deficiencies

The vitamin B complex comprises eight distinct molecules with different properties and functions. Vitamin B_1 (thiamine) is a coenzyme in carbohydrate metabolism, with the triphosphate derivative particularly active in neurons. Deficiency causes beriberi, a nervous system disorder resulting in weight loss, various degrees of amnesia and psychosis (in its severest form known as Korsakoff's syndrome), impaired perception, limb weakness, arrhythmia, and swelling, possibly leading to heart failure and death.

Vitamin B_2 (riboflavin) is the central component of the important enzyme cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), whereas vitamin B_3 (niacin) is converted into the cofactors nicotinamide adenine dinucleotide (NAD) and its phosphate derivative NADP. Therefore, both vitamins are required in many enzymes that take part in carbohydrate, fat, and protein metabolism as well as other functions. Riboflavin deficiency causes ariboflavinosis, which is characterized by sensitivity to light, cracked lips, dermatitis, and swelling of the tongue, pharyngeal and oral mucosa, and genitals. Niacin deficiency causes pellagra, which has varied symptoms including diarrhea, dermatitis, insomnia, fatigue, and mental confusion leading in severe cases to dementia. Vitamin B_5 (pantothenic acid) is needed for the synthesis of coenzyme A (CoA), and is therefore critical in the metabolism and synthesis of carbohydrates, proteins, and fats. Deficiency is rare and complete deficiency in humans has not been observed. Vitamin B_6 (pyridoxal, pyridoxine, or pyridoxamine) helps to balance sodium and potassium levels; it is also the precursor of pyridoxal phosphate, a cofactor required for the synthesis of heme and several important neurotransmitters. Deficiency may therefore lead to anemia due to the lack of heme, depression due to its impact on neurotransmitter production, high blood pressure and water retention due to the impact on electrolyte balance, and also elevated levels of homocysteine. Vitamin B_7 (biotin) is a coenzyme in the metabolism of fatty acids and leucine, and it plays a role in gluconeogenesis. Deficiency may lead to stunted growth and neurological disorders in infants. Vitamin B_9 (folic acid) is the source of tetrahydrofolate which is essential in DNA synthesis and many other core metabolic reactions. Deficiency in adults causes macrocytic anemia and elevated levels of homocysteine, but the impact in pregnant women is much more severe, leading to neural tube defects in the fetus. Spina bifida, in which bones of the spine do not completely enclose the spinal cord, is the most common congenital abnormality associated with folate deficiency [10]. Vitamin B_{12} (cobalamin) is involved in the regeneration of folate, which means that deficiency in many cases mimics folic acid deficiency and can be alleviated by folate in the diet. However, it is also the cofactor for two specific (non-folate-dependent) enzymes, methylmalonyl coenzyme A mutase

(MUT), and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR). Therefore, even in the presence of adequate folate, cobalamin deficiency can result in the failure of these reactions, leading to the accumulation of metabolites that weaken and destabilize myelin, resulting in neurological symptoms associated with demyelination. Finally, choline is a B vitamin that has three primary roles: structural integrity and signaling in cell membranes, cholinergic neurotransmission (acetylcholine synthesis), and the provision of methyl groups via its metabolite, trimethylglycine (betaine), for the synthesis of S-adenosylmethionine. Choline deficiency is rare because, like vitamin D, humans can synthesize some choline although not always adequate amounts.

Vitamin C Deficiency

Vitamin C (ascorbic acid) is a powerful electron donor (antioxidant) and a cofactor in several metabolic pathways, including those forming the mature form of collagen. It plays an important role in the synthesis and stabilization of neurotransmitters, and also reduces iron compounds, enhancing the gastrointestinal absorption of dietary nonheme iron [11]. Insufficient vitamin C in the diet causes scurvy, which involves the breakdown of connective tissue fibers and muscular weakness [11]. High levels of vitamin C are found in citrus fruits and green vegetables [4].

Vitamin D Deficiency

Vitamin D is required for normal calcium and phosphorus homeostasis. It is a ligand that, when bound to its receptor, acts as a transcription factor controlling genes that affect calcium and phosphorus absorption; it is therefore particularly important for bone growth and maintenance. Deficiency results in impaired bone mineralization, which leads to bone-softening diseases such as rickets, a childhood disease characterized by stunted growth and deformity of the long bones, and osteomalacia, an adult bone-thinning disorder characterized by proximal muscle weakness and bone fragility, with chronic musculoskeletal pain.

Vitamin E Deficiency

Vitamin E is a group of compounds with powerful antioxidant activity. It protects fatty acids, low-density lipoproteins (LDLs), and other components of cell membranes from oxidation by free radicals [4]. Although it protects all cells, vitamin E is particularly important in red blood cells and neurons, so severe vitamin E deficiency results in anemia and neurological problems associated with nerve degeneration in the hands and feet. Vegetable oils, nuts, and green leafy vegetables are the major sources of vitamin E, and the consumption of foods rich in vitamin E is thought to reduce the risk of cancer, cardiovascular disease, and cataracts [4].

Vitamin K Deficiency

Vitamin K is a group of fat-soluble vitamins derived from 2-methyl-1,4-naphthoquinone that are needed for γ-carboxylation, a form of protein posttranslational modification. This is particularly important for the formation of the calcium-binding Gla domain, which is present in several blood-clotting proteins and osteocalcin. Vitamin K deficiency therefore affects blood coagulation as well as bone mineralization. Vitamin K1 (phylloquinone) needs to be sourced from the diet whereas vitamin K2 (menaquinone) is produced by bacteria in the large intestine and deficiency is rare except in maladsorption disorders or in patients with reduced gut flora (e.g., after treatment with broad-spectrum antibiotics). There are also several synthetic forms of the vitamin.

Iron Deficiency and Toxicity

Iron is the component of heme that binds oxygen, allowing oxygen to be transported from the lungs to peripheral tissues as part of oxyhemoglobin and carbon dioxide to be transported in the opposite direction. Iron also acts as an intracellular electron transporter to transfer energy (especially to the mitochondria) and it is a part of enzyme systems involved in the synthesis of hormones and neurotransmitters [4]. Iron deficiency is the most widespread nutritional disorder in the world, affecting an estimated 1.2 billion people [12]. In children, low iron intake is associated with cognitive dysfunction whereas in adults it causes iron deficiency anemia (IDA), oxidative DNA damage, reduced immunity, and (in pregnant women) premature births/low birth weight [12]. The true prevalence of iron deficiency in a population is greater than the level of clinically detectable IDA because most

individuals are likely to be iron deficient long before a detectable drop in hemoglobin levels [12]. Iron deficiency is often caused by inadequate intake, but it can also result from the consumption of antinutritional molecules such as phytic acid/phytate which inhibit the uptake of bioavailable iron even if sufficient amounts are present in the diet. Foods rich in phytate include many cereal grains, so people with cereal-rich diets are particularly susceptible to iron-deficiency diseases.

The upper limit for iron consumption is 25–50 mg/day. Symptoms of iron toxicity include fatigue, anorexia, dizziness, nausea, vomiting, headache, weight loss, shortness of breath, and possibly a grayish color to the skin [13]. Chronic excess iron intake results in symptoms such as liver cirrhosis, diabetes, and heart failure, and can promote some cancers. Acute overdose (e.g., iron supplements) is toxic and may induce gastrointestinal side effects as well as causing secondary hemochromatosis [13].

Zinc Deficiency and Toxicity

Zinc is a key functional component of over 300 enzymes and also coordinates the functional domains of numerous transcription factors. Zinc deficiency therefore affects many aspects of metabolism and gene expression, and has notable detrimental effects on the immune system, basal metabolism, and development, particularly spermatogenesis and testosterone steroidogenesis [14]. Severe and prolonged zinc deficiency in humans reduces appetite and bone growth, delays sexual maturation, causes skin lesions, diarrhea, and alopecia (hair loss), and reduces the ability of the cellular immune system to fight infections [14]. Zinc, like iron, is affected by the amount of phytate in the diet, so deficiency is particularly prevalent in populations that subsist on cereal-rich diets.

The upper level of Zn intake in adults is 45 mg/day, although tolerance is lower in women, children, and the elderly. Excessive long-term zinc intake interferes with iron and copper metabolism, so the symptoms of zinc toxicity overlap with those of iron and copper deficiency, e.g., anemia, neurological symptoms, and lower immunity [14]. Excessive levels of zinc also affect the relative levels of LDL and HDL cholesterol and increase the risk of cardiovascular disease [14].

Iodine Deficiency and Toxicity

Iodine is an essential component of the thyroid hormones thyroxine (T4) and triiodothyronine (T3), whose function is to increase the basal metabolic rate (therefore affecting carbohydrate, fat, and protein metabolism), stimulate protein synthesis, regulate long bone growth in concert with growth hormone, stimulate neuronal maturation, and regulate how cells respond to catecholamines [15, 16]. For some of these functions, iodine acts in concert with iron, zinc, and selenium [16]. Iodine deficiency disorder (IDD) generally reflects a lack of iodine in the soil, which places more than 1.5 billion people in the world at risk, particularly children and pregnant or lactating women [4, 15]. The initial effects of IDD are to suppress the synthesis of thyroid hormones, which leads the pituitary gland to produce more thyroid-stimulating hormone and causes the thyroid to enlarge in an attempt to capture more iodine from the blood. This condition, known as goiter, can be reversed by iodine supplementation (see below). The effects of long-term iodine deficiency in pregnancy and childhood cannot be reversed – these include stunted physical and mental development, resulting in a condition known as cretinism [16].

Iodine toxicity is unusual because a daily intake of 1 mg or more appears to be safe. However, where there is prolonged excessive consumption or an underlying disease that prevents iodine metabolism, the excess iodine can inhibit thyroid hormone synthesis, a phenomenon known as the Wolff–Chaikoff effect [4].

Selenium Deficiency and Toxicity

Selenium is a component of the nonstandard amino acids selenocysteine and selenomethionine, which are required for the activity of a number of enzymes (selenoenzymes) such as glutathione peroxidase, tetraiodothyronine 5′ deiodinase, thioredoxin reductase, formate dehydrogenase, and glycine reductase. Its presence in thyroid hormone deiodinase means that selenium is necessary for the normal function of the thyroid gland. Many of the other selenoenzymes act as antioxidants with a range of protective roles in the body, helping to prevent cancer and cardiovascular disease, often in concert with vitamin E [4, 16, 17]. Selenium may also play a role in the regulation of other micronutrients, such as iron and zinc [16].

Selenium deficiency can lead to Keshan disease, a potentially fatal cardiomyopathy associated with increased susceptibility to infectious diseases, and also Kashin–Beck disease (usually if selenium and iodine deficiency occur concurrently) which is characterized by cartilage atrophy and necrosis leading to damaged joints [4, 18]. Selenium deficiency is unusual, but occurs where the mineral is particularly scarce in the soil, as in some parts of China. Interestingly, in other parts of China, the soil is so rich in selenium (>9% selenium content) that eating corn grown on this soil can result in acute selenium toxicity (selenosis). The maximum safe dietary intake of selenium is thought to be 400–800 μg/day, and most people consume only 10% of this amount, but in villages surrounding this selenium-rich area, a typical diet yields 3,200–6,690 μg of selenium per day, which is 100-fold the normal level. In these villages, morbidity can approach 50% with typical symptoms including a garlic-like odor to the breath, gastrointestinal disorders, loss of hair and nails, fatigue, irritability, and progressive neurological damage, leading eventually to liver cirrhosis and death [4, 18].

Other Microminerals

Whereas iron, zinc, iodine, and selenium are the "big four" micronutrients with diverse functions in the body, a variety of additional mineral ions are required for specific purposes. Copper is an essential component of several enzymes including cytochrome c oxidase, cobalt is a component of vitamin B_{12}, manganese is an essential cofactor for enzymes such as the antioxidant superoxide dismutase, molybdenum is a component of several redox enzymes, and nickel is present in urease. Fluorine is a special case in that it is not essential for life, but the addition of fluoride to drinking water helps to prevent dental caries, so it certainly contributes to human health and well-being. Other minerals, such as boron, vanadium, silicon, and arsenic, are thought to have biological roles although this has yet to be demonstrated in humans. Many of these microminerals are known to cause rare deficiency disorders as well as toxicity effects. Chronic copper deficiency, e.g., has multiple effects on metabolism because the corresponding enzymes cannot function, and also leads to anemia because copper is required for efficient iron absorption. Excess copper competes with zinc so the symptoms overlap with zinc deficiency, but acute copper toxicity is rare other than in individuals with metabolic disorders due to the efficiency of copper secretion.

Responses to Malnutrition

The most effective intervention to alleviate micronutrient malnutrition is the implementation of a varied diet including fresh fruit, vegetables, fish, and meat. This is impractical in many countries because food is not widely available, but even where fresh food is abundant, there can be compliance issues that result in persistent low-level malnutrition. Where infrastructure allows, micronutrient nutrition can be improved using supplements (usually in tablet/sachet form) or conventional fortification (where micronutrients are added to processed foods, such as packaged cereals). Unfortunately, such strategies have been largely unsuccessful in developing countries because of insufficient funding, poor governance, and a poor distribution network. There has been some success in a limited number of cases discussed later, including mineral fortification (iodized salt) and vitamin supplementation (vitamin A), although coverage has been incomplete. Developing country governments must address the risk of micronutrient malnutrition and should actively participate in the establishment of intervention programs, seeking international expertise and assistance where necessary. A more recent development is biofortification, where intervention takes place before plants are harvested. Different strategies to alleviate micronutrient malnutrition have been ranked according to their cost-effectiveness by the Copenhagen Consensus, a panel of expert economists that decide the most cost-effective strategies for addressing global challenges (Table 4). In this section, the different interventions for improving micronutrient nutrition are summarized, mainly in the context of developing countries where malnutrition has the greatest impact.

Emergency Measures (Short-Term Relief): Supplementation Programs

Supplementation is the distribution of pills or mineral solutions for immediate consumption and is the most effective short-term intervention to improve nutritional health. Supplementation helps to alleviate acute

Biotechnology and Nutritional Improvement of Crops.

Table 4 Copenhagen Consensus Center strategies [23]. The Copenhagen Consensus Center is a think-tank based in Denmark that advises governments and philanthropists about the best ways aid and development money can be used to address the world's greatest challenges. Currently, strategies to address malnutrition represent five of the top ten strategies providing the best value

Rank	Solution	World's greatest challenges
1	Micronutrient supplements for children (vitamin A and zinc)	Malnutrition
2	The Doha development agenda	Trade
3	Micronutrient fortification (iron and salt iodization)	Malnutrition
4	Expanded immunization coverage for children	Diseases
5	Biofortification	Malnutrition
6	Deworming and other nutrition programs at school	Malnutrition and Education
7	Lowering the price of schooling	Education
8	Increase and improve girls' schooling	Gender equality
9	Community-based nutrition promotion	Malnutrition
10	Provide support for women's reproductive role	Gender equality

mineral shortages but is unsustainable for large populations and should be replaced with fortification at the earliest opportunity [19]. In developed countries, where mineral malnutrition is rare, supplementation is focused on a small subset of the population with specific deficiencies resulting from medical conditions. In developing countries, where acute and chronic deficiencies are commonplace, supplementation is highly recommended as a complement to the diet (fortified or otherwise) for the entire population. Supplementation has also been recommended by WHO, the World Food Program (WFP), and UNICEF [20] for extreme situations such as refugee camps, where it can also help to address diseases such as acute diarrhea.

The distribution of vitamin A supplements has been one of the most cost-effective and successful acute intervention programs in the developing world [19], but this is a rarity. Like fortification, successful supplementation strategies require a robust infrastructure and a government determined to improve the nutritional health of its population [19]. Supplementation requires compliance monitoring because people often neglect to take regular supplements at prescribed intervals. For example, in some communities with both chronic iodine deficiencies and no access to iodized salt, the distribution of iodine capsules to women is a short-term but more expensive measure to avoid IDD [21]. Many studies have shown that zinc supplements are beneficial, particularly in areas with zinc-depleted soil [4, 22]. Zinc supplementation has been adopted by WHO and UNICEF in their guidelines for the treatment of acute diarrhea [20]. Zinc supplements are normally formulated as tablets or as oral rehydrated solutions, requiring the active compliance of families and communities. The supplements must be administered frequently, and may be more efficient when other micronutrients such as vitamin A are administered simultaneously [14, 19].

Long-Term Measures (Sustainable Relief): Fortification Programs

Food fortification is one of the most cost-effective long-term strategies for micronutrient nutrition [23] and ranks third in terms of cost-benefit balance according to the Copenhagen Consensus (Table 4). Fortification takes place during food processing and increases the product price. These factors make fortified products unaffordable to the most impoverished people living in remote rural areas. Even in more accessible areas, fortification requires government awareness and policies for implementation and compliance monitoring. The Flour Fortification Initiative (FFI) claims that food fortification must be implemented at a national level to be successful, and must involve the public, private business and government sectors. The FFI supports mandatory fortification based on scale, equity, business, cost, and sustainability. The creation of partnerships among countries with established fortification policies would help other countries to establish their own infrastructure.

Although the implementation of mandatory fortification would take time, there are enormous benefits for the country and its population [24].

Before considering case studies, it is important to note that food fortification targets must be selected carefully. Research must be carried out to identify the best vehicles to deliver micronutrients, i.e., those with the widest and most frequent consumption reflecting the staple diet of individual countries and/or regions. Also, a suitable fortification agent must be identified – one that is stable but easily mobilized in the gut, one that does not alter the quality of the food to which it is added, and one that can be stored and distributed easily. Since many parts of the world suffer from multiple deficiencies, strategies must also be developed to fortify foods simultaneously with several micronutrients, without adverse interactions among them. The addition of a single micronutrient would have approximately the same cost implications as the addition of several, but more research is needed to determine the most cost-effective way to make nutritionally complete foods [23, 24].

Iodization of Salt Iodine fortification of salt is one of the nutrition success stories of the twentieth century, helping to eliminate IDD in many parts of the world. Even though alternative food vehicles have been proposed, salt is thus far the most economical and sustainable fortification target. In 1990, 20% of households in the developing world consumed iodized salt. In 1994, WHO and UNICEF recommended mandatory salt iodization in all countries to ensure consumption and prevent access to unfortified salt. By the year 2000, iodized salt had reached 70% of households in the developing world. Although some countries still lack access to iodized salt, the reach is improving all the time, with many countries becoming self-sufficient and no longer needing donations to support their fortification programs [25]. Countries with no nutrition policies or monitoring are the main challenges for intervention strategists, because fortification requires external funding and IDD reappears if salt iodization is discontinued.

Organizations such as WHO, UNICEF, and The International Council for the Control of Iodine Deficiency Disorders (ICCIDD) have created and distributed guidelines for the implementation of iodine fortification. They work together to eliminate iodine deficiency through the Universal Salt Iodization (USI) in partnership with the Micronutrient Initiative (MI), the World Bank, Kiwanis International, and with public and private sector involvement including consumer organizations, schools, and medical authorities around the world.

Iron and Folate Fortification of Wheat Flour Another successful fortification approach is the inclusion of minerals in wheat flour, so that widely consumed products such as bread become nutritionally beneficial as well as providing calories. Indeed, bread is often double fortified with iron and folate, most recently in Iran [26]. The mandatory fortification of wheat flour has been established in several developing countries in Latin America, leading to a reduction in IDA [19]. The FFI has created a network that involves the participation of public, private, and government sectors, resulting in an increase in the global prevalence of iron-fortified wheat flour from 18% in 2004 to 27% in 2007, helping 540 million people to avoid IDA [26]. The MI also supports this strategy and works with FFI in developing countries.

Iron fortification can be technically challenging because iron compounds that are easily absorbed by the gut tend to leach easily and also change the taste of food, whereas those with less impact on taste are the most difficult to absorb [27, 28]. Encapsulation has been investigated as a potential solution and this is an active area of current research [29].

Other Examples Zinc fortification has been implemented in the industrialized world but rarely in developing countries. One exception is zinc-fortified wheat and corn flour in Mexico, which is used to make bread and tortillas, the two principal staples [30]. Organizations such as the Zinc Task Force (ZTF) and the International Zinc Nutrition Consultative Group (IZINCG) are fighting zinc deficiency by promoting diverse strategies to eliminate it. As zinc and iron deficiency tend to go hand in hand, it has been suggested that double fortification would be effective with little additional cost, particularly if iron fortification were already in place.

Selenium-fortified products such as salt, margarine, cereals, and soft drinks have been produced although not widely distributed [18]. Salt fortified with selenium has been used in parts of China where the soil is naturally depleted but it has not been supported to the same degree as iodized salt [17]. The multi-micronutrient fortification of biscuits with iron, zinc, iodine, and vitamin A has been implemented with Vietnamese schoolchildren, reducing the risk of IDA, IDD, and VDA, and increasing the effectiveness of deworming [31].

Long-Term Measures (Sustainable Relief): Biofortification

Conventional interventions have a limited impact, so biofortification has been proposed as an alternative long-term approach for improving nutrition [23, 32]. Biofortification focuses on enhancing the micronutrient qualities of crops *at source*, encompassing processes that increase both micronutrient levels and their bioavailability in the edible parts of staple crops. The former can be achieved by agronomic intervention (in the case of minerals), plant breeding or genetic engineering, whereas only plant breeding and genetic engineering can influence nutrient bioavailability.

Plant breeding and genetic engineering are often regarded as similar means to achieve a common goal because, in contrast to agronomic interventions, both involve changing the genotype of a target crop. The two processes are similar in aim, albeit different in scope. Both attempt to create plant lines carrying genes that encourage the efficient accumulation of bioavailable micronutrients – plant breeding achieves this by crossing the best-performing plants and selecting those with favorable traits over many generations, whereas genetic engineering accesses genes from any source and introduces them directly into the crop. Plant breeding is restricted to genes that can be sourced from sexually compatible plants and the limited amount of genetic variability therein, whereas genetic engineering has no taxonomic constraints and even synthetic genes can be used.

The main advantage of genetic engineering and plant breeding approaches for mineral enhancement is that investment is only required at the research and development stage, and thereafter the nutritionally enhanced crops are entirely sustainable. Furthermore, as stated above, mineral-rich plants tend to be more vigorous and more tolerant of biotic stress, so the overall yields are likely to improve in line with mineral content [27]. Unlike conventional intervention strategies, genetic engineering and plant breeding are both economically and environmentally sustainable. A combination of both strategies has also been proposed [33] and can produce significant synergic improvements compared to each strategy applied individually [34]. Although there are no commercial nutritionally enhanced plants derived from either method at the current time, this approach has the greatest cost-effectiveness in the long term and is likely to have the most important impact over the next few decades.

Biofortification is also likely to be more accessible than conventional interventions in the long term because it removes hurdles such as the reliance on infrastructure and compliance. Moreover, plants assimilate minerals into organic forms that are naturally bioavailable and contribute to the natural taste and texture of the food. Economic studies have shown the many potential health benefits of biofortification, especially in combination with conventional strategies [35].

Nutritional Improvement Through Agronomic Approaches Farmers have applied mineral fertilizers to soil for hundreds of years in order to improve the health of their plants, but within certain limits the same strategy can also be used to increase mineral accumulation within cereal grains for nutritional purposes. This strategy only works if the mineral deficiency in the grain reflects the absence of that mineral in the soil, and if the mineral fertilizer contains minerals that can be mobilized rapidly and easily. Also, even if plants can absorb minerals efficiently from the soil, they may store the mineral in inedible organs (e.g., leaves but not fruits or seeds), or they may accumulate the mineral in a form that is not bioavailable, thus having no impact on nutrition [27].

In industrialized countries such as Finland and New Zealand, this strategy has been applied successfully to increase the amount of selenium in the diet [18]. It is difficult to apply to iron, because most of the inorganic iron in the soil is inaccessible to plants. Like supplements and fortification, agronomic intervention is probably best applied in niche situations or in combination with other strategies [36].

One drawback of agronomic intervention in developing countries is the cost and impact of fertilizers. Fertilizer use increases the cost of food, thus reducing its availability to the most impoverished people. To be effective, fertilizers must be applied regularly, but impoverished farmers would be under financial pressure to "cut corners" to save costs, even though seeds produced from mineral-rich soil tend to germinate more vigorously than those in poor soils, thus increasing yields [36].

Iodine fertilizers have received comparatively little attention due to the success of fortification programs, but they have been used in regions of China where the soil has low iodine levels. The addition of iodine to irrigation water (fertigation) in China has successfully increased the level of iodine in rice, but despite the technical success such projects have not addressed IDD due to poor infrastructure [16]. Iron has a very low mobility in soil because it binds rapidly to soil particles when applied as fertilizer in the form of $FeSO_4$. It is converted rapidly from Fe^{2+} to Fe^{3+} under these circumstances, rendering it unavailable for absorption [27]. In poor soils lacking the macronutrients nitrogen, phosphorus, and potassium, the application of NPK fertilizers can promote the capture of iron, although this also depends on the soil pH. Alkaline conditions in the rhizosphere prevent the uptake of iron and zinc, whereas slightly acidic soil promotes the absorption of these minerals [27]. Foliar sprays of $FeSO_4$ or chelates allow the direct uptake of iron.

Unlike iron, zinc is very mobile in soil and is easily absorbed, especially under slightly acidic conditions. Zinc fertilizers such as $ZnSO_4$ can increase the yield of cereals and legumes in zinc-deficient soils, and can also increase zinc levels in the grain (although this is dependent on genotype). Zinc fertilization has been used in Turkey, where NPK fertilizers are enriched with zinc and applied normally for crop production. Soils in Turkey are extremely deficient in zinc and this program has successfully increased plant growth and yield. Although it is likely that the population has benefited from the nutritional properties of crops grown with zinc fertilizers, no population studies have yet been carried out to determine the impact on human health [36].

Finally, selenium provides probably the most successful example of agronomic intervention by mineral fertilization because it fulfills the three major requirements for such a strategy to work: (1) sodium selenate is highly mobile in many soil types; (2) it is absorbed easily by plants and, in the case of cereals, accumulates in the grain; and (3) it accumulates in a readily bioavailable form, selenomethionine [17]. As is the case for zinc, selenium added to NPK fertilizers has been used successfully in Finland and New Zealand to increase the selenium content of grains, with a positive impact on the general health and well-being in the population [18].

Nutritional Improvement Through Conventional Breeding Plant breeding programs focus on improving the level and bioavailability of nutrients in staple crops using their natural genetic variation [37]. The HarvestPlus program was established by the Consultative Group on International Agricultural Research (CGIAR) to improve human nutrition by breeding new varieties of the staple food crops consumed by the poor. It is a global alliance of institutions such as the International Rice Research Institute (IRRI), the Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT), the Centro Internacional de Agricultura Tropical (CIAT) and the International Institute of Tropical Agriculture (IITA) and other academic institutions world-wide. Its aims include the discovery of genetic variation affecting heritable mineral traits, verification of its stability under different conditions and the feasibility of breeding to increase mineral content in edible tissues without affecting yields or other quality traits. Feasibility data, mainly for iron and zinc, has been collected and a summary has been published [38].

Although breeding for increased mineral levels is more sustainable than conventional interventions, no high-mineral varieties have been introduced to the market thus far. This reflects the long development times, particularly if the mineral trait needs to be introgressed from a wild relative. Breeders utilize molecular biology techniques such as quantitative trait locus (QTL) maps and marker-assisted selection (MAS) to accelerate the identification of high-mineral varieties, but they have to take into account differences in soil properties (e.g., pH, organic composition) that may interfere with mineral uptake and accumulation. For example, the mineral pool available to plant roots

may be extremely low in dry, alkaline soils with a low content of organic matter [36].

Conventional breeding could also be useful for the enhancement of organic nutrients, although there has been no coordinated international effort to achieve progress in this area. Even so, crosses between inbred lines have been used to identify QTLs affecting carotenoid and tocopherol levels in corn [39], as well as carotenoid levels in carrot and tomato [40], and these could be used in the future for nutritional improvement programs. Recently, natural genetic variation at several loci in corn has been exploited to generate lines with increased levels of specific carotenoids [41, 42].

Although some variation in iodine content has been found in staple crops [43], the focus remains on manual intervention strategies because salt iodization has been so successful. However, the HarvestPlus initiative has proposed the inclusion of iodine in its program because of widespread iodine deficiency in some countries and the interaction between iodine and other micronutrients (mainly iron, zinc, and vitamin A [16]). Iron and zinc levels vary significantly in many crops, and CGIAR is looking into the possibility of breeding high-mineral varieties of cereals using this genetic diversity. The amount of iron in rice grains varies between 6 and 22 mg kg^{-1}, whereas in corn it varies between 10 and 160 mg kg^{-1}, and in wheat the range is 15–360 mg kg^{-1} [44]. Zinc levels are similarly broad – between 14 and 61 mg kg^{-1} in rice, 14 and 190 mg kg^{-1} in wheat, and between 12 and 96 mg kg^{-1} in corn [43, 44]. Despite this natural variation, commercial varieties of wheat still have low mineral levels compared to wild wheat, where the grain may contain up to tenfold the normal amount of zinc. Furthermore, wild emmer wheat accessions have been identified in which the seeds contain up to 139 mg kg^{-1} zinc, up to 88 mg kg^{-1} iron, and up to 380 g kg^{-1} protein, as well as showing strong tolerance of drought stress and low zinc levels in the soil [44]. There also appears to be moderate genetic variation in the selenium content of modern cereals although wild wheat varieties, with higher selenium levels, are potential sources of additional diversity [43, 44].

Nutritional Improvement Using Biotechnology

The major advantages of genetic engineering over conventional breeding are the diversity of the potential sources of genetic information, the speed with which improved elite varieties can be generated and, perhaps most important, the fact that nutritional traits for different vitamins and minerals can be stacked in the same plant without highly complex breeding programs [32]. Genetic engineering currently offers the only opportunity to produce nutritionally complete staple foods.

Despite these advantages, there are no high-micronutrient, genetically engineered crops on the market thus far. Whereas with conventional breeding this reflects the long development phase, in the case of genetic engineering this reflects the current uncertain regulatory environment, particularly trade barriers and differences in national regulatory systems that inhibit the production, transport, and use of transgenic products. Trade barriers for transgenic crops have been established de facto in the EU because of the precautionary approach to regulatory oversight, so developing countries (such as China and India) are pressured not to grow such products for export even though they may still benefit the domestic population by improving health and wealth [2, 3].

Biotechnology Strategies to Increase Nutritional Content

Many biotechnology strategies can be used to enhance the nutritional value of crops and these offer a rapid way to introduce such traits into elite varieties. The best approach for a given nutrient depends predominantly on whether the plant synthesizes the nutritional compound de novo or obtains it from the environment. Organic molecules such as amino acids, fatty acids, and vitamins are synthesized by the plant, and increasing the nutritional content therefore requires some form of metabolic engineering with the aim of increasing the amount of the desirable compound, decreasing the amount of a competitive compound (e.g., one that uses the same precursors but diverts them into a different pathway) or even extending an existing metabolic pathway to generate a product that is not usually made in that species [32]. In contrast, mineral nutrients are obtained from the environment and mineral enhancement therefore involves strategies to increase uptake, transport and/or to increase accumulation in harvestable tissues [23].

Increasing the Availability of Essential Amino Acids

Among the eight essential amino acids discussed above, methionine, lysine, and tryptophan are the most limiting in legumes and cereals, and since these are the major types of staple crops they are also the primary targets for nutritional enhancement. Generally, four strategies have been applied to increase the content of essential amino acids in plants: (1) mimicking natural mutants, (2) the expression of recombinant storage proteins with desirable amino acid profiles, (3) genetic engineering to enhance the free amino acid pool, and (4) combination approaches.

Mimicking Natural Mutants The naturally occurring *opaque2* mutant in corn has higher lysine and tryptophan levels than wild-type corn but has a soft, chalky kernel which is unsatisfactory for cooking. The higher content of essential amino acids in the mutant results from lower levels of certain storage proteins (e.g. α- and β-zeins) allowing other proteins (e.g. 27-kDa γ-zein) to replace them and increase the prevalence of the two limiting amino acids. Breeding programs have resulted in the development of quality protein maize (QPM) which benefits from the higher lysine and tryptophan levels of the *opaque2* mutant but combines this with a hard kernel for superior cooking qualities [45]. Although QPM has lower levels of α- and β-zeins compared to wild-type corn, it has higher levels of γ-zein. Zarkadas et al. [46] showed that QPM may provide up to 73% of human protein requirements, compared to 28–50% for common corn. Success with *opaque2* corn has stimulated extensive research to identify similar mutants in other cereals such as barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*).

The *opaque2* corn mutants have inferior agronomic traits that cannot be overcome easily, but the laborious breeding program that gave rise to QPM can be replicated much more rapidly by genetic engineering. The transformation of corn with an RNAi construct to suppress target storage protein genes resulted in a phenocopy of the *opaque2* phenotype; transgenic seeds contained less α-zein than normal but higher levels of lysine [47, 48].

Expression of Recombinant Storage Proteins The main source of essential amino acids in sink tissues such as developing seeds is the storage proteins; therefore, one reason for the lack of certain amino acids in some staple crops is the absence of major storage proteins containing them. Thus, one of the main strategies for improving the levels of limiting amino acids is to express heterologous storage proteins that are rich in these specific amino acids.

One of the earliest attempts using this approach was the expression of pea legumin, which is rich in lysine, in rice endosperm, where lysine is limiting; in the best lines, heterologous legumin represented up to 4.2% of the total protein content [49]. Similarly, Stöger et al. [50] generated transgenic wheat plants expressing pea legumin using the low molecular weight (LMW) glutenin promoter, with legumin representing 1.5% of total soluble protein in the best transgenic lines. More significant increases in lysine content have been achieved by modifying cereal storage protein genes before deployment to increase the number of lysine codons. For example, Jung and Carl [51] modified the barley hordothionine protein to include 12 lysine residues (hordothione-12, HT12) and the barley high lysine protein to include eight lysine residues (barley high lysine-8, BHL8) and expressed them in corn along with the bacterial enzyme dihydrodipicolinate synthase (DHPS, see below). This resulted in a total lysine content of nearly 0.8%, about four times the level in wild-type seeds. The expression of HT12 in sorghum increased the lysine content by 50% compared to wild-type grain, and the expression of *sb401*, encoding another high-lysine storage protein, increased lysine levels in corn by 54.8% and total protein content by up to 39% [52].

Lysine levels can also be increased by expressing the animal equivalent of storage proteins, such as the nutritional proteins found in milk. The principle was established when porcine α-lactalbumin was expressed in corn and either targeted for secretion to the apoplast or for retention in the endoplasmic reticulum (ER), but individual amino acid levels were not reported [53]. More recently, Bicar et al. [54] repeated the experiment and showed that α-lactalbumin expression in corn increased the lysine content up to 47%. The lysine content of transgenic corn has also been increased up to 26% by expressing a heterotypical Arabidopsis lysyl tRNA synthetase, which inserts lysine residues in place of other amino acids during the synthesis of seed-storage proteins [55].

The expression of heterologous storage proteins has also been used as a strategy to increase the levels of methionine in transgenic crops, focusing on the 2S storage proteins which are unusually methionine rich. For example, expression of Brazil nut 2S albumin in soybean and narbon bean doubled the methionine content of the seeds, and increased methionine content by 33% in canola [56]. Similarly, the expression of sunflower 2S albumin increased total methionine content by up to sevenfold in potato tubers [57]. This approach does not always work well, e.g., the expression of the sunflower albumin SFA8 in rice and chickpea [58] merely redistributed the sulfur-containing amino acids (more methionine, less cysteine) with no net improvement in nutritional properties. The 2S sulfur-rich albumins are allergenic in some human populations, reducing their usefulness for improving nutritional quality [59].

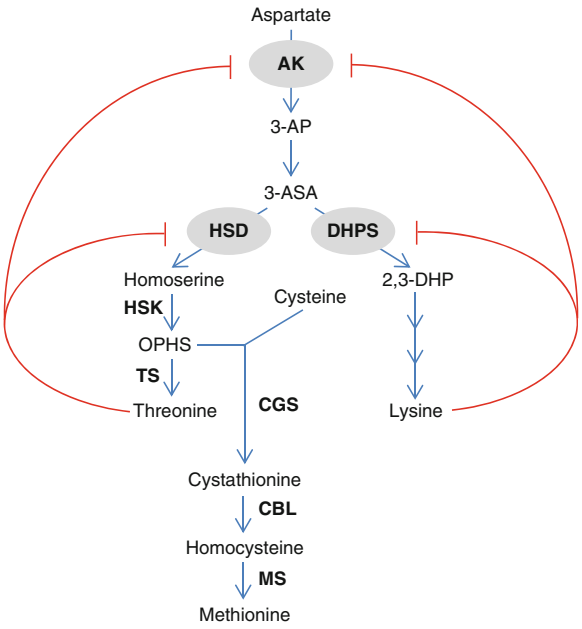
Ideally, a single protein would provide nutritional completeness in terms of essential amino acids. Grain from the pseudo-cereal amaranth (*Amaranthus hypochondriacus*) not only has a high protein content compared to traditional crops (17–19% of seed dry weight (DW) compared to ~10%), but that protein is rich in essential amino acids such as lysine (5%, more than twice the amount in wheat flour), threonine (2.9%), tyrosine (3.4%), and the sulfur-containing amino acids cysteine and methionine (4.4%). This provides a nutritional composition fairly close to the ideal as recommended by WHO, and the protein is not allergenic [5]. The cDNA for this protein has therefore been expressed in a number of crops with nutritionally incomplete proteins to increase overall protein levels and provide greater amounts of limiting amino acids. Rascón-Cruz et al. [60] expressed amaranthin in corn using the rice glutelin-1 promoter and increased the amount of protein by up to 32% while simultaneously boosting the levels of the three most limiting amino acids, lysine, tryptophan, and isoleucine. Chakraborty et al. [57] expressed the protein in potato tubers using the granule-bound starch synthase (GBSS) promoter, and increased total protein levels by up to 45%. Most recently, the protein was expressed in wheat using the LMW glutenin promoter with the amaranthin protein representing nearly 2.5% of total seed protein in some lines, increasing the levels of lysine to 6.4%, and tyrosine to 3.8% [61].

As well as natural heterologous storage proteins, completely synthetic proteins (i.e., proteins designed from first principles) can also be expressed to boost the levels of particular amino acids. For example, a synthetic protein matched to human amino acid requirements was expressed in cassava [62]. Even in these ideal cases, however, the levels of essential amino acids in the resulting transgenic crops tend to fall below expectations given the composition of the heterologous proteins. The inability of heterologous proteins to change the essential amino acid content of target crops abruptly and predictably often reflects the limited free amino acid pool, which provides the substrates for protein synthesis.

Engineering the Free Amino Acid Pool

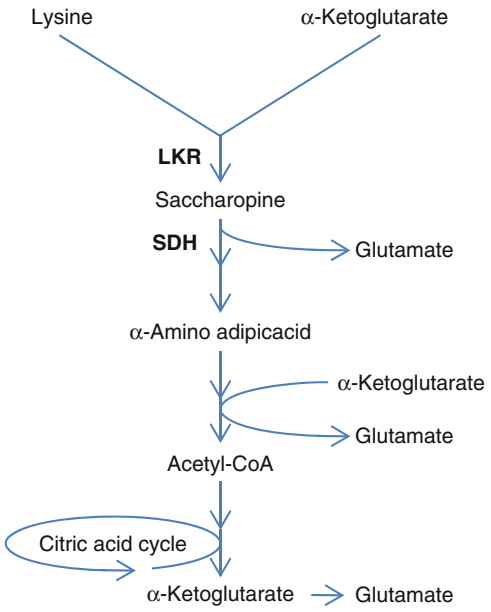
In higher plants, lysine, threonine, and methionine are synthesized from aspartic acid via a pathway that is highly branched and under complex feedback control (Fig. 1) [63]. Two key enzymes are aspartate kinase (AK), which functions early in the pathway and is inhibited by both lysine and threonine, and dihydrodipicolinate synthase (DHPS), which is the first enzyme specifically committed to lysine biosynthesis and is inhibited by lysine alone. Feedback-insensitive versions of the bacterial enzymes have been expressed in model plants with encouraging results, e.g., the free lysine content in tobacco and Arabidopsis seeds was increased by expressing feedback-insensitive DHPS [63]. Similarly, the expression of feedback-insensitive DHPS in corn embryos increased the levels of free lysine from <2% to almost 30% of the free amino acid pool, with concomitant increases in threonine [64]. Analogous approaches have increased the lysine levels in canola and soybean [65, 66]. However, high levels of lysine in all plant tissues can cause abnormal vegetative growth and flower development that reduce seed yield [63].

The increased accumulation of lysine in tobacco seeds correlated with the enhanced activity of a bifunctional enzyme, lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), which controls the first two reactions of the α-amino adipic acid pathway of lysine catabolism (Fig. 2) [67, 68]. To determine the impact of lysine catabolism on the levels of free lysine in the amino acid pool, a feedback-insensitive DHPS was expressed in wild-type



Biotechnology and Nutritional Improvement of Crops. Figure 1

Synthesis of the essential amino acids lysine, threonine, and methionine [63]. The three key enzymes (*gray circles*) are aspartokinase (AK), dihydrodipicolinate synthase (DHPS), and homoserine dehydrogenase (HSD), all of which are subject to end-product feedback inhibition (*red arrows*). Abbreviations for substrates: 3-AP, aspartyl-3-phosphate; 3-ASA, aspartate semialdehyde; OPHS, *O*-phosphohomoserine; 2,3-DHP, 2,3- dihydrodipicolinate. Abbreviations for other enzymes: HSK, homoserine kinase; TS, threonine synthase; CGS, cystathionine-γ-synthase; CBL, cystathionine β-lyase; MS, methionine synthase. Multiple arrows indicate several unspecified reactions



Biotechnology and Nutritional Improvement of Crops. Figure 2

Lysine catabolism in plants, showing the production of three glutamate molecules for every molecule of lysine [63]. Enzyme abbreviations: LKR, lysine-ketoglutarate reductase; SDH, saccharopine dehydrogenase. Multiple arrows indicate several unspecified reactions

Arabidopsis seeds and those of a LKR/SDH knockout mutant [69]. Whereas transgenic seeds without the mutation contained 12 times the normal levels of lysine, transgenic mutant seeds contained 80 times normal lysine levels, showing the importance of lysine catabolism in strategies to increase the accumulation of this amino acid. Similarly, it has been possible to cross transgenic corn lines where one parent expresses a feedback-insensitive enzyme for lysine synthesis that doubles the amount of free lysine [70] and the other expresses an RNAi construct against LKR/SDH thus inhibiting lysine catabolism [71]. In the double transgenic progeny, the amount of lysine was 40 times the level present in wild-type corn [64].

The major enzyme-controlling methionine synthesis is cystathionine γ-synthase (CGS), and its activity in Arabidopsis is under feedback control reflecting the abundance of the important metabolic intermediate S-adenosylmethionine (SAM) [72]. Mutations in the N-terminal portion of CGS that affect this feedback regulation result in methionine overproduction [72, 73], and this can be combined with the expression of a feedback-insensitive AK to increase methionine levels even further [74].

The expression of feedback-insensitive AK also increases the abundance of threonine. In some cases, the increases in threonine levels were matched by smaller, although still significant, increases in the levels of isoleucine, methionine, and lysine, indicating that AK activity is more important for threonine synthesis than it is for lysine synthesis [63].

Tryptophan synthesis in plants is strongly regulated by high levels of tryptophan inhibiting the enzyme anthranilate synthase, which catalyzes the conversion of chorismate to anthranilate. As is the case for other amino acids, a preferred strategy to overcome this limitation is the expression of a feedback-insensitive version of this key enzyme, as has been achieved in tobacco leaves and the roots of the forage legume *Astragalus sinicus* [75]. A mutant anthranilate synthase from rice has been shown to increase the amount of free tryptophan in transgenic rice seeds by over 400-fold [76], in potato tubers by approximately 30-fold [77], and in soybean seeds by approximately 20-fold [78]. A similar mutant gene from tobacco increased free tryptophan levels by sixfold in transgenic soybean leaves and twofold in seeds [79].

Tabe et al. [80] have recently shown that cysteine levels can be increased by up to 26-fold in developing lupin seeds by expressing a feedback-insensitive serine acetyltransferase (SAT). The levels of glutathione were also higher in developing seeds, but methionine levels were unaffected. Interestingly, the overall levels of cysteine and methionine in mature seeds did not change, suggesting feedback to counter the accumulation of cysteine later in development. The above strategy was also combined with the overexpression of a seed storage protein by crossing transgenic lines expressing the SAT enzyme with those expressing a sunflower albumin gene. Again, this resulted in higher levels of cysteine during development but no change in the amount of methionine; there was no significant change in either amino acid in mature seeds [80].

Increasing the Availability of Essential Fatty Acids

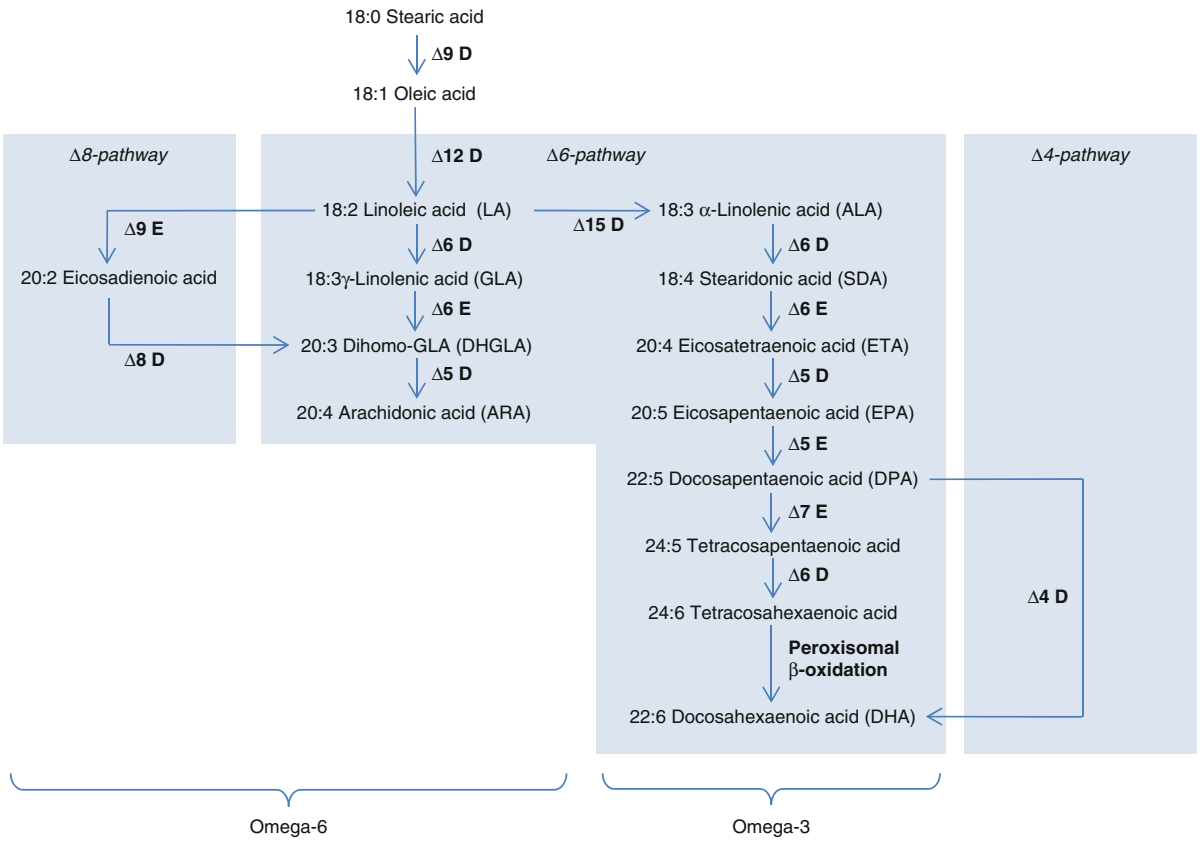
Polyunsaturated fatty acids (PUFAs) are synthesized from saturated fats through an alternating sequence of desaturation and elongation reactions, each requiring a different class of enzymes (Fig. 3). Humans are unable to synthesize PUFAs because they lack the necessary Δ12 and Δ16 desaturases to convert oleic acid into α-linolenic acid (omega-3) and linoleic acid (omega-6). However, they do possess Δ6 and Δ5 desaturases, so if adequate amounts of α-linolenic acid and linoleic acid can be sourced in the diet they can be converted into longer-chain molecules in the liver, yielding the very-long-chain (VLC) PUFAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA) [81]. Even so, this is a slow process because the Δ6 and Δ5 desaturases are inefficient, and these molecules can become limiting. Strategies to increase the availability of essential fatty acids in plants therefore not only concentrate on the accumulation of α-linolenic acid and linoleic acid but also on the VLC-PUFAs EPA, DHA, and ARA.

As shown in Fig. 3, the synthesis of EPA and ARA requires three enzyme activities: Δ6-desaturase, Δ6-elongase, and Δ5-desaturase; DHA synthesis requires an additional β-oxidation step which occurs in peroxisomes [82]. The elongation steps require an acyl-CoA substrate, whereas the desaturations require the presence of phosphatidylcholine. The switching of fatty acids between the phosphatidylcholine and CoA pools is facilitated by acyltransferases and these are often the limiting step in VLC-PUFA synthesis [83] (Fig. 4). However, this bottleneck can be alleviated using the alternative Δ8-pathway, in which the elongation and saturation steps are realized by a Δ9-elongase, Δ8-desaturase, and Δ5-desaturase [84] (Fig. 3). This pathway is more efficient for ARA and EPA synthesis because there is less reliance on acyl exchange [82].

Four main strategies have been used to enhance VLC-PUFA biosynthesis in plants: expressing enzymes that increase the availability of precursors for α-linolenic acid and linoleic acid synthesis, enhancing the typical Δ6-pathway, introducing the alternative Δ8-pathway and importing the microbial Δ4-pathway (Fig. 3). Thus far, enhancing the Δ6-pathway has been most successful (Table 5), increasing the levels of ARA, EPA, and DHA by up to 25%, 15%, and 0.5%, respectively [85, 86]. However, Kinney et al. [87] showed that the levels of EPA and DHA could be increased by 19.5% and 3%, respectively, in soybean by enhancing the Δ4- and Δ6-pathways simultaneously, and Qi et al. [88] increased EPA levels to 3% and ARA levels by 6.6% in *Arabidopsis* by importing the Δ8-pathway and removing the acyl exchange bottleneck.

Increasing Vitamin Levels

Vitamin A In plants, carotenoids such as β-carotene (pro-vitamin A) are synthesized in the plastids via the

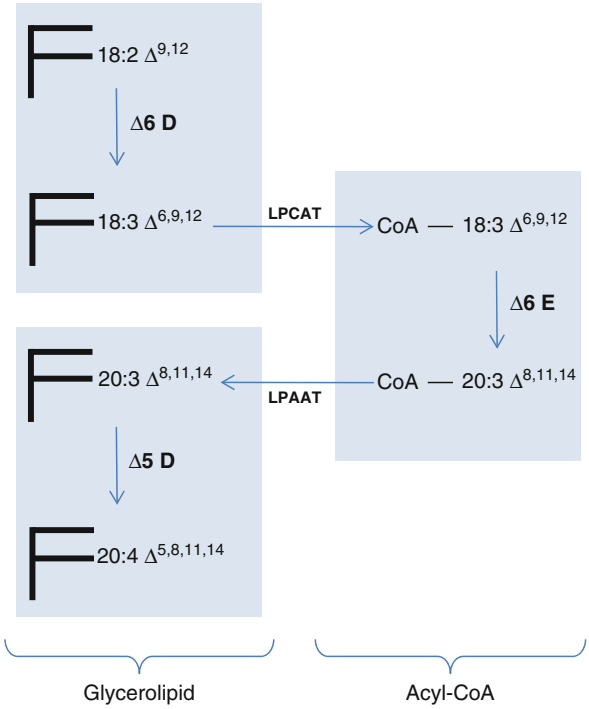


Biotechnology and Nutritional Improvement of Crops. Figure 3

Overview of very-long-chain polyunsaturated fatty acid synthesis focusing on the strategies that have been used in transgenic plants either by enhancing the standard Δ6-pathway or importing enzymes from the alternative Δ8-pathway and microbial Δ4-pathway [82]. Enzymes identified by "D" and "E" are desaturases and elongases, respectively. Humans lack the Δ12 desaturase that converts oleic acid into linoleic acid

methylerythritol-4-phosphate (MEP) pathway, also known as the non-MVA pathway. Initially, pyruvate and D-glyceraldehyde-3-phosphate are converted into 1-deoxy-D-xylulose-5-phosphate (DXP) by DXP synthase (DXS), and DXP is then converted into the isomeric C5 precursors isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Three molecules of IPP condense with one molecule of DMAPP to form the C20 precursor geranylgeranyl diphosphate (GGPP), which is used for the synthesis of carotenoids, tocopherols, chlorophylls, plastoquinones, and gibberellins [89]. The first committed reaction in carotenoid biosynthesis is the conversion of GGPP to phytoene by phytoene synthase (PSY). In plants, phytoene then undergoes four desaturation steps catalyzed by phytoene desaturase (PDS) and ζ–carotene desaturase (ZDS) to generate the first colored carotene, *cis*-lycopene, which is converted to all-*trans*-lycopene by carotene isomerase (CRTISO) [90]. Lycopene is the substrate for two different enzymes – lycopene β-cyclase (LYCB), which adds β-ionone rings to both ends generating β-carotene; and lycopene ε-cyclase (LYCE), which adds an ε-ionone ring to one end, the other being cyclized by LYCB to generate α-carotene [90]. Both molecules can be hydroxylated to produce xanthophylls such as lutein, zeaxanthin, and violaxanthin (Fig. 5) although these do not have pro-vitamin A activity [90].

The amount of β-carotene produced by plants can be enhanced by increasing the availability of carotenoid



Biotechnology and Nutritional Improvement of Crops. Figure 4

Fatty acid desaturation in plants requires glycerolipid substrates, whereas elongation requires acyl-CoA substrates. Successive desaturation and elongation steps therefore require substrates to be shuttled between phosphatidylcholine and coenzyme A (CoA) using enzymes known as acyltransferases. Enzymes identified by "D" and "E" are desaturases and elongases, respectively. Abbreviations: 18:2 $\Delta^{9,12}$, linoleic acid; 18:3 $\Delta^{6,9,12}$, γ-linolenic acid; 20:3 $\Delta^{8,11,14}$, dihomo-γ-linolenic acid; 20:4 $\Delta^{5,8,11,14}$, arachidonic acid; LPCAT, lysophosphatidylcholine acetyltransferase; LPAAT, lysophosphatidic acid acyltransferase

precursors, by expressing enzymes in the common part of the pathway (between GGPP and lycopene), by biasing the pathway toward the β-branch through the expression of LYCB at the expense of LYCE, or by increasing the storage capacity for carotenoids (Table 6) [90].

The first approach has been successful in producing plants that synthesize high levels of GGPP, but because this is used in several pathways not all of the flux is directed toward carotenoid synthesis. For example, the overexpression of DXP synthase in tomato and potato increased the total carotenoid content by up to 1.6-fold compared to wild type, but the levels of tocopherols and plastoquinones were also affected [91].

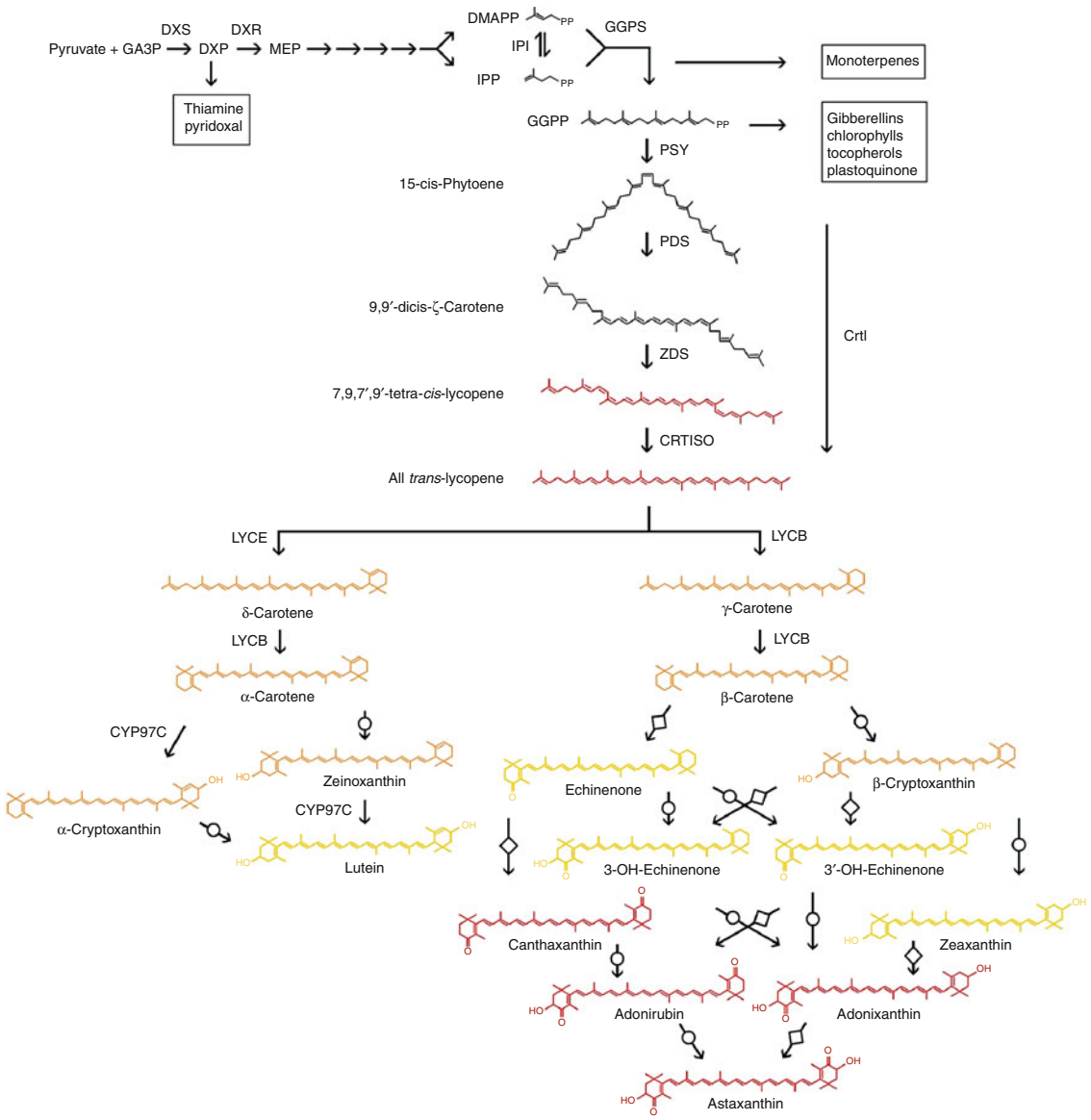
The expression of enzymes in the committed part of carotenoid pathway is a more targeted approach, and is particularly necessary in cereal grains where the pathway is blocked at the first committed step. In rice endosperm, the carotenoid pathway terminates at GGPP because there is very limited PSY activity. "Golden Rice" is a transgenic variety expressing daffodil PSY and LYCB as well as the multifunctional bacterial enzyme CrtI, which carries out all the desaturation steps between phytoene and lycopene. The β-carotene content of the original Golden Rice was 1.6 μg/g DW [92], but the replacement of daffodil PSY with the more active corn enzyme resulted in Golden Rice 2, in which the β-carotene levels reached 31 μg/g DW [93]. Similar results have been achieved in corn endosperm. For example, Aluru et al. [94] expressed the bacterial enzymes CrtB (PSY) and CrtI under the control of an enhanced seed-specific promoter, increasing the total carotenoid level to 33.6 μg/g DW and the β-carotene level to 9.8 μg/g DW. More recently, Zhu et al. [95] used a combinatorial transformation strategy to introduce up to five carotenogenic transgenes into corn, with the best-performing line (Ph-3, expressing PSY and CrtI), producing over 60 μg/g DW of β-carotene. Another recent breakthrough in this area was the creation of transgenic corn plants transformed with four genes enabling the simultaneous modulation of three metabolic pathways [96]. As above, the carotenoid pathway was engineered with the genes encoding PSY and CrtI, resulting in a 169-fold increase in β-carotene levels to 57 μg/g DW. These combinatorial and stacked transgene approaches are discussed later in detail.

Similar progress has been made in the brassicas. The expression of bacterial CrtB (PSY) in canola resulted in a 50-fold increase in total carotene levels compared to wild-type seeds (1,617 μg/g fresh weight (FW)), with a β-carotene content of 949 μg/g FW [97]. Ravanello et al. [98] achieved 1,341 μg/g FW total carotenoids with the same gene. The combined expression of CrtB (PSY) and CrtI boosted levels to 1,412 μg/g FW, but further addition of CrtY (LYCB) reduced total

Biotechnology and Nutritional Improvement of Crops. Table 5 Transgenic plants with enhanced levels of essential fatty acids [82]

Species	Genes (source)	Promoters	% Fatty acids
Arabidopsis (*Arabidopsis thaliana*)	*Δ9-elongase* (*Isochrysis galbana)*	Constitutive	ARA: 6.6
	Δ8-desaturase (*Euglena gracilis)*	CaMV 35S	EPA: 3
	Δ5-desaturase (*Mortierella alpine*)		
Arabidopsis	*Δ5/Δ6-desaturase* (*Danio rerio*)	Seed-specific	ARA: 1.2
	Δ6-elongase (*Caenorhabditis elegans*)	Napin	EPA: 2.5
	Δ4-desaturase (*Pavlova salina*)		
	Δ5-elongase (*P. salina*)		
Linseed (*Linum usitatissimum*)	*Δ6-desaturase* (*Phaeodactylum tricornutum)*	Seed-specific	ARA: 1.5
	Δ6-elongase (*Physcomitrella patens*)	USP	EPA: 1
Tobacco (*Nicotiana tabacum*)	*Δ5-desaturase* (*P. tricornutum*)		ARA: 2
Soybean (*Glycine max*)	*Δ6-desaturase* (*Saprolegnia diclina)*	Different seed-specific promoters	EPA: 19.5
	Δ6-elongase (*M. alpina*)		ARA: 5.3
	Δ5-desaturase (*M. alpina*)		
	Fad3 (Arabidopsis)		
	Δ17-desaturase (*Saprolegnia diclina*)		
	Δ4-desaturase (*Syzygium aggregatum*)		
	Elongase (*P. salina*)		
Mustard (*Brassica juncea*)	*Δ6-desaturase* (*Pythium irregulare)*	Seed-specific	ARA: 25
	Δ6-elongase (*P. patens*)	Napin	EPA: 15
	Δ5-desaturase (*Thraustochytrium* sp.)		
	Δ12-desaturase (*Calendula officinalis*)		
	Elongase (*Thraustochytrium* sp.)		
	ω3-desaturase (*Phytophthora infestans*)		
Marchantia polymorpha	*Δ6-desaturase (Marchantia polymorpha)*	Constitutive	ARA: 11.4 (3.6-fold)
	Δ6-elongase (M. polymorpha)	CaMV35S	EPA: 12.1 (2-fold)
	Δ5-desaturase (M. polymorpha)		
Tobacco	*Δ6-desaturase (M. polymorpha)*	Constitutive modified CaMV35S	ARA: 13.4
	Δ6-elongase (M. polymorpha)		EPA: 3.2
	Δ5-desaturase (M. polymorpha)		
Soybean	*Δ6-desaturase (M. polymorpha)*	Seed-specific α′subunit of β-conglycin	ARA: 3
	Δ6-elongase (M. polymorpha)		EPA: 0.3
	Δ5-desaturase (M. polymorpha)		

Source: Data updated from [82]



Biotechnology and Nutritional Improvement of Crops. Figure 5

The extended carotenoid biosynthetic pathway in plants [171]. The precursor for the first committed step in the pathway is GGPP (geranylgeranyl pyrophosphate), which is converted into phytoene by phytoene synthase (PSY, CrtB). GGPP is formed by the condensation of IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate), which are derived predominantly from the plastidial MEP (methylerythritol 4-phosphate) pathway as shown in the upper part of the figure. The pathway is linear until lycopene, involving three steps catalyzed by separate enzymes in plants but by the single, multifunctional enzyme CrtI in bacteria. Lycopene is the branch point for the α- and β-carotene pathways, which usually end at lutein and zeaxanthin, respectively, through the expression of β-carotene hydroxylases (*arrows* with *circles*). An elaborated ketocarotenoid pathway can be introduced by expressing β-carotene ketolases (*arrows* with *diamonds*) since these compete for substrates with β-carotene hydroxylases and generate diverse products. Other abbreviations: GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; IPI, IPP isomerase; GGPPS, GGPP synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; HydE, carotene ε-hydroxylase

Biotechnology and Nutritional Improvement of Crops. Table 6 Transgenic plants with enhanced carotenoid levels [171]

Species	Genes (source)	Promoters	Carotenoid levels in transgenic plants
Rice (*Oryza sativa*)	*psy1* (daffodil; *Narcissus pseudonarcissus*)	CaMV35S (constitutive)	0.3 μg/g dry weight (DW) phytoene in seeds
		Gt1 (seed specific)	0.74 μg/g DW phytoene in seeds
	psy1 and *lycb* (daffodil)	Gt1 (*psy1* and *lycb*) and CaMV35S (*crt*I)	1.6 μg/g DW total carotenoids in endosperm
	*crt*I (*Pantoea ananatis*)		
	psy1 (corn)	Gt1	37 μg/g DW total carotenoids in seeds
	*crt*I (*P. ananatis*)		
Canola (*Brassica napus*)	*crt*B (*P. ananatis*)	Napin (seed specific)	1,617 μg/g fresh weight (FW) total carotenoids in seeds (50-fold)
	*crt*B (*P. ananatis*)	Napin	1,341 μg/g FW total carotenoids in seeds
	*crt*E and *crt*B (*P. ananatis*)		1,023 μg/g FW total carotenoids in seeds
	*crt*B (*P. ananatis*)		1,412 μg/g FW total carotenoids in seeds
	*crt*I *(P. ananatis)*		
	*crt*B and *crt*Y (*P. ananatis*)		935 μg/g FW total carotenoids in seeds
	*crt*B and *β-cyclase* (*B. napus*)		985 μg/g FW total carotenoids in seeds
	*crt*B and *crt*Y (*P. ananatis*)		1,229 μg/g FW total carotenoids in seeds
	*crt*I *(P. ananatis)*		
	idi, *crt*E, *crt*B, *crt*I, and *crt*Y (*P. ananatis*)	CaMV35S, napin and Arabidopsis *FAE1* (seed specific)	412–657 μg/g FW total carotenoids in seeds (30-fold)
	*crt*Z , *crt*W (*Brevundimonas* sp.)		60–190 μg/g FW total ketocarotenoids in seeds

Biotechnology and Nutritional Improvement of Crops. Table 6 (Continued)

Species	Genes (source)	Promoters	Carotenoid levels in transgenic plants
Tomato (*Solanum lycopersicum*)	*psy1* (tomato)	CaMV35S	3,615 μg/g DW total carotenoids in vegetative tissue (1.14-fold)
	psy1 (tomato)	CaMV35S	2,276.7 μg/g DW total carotenoids in fruit (1.25-fold)
			819 μg/g DW β-carotene in fruit (1.4-fold)
	*crt*I (*P. ananatis*)	CaMV35S	520 μg/g DW (1.9-fold) β-carotene in fruit
	lycb (Arabidopsis) *chyb* (pepper; *Capsicum annuum*)	pds	63 μg/g FW β-carotene in fruit (12-fold)
	*crt*B (*P. ananatis*)	Polygalacturonase (fruit specific)	825 μg/g DW β-carotene in ripe fruit (2.5-fold)
	dxs (*Escherichia coli*)	Fibrillin	7,200 μg/g DW total carotenoids in fruit (1.6-fold)
	det-1 (tomato, antisense)	P119, 2A11, and TFM7 (fruit specific)	130 μg/g DW β-carotene (8-fold) in red-ripe fruit (assuming a water content of 90%)
	CRY2 (tomato)	CaMV35S	1,490 μg/g DW total carotenoids ripe fruit pericarps (1.7-fold)
			101 μg/g DW β-carotene ripe fruit pericarps (1.3-fold)
	chrd (cucumber; *Cucumis sativus*)	CaMV35S	Reduced carotenoid levels in flower
	*crt*Y (*P. ananatis*)	aptI	286 μg/g DW β -carotene in fruit (4-fold)
	Fibrillin (pepper)	Fibrillin	150 pg/g FW β -carotene in fruit
	lycb (Arabidopsis)	pds (fruit specific)	546 μg/g DW FW total carotenoids in fruit (7-fold) (assuming a water content of 90%)
	lycb (tomato)	CaMV35S	2,050 μg/g DW total carotenoids in fruit (31.7-fold) (assuming a water content of 90%)
	lycb (daffodil)	Ribosomal RNA	950 μg/g DW β-carotene in fruit

Biotechnology and Nutritional Improvement of Crops. Table 6 (Continued)

Species	Genes (source)	Promoters	Carotenoid levels in transgenic plants
Potato (*Solanum tuberosum*)	*ZEP* (Arabidopsis)	GBSS (tuber specific)	60.8 μg/g DW total carotenoids in tubers (5.7-fold)
	*crt*B (*P. ananatis*)	Patatin (tuber specific)	35 μg/g DW total carotenoids in tubers (6.3-fold)
			11 μg/g DW β-carotene in tubers (19-fold)
	lyce (potato, antisense)	Patatin	12.27 μg/g DW total carotenoids in tubers (2.5-fold)
			0.043 μg/g DW β-carotene in tubers (14-fold)
	*crt*O (*Synechocystis* sp.)	CaMV35S	39.76 μg/g DW total carotenoids in tubers
			ketocarotenoids represented 10–12% of total carotenoids in tubers
	dxs (*E. coli*)	Patatin	7 μg/g DW total carotenoids in tubers (2-fold)
	*crt*B (*P. ananatis*) *bkt1* (*Haematococcus pluvialis*)	Patatin	5.2 μg/g DW total carotenoids in tubers
			1.1 μg/g DW total ketocarotenoids in tubers
	bkt1 (*H. pluvialis*)	Patatin	30.4 μg/g DW total carotenoids in tubers
			19.8 μg/g DW total ketocarotenoids in tubers
	or (cauliflower; *Brassica oleracea* var *botrytis*)	GBSS	25 μg/g DW total carotenoids (6-fold) in tubers
	or (cauliflower)	GBSS	31 μg/g DW total carotenoids in tubers (5.7-fold)
	crtB, *crtI*, and *crtY* (*P. ananatis*)	Patatin	114 μg/g DW total carotenoids in tubers (20-fold)
			47 μg/g DW β-carotene in tubers (3,600-fold)
	bch (potato, antisense)	Patatin	21.7 μg/g DW total carotenoids in tubers (4.5-fold)
			0.085 μg/g DW β-carotene in tubers (38-fold)
	bch (potato, antisense)	GBSS and CaMV35S	3.31μg/g DW β-carotene in tubers

Biotechnology and Nutritional Improvement of Crops. Table 6 (Continued)

Species	Genes (source)	Promoters	Carotenoid levels in transgenic plants
Corn (*Zea mays*)	*psy1* (corn)	Wheat LMW glutelin, barley (*Hordeum vulgare*) D-hordein, corn γ-zein, rice prolamin (all endosperm-specific)	146.7 μg/g DW total carotenoids in seeds
	*crt*I (*P. ananatis*)		35.85 μg/g DW total ketocarotenoids in seeds
	*crt*W (*Paracoccus* spp.)		
	lycb (*Gentiana lutea*)		
	*crt*B *and crt*I (*P. ananatis*)	super γ -zein	33.6 μg/g DW total carotenoids in seeds (34-fold)
	psy1 (corn)	Wheat LMW glutelin and barley D-hordein	163.2 μg/g DW total carotenoids in seeds (112-fold)
	*crt*I (*P. ananatis*)		59.32 μg/g DW β-carotene in seeds (169-fold)
Lotus japonicus	*crt*W (*Agrobacterium aurantiacum*)	CaMV35S	387 μg/g FW total carotenoids in flower petals (1.5-fold)
			89.9 μg/g FW total ketocarotenoids in flower petals (2.2-fold)
Carrot (*Daucus carota*)	*bkt1* (*H. pluvialis*) *chyB* (Arabidopsis)	CaMV35S and *Agrobacterium rhizogenes rol*D (root specific)	345.5 μg/g FW total carotenoids in root
			2,400 μg/g root DW novel ketocarotenoids
	psy (Arabidopsis)	CaMV35S	858.4 μg/g DW total carotenoids in roots
Tobacco (*Nicotiana tabacum*)	*crt*W and *crt*Z (*Paracoccus* sp.)	CaMV35S	1,275 μg/g DW total carotenoids in leaves
			64 μg/g FW total ketocarotenoids in leaves
	*crt*O (*Synechocystis* sp.) *crt*Z (*P. ananatis*)	CaMV35S	839 μg/g DW total carotenoids in leaves (2.5-fold)
			342.4 μg/g DW total ketocarotenoid in leaves
	*crt*O (*Synechocystis* sp.)	CaMV35S	429 μg/g DW total carotenoids in leaves
			156.1 μg/g DW total ketocarotenoid in leaves
	*crt*W and *crt*Z (*Brevundimonas* sp.)	*rrn*	7,380 μg/g FW total carotenoids in leaves (2.1-fold)
			7,290 μg/g FW total ketocarotenoids in leaves
Wheat (*Triticum aestivium*)	*psy1* (corn) *crt*I (*P. ananatis*)	CaMV35S and 1D × 5 (constitutive)	4.96 μg/g DW in seeds

Biotechnology and Nutritional Improvement of Crops. Table 6 (Continued)

Species	Genes (source)	Promoters	Carotenoid levels in transgenic plants
Arabidopsis (*Arabidopsis thaliana*)	*bkt1* (*H. pluvialis*)	Napin	4-keto-lutein, canthaxanthin, and adonirubin seeds up to 13-fold
	bch (Arabidopsis)	CaMV35S	2,274.8 nmol/g DW total carotenoids
	psy (Arabidopsis)	Napin	260 μg/g FW β-carotene in seeds
	psy (Arabidopsis)	CaMV35S	1,600 μg/g DW (10-fold) in seed-derived calli and 500 μg/g DW (100-fold) of total carotenoids in roots
	chyB (Arabidopsis)	CaMV35S	285 mmol/chl a(mol) violaxanthin (2-fold)
			728 mmol/chl a(mol) of total carotenoid
	AtB1 (Arabidopsis)	CaMV35S	38.2 μg/g β-carotene leaf tissue
	CYP97A3 (Arabidopsis)	CaMV35S	41.7 μg/g β-carotene leaf tissue
	CYP97B3 (Arabidopsis)	CaMV35S	36.7 μg/g β-carotene leaf tissue
	CYP97C1 (Arabidopsis)	CaMV35S	41.3 μg/g β-carotene leaf tissue

Source: Data updated from [171]

carotenoid levels to 1,229 μg/g FW although it increased the relative amount of β-carotene to 846 μg/g FW. Recently, Fujisawa et al. [99] introduced seven bacterial transgenes into canola encoding the enzymes isopentenyl pyrophosphate isomerase (which interconverts IPP and DMAPP), CrtE (GGPP synthase), CrtB (PSY), CrtI (carotene desaturase), CrtY (LYCB), and two additional enzymes (CrtZ and CrtW) that catalyze downstream steps converting β-carotene into ketocarotenoids. Although this resulted in the production of several ketocarotenoids, the predominant carotenoid was still β-carotene, which accumulated to 214.2 μg/g FW, 1,074-fold higher than in wild-type seeds.

Diretto et al. [100, 101] expressed CrtB, CrtI, and CrtY in potato tubers, increasing total carotenoid levels to 114 μg/g DW and β-carotene levels to 47 μg/g DW. They also silenced the endogenous *lyce* and *bch* genes, thereby eliminating competition at the branch point between the α- and β-carotene pathways and preventing the further metabolism of β-carotene.

Ripening tomatoes accumulate low levels of β-carotene and in order to address this deficiency, several investigators have attempted to overexpress either the endogenous *lycb* gene or equivalent heterologous genes [102–106]. D'Ambrosio et al. [103] have been the most successful with this approach, achieving a 32-fold increase in β-carotene levels and generating orange-colored tomato fruits. Another successful strategy was the suppression of the endogenous *DET1* gene, which regulates photomorphogenesis. The expression of a *det1* RNAi construct in tomato chromoplasts increased β-carotene levels eightfold to 130 μg/g DW [107].

An alternative strategy to achieve β-carotene accumulation in plants is to modify the storage capacity of chromoplasts, where β-carotene accumulates in specialized lipoprotein-sequestering structures. A spontaneous mutation in the cauliflower *Orange* (*Or*) gene resulted in deep orange cauliflower heads associated with the hyperaccumulation of carotenoids in

chromoplasts [108, 109] and the mutant allele has been cloned and expressed in potato tubers, where it increased the level of β-carotene tenfold and turned the tuber flesh orange [110].

Vitamin E Vitamin E is a collection of four tocopherols and four tocotrienols (each as α, β, γ, and δ derivatives), collectively known as tocochromanols [111]. All eight isomers can be absorbed equally efficiently during digestion but the hepatic α-tocopherol transfer protein (α-TTP) shows preferential retention of α-tocopherol, making it the most important form in terms of vitamin E activity in the human body [111].

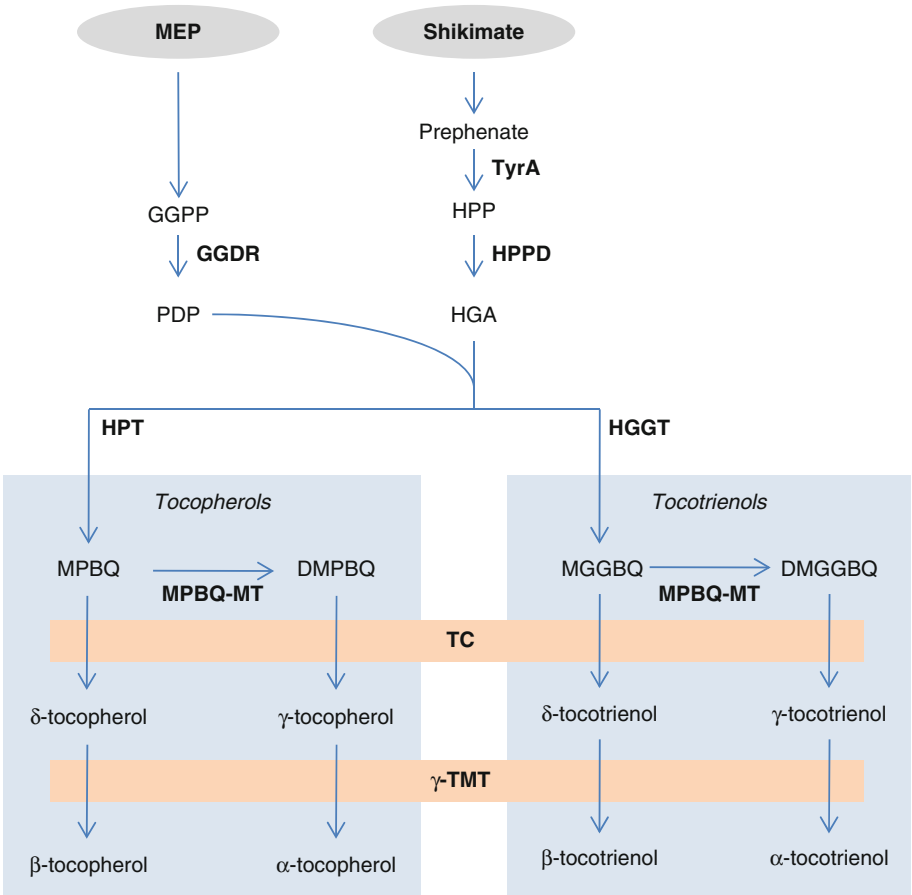
Tocochromanol biosynthesis in higher plants (Fig. 6) occurs in the plastids and requires precursors from the cytosolic shikimate pathway and the plastidial MEP pathway [111]. The tocochromanol head group is derived from the shikimate pathway, and this involves the conversion of p-hydroxyphenylpyruvic acid (HPP) to homogentisic acid (HGA), by HPP dioxygenase (HPPD); the MEP pathway contributes the side chain [111]. The first committed step in tocochromanol biosynthesis is the prenylation of HGA. There are two enzymes that carry out this reaction. If prenylation is carried out by homogentisate phytyltransferase (HPT), the product is 2-methyl-6-phytylplastoquinol (MPBQ), which leads to the synthesis of tocopherols. Alternatively, HGA can be prenylated by homogentisate geranylgeranyl transferase (HGGT), producing 2-methyl-6-geranylgeranylplastoquinol (MGGBQ), which leads to the synthesis of tocotrienols [111].

The eight isomers form through a complex series of reactions involving enzymes with multiple substrates at each stage. Both MPBQ and MGGBQ are substrates for tocopherol cyclase (TC), leading to the production of δ-tocopherol and δ-tocotreinol, respectively. They are also substrates for the enzyme MPBQ methyltransferase (MPBQ-MT), which adds a second methyl group to form 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) and 2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinone (DMGGBQ), respectively. These methylated derivatives are also substrates for TC, leading to the production of γ-tocopherol and γ-tocotreinol, respectively. Finally, all four of these products (the δ- and γ-tocopherols, and the δ- and γ-tocotrienols) can be methylated by γ-tocopherol methyltransferase (γ-TMT) to produce their β- and α- counterparts [111].

Plants can be engineered to accumulate higher levels of vitamin E by overexpressing the genes involved in tocochromanol synthesis, and this can be achieved either by increasing the total tocochromanol content or skewing tocochromanol synthesis toward the more potent vitamers, particularly α-tocopherol (Table 7). In the first approach, the overexpression of HPT increased tocopherol levels by up to 1.6-fold in the seeds and 4.4-fold in the leaves of transgenic Arabidopsis plants [112]. The overexpression of HPPD increased tocotrienol levels tenfold in Arabidopsis leaves [113], twofold in tobacco leaves, and 1.5-fold in tobacco seeds [114]. In the second approach, the expression of Arabidopsis γ-TMT in lettuce increased the α/γ tocopherol ratio but had no effect on the total tocopherol content [115]. However, by crossing the lines expressing HPT and γ-TMT, both the total tocopherol content and the α/γ tocopherol ratio were increased [115]. In canola, total tocochromanol levels have been doubled by expressing genes encoding HPT, HPPD, and TC [116].

The impact of HPPD expression can be enhanced by the simultaneous expression of TyrA, the enzyme responsible for the synthesis of HPP from prephenate (Fig. 6). TyrA expression has little effect on its own, but when combined with HPPD in tobacco, tocochromanol levels in the leaves increased eightfold [117]. Similar results have been achieved in Arabidopsis, canola, and soybean seeds using the same combination of genes, although the total tocochromanol content increased only 2–2.5-fold in these cases [118]. The expression of HPT, HPPD, and TyrA increased the tocochromanol content of seeds fivefold, and the further addition of geranylgeranyldiphosphate reductase (GGDR), which provides the precursor phytyldiphosphate, increased the tocochromanol content by up to 15-fold [118].

The seed-specific expression of Arabidopsis MPBQ-MT and γ-TMT in soybean resulted in a complete conversion of β-, γ-, and δ-tocopherols to α-tocopherol, with a fivefold enhancement in the vitamin E activity of soybean oil [119]. Similarly, Tavva et al. [120] achieved a 10.4-fold increase in α-tocopherol levels and a 14.9-fold increase in β-tocopherol levels in soybean seeds expressing *Perilla frutescens* γ-TMT.



Biotechnology and Nutritional Improvement of Crops. Figure 6
Vitamin E synthesis in plants [111]. The pathway involves the prenylation of homogentisic acid (HGA), derived from the shikimate pathway, with phytyldiphosphate (PDP), derived from the methylerythritol phosphate (MEP) pathway. Prenylation may be carried out by two different enzymes – HPT (homogentisate phytyltransferase) or HGGT (homogentisate geranylgeranyl transferase) – to generate alternative intermediates that give rise to the tocopherol and tocotrienol branches of the pathway, respectively. These intermediates are substrates for the same three enzymes, yielding eight different products. Additional substrate abbreviations: GGPP, geranylgeranyl pyrophosphate; HPP: ρ-hydroxyphenylpyruvic acid; MPBQ, 2-methyl-6-phytylbenzoquinol; DMPBQ, 2,3-dimethyl-5-phytylbenzoquinol; MGGBQ, 2-methyl-6-geranylgeranylplastoquinol; DMGGBQ, 2,3-dimethyl-5-geranylgeranylplastoquinol. Additional enzyme abbreviations: GGDR, geranylgeranyl diphosphate reductase; HPPD, HPP dioxygenase; MPBQ-MT: MPBQ methyltransferase; TC, tocopherol cyclase; γ–TMT: γ-tocopherol methyltransferase

Vitamin C Most vitamin C (ascorbic acid) in plants is synthesized through the L-galactose pathway [121], although other routes through galacturonic acid, L-glucose, and *myo*-inositol have been proposed [122]. Oxidation of ascorbic acid produces the short-lived radical monodehydroascorbate (MDHA), which is either converted to ascorbic acid by MDHA reductase (MDHAR) or undergoes spontaneous degradation into ascorbic acid and dehydroascorbate (DHA). DHA is then either recycled to ascorbic acid by dehydroascorbate reductase (DHAR), using glutathione as the reductant, or undergoes irreversible hydrolysis to generate 2,3-diketogulonic acid (Fig. 7).

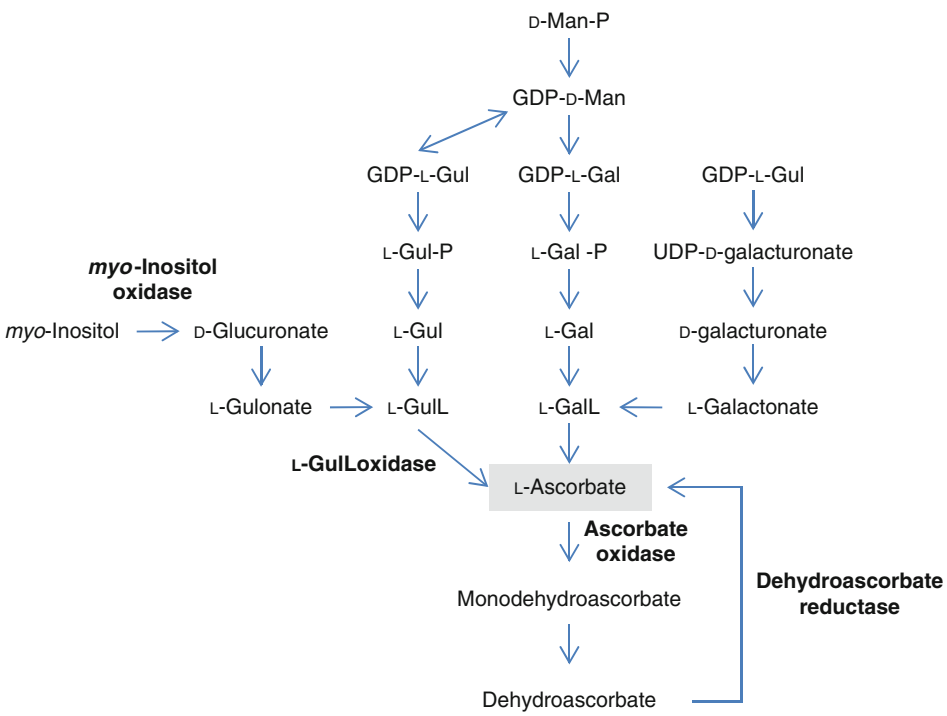
Biotechnology and Nutritional Improvement of Crops. Table 7 Transgenic plants with enhanced tocochromanol levels [111]

Species	Genes (source)	Promoters	Tocochromanol levels in transgenic plants
Tobacco (*Nicotiana tabacum*)	*hppd* (barley; *Hordeum vulgare*)	CaMV 35S	50 μg/g DW in seeds (1.5–2-fold)
	tyrA (*Erwinia uredovora*)	Arabidopsis histone gene H4748	67 μg/g DW in leaf (1.3-fold)
	tyrA (*E. uredovora*), *hppd* (Arabidopsis)		14.3 μg/g DW of α-tocotrienols
			551 μg/g DW in leaf (10-fold)
Corn (*Zea mays*)	*hggt* (barley)	Embryo-specific	>800 nmol/g DW in seeds (6-fold)
	hppd and *vte3* (Aradidopsis)	CaMV 35S	9.5 μg/g DW (3-fold)
Lettuce (*Lactuca sativa*)	*vte4* (Arabidopsis)	CaMV 35S	Improved α-/γ –tocopherol ratio up to 0.4–544 as compared to α/γ ratio in wild type, which is 0.6–1.2
Mustard (*Brassica juncea*)	*vte4* (Arabidopsis)	CaMV 35S	62.29 μg/g of α-tocopherol levels in seeds (6-fold)
Canola (*Brassica napus*)	*hppd* (Arabidopsis)	DC3Ω	819 μg/g oil in seeds (1.2-fold)
	hppd, *hpt1*, *vte1* (Arabidopsis)	DC3Ω (*hppd*), napin (*hpt1, vte1)*	1,850 μg/g oil in seeds (2-fold)
	tyrA (*E. uredovora*)	Lac	540 μg/g of total tocochromanols in seeds (2.3-fold)
	tyrA (*E. uredovora*), *hppd*, *hpt1* (Arabidopsis)	Lac	3.7-fold increase in seeds
	vte1 (Arabidopsis)	Napin	1,018 μg/g oil of total tocochromanols in seeds (20–50% increase)
	vte1 (corn)	Napin	1,159 μg/g oil of total tocochromanols in seeds
Soybean (*Glycine max*)	*tyrA* (*E. uredovora*), *hppd*, *hpt1* and *ggh* (Arabidopsis)	Lac	4,806 μg/g of total tocochromanols in seeds (15-fold)
	vte3 (Arabidopsis)	7Sα	The majority of the tocopherol accumulated as γ-tocopherol (75–85%) with increased α-tocopherol as well. By contrast, these seeds had very low levels of β- and δ-tocopherol, only 0.5–1.5% of total tocopherols
	vte4 (Arabidopsis)	7Sα	100% of α-tocopherols in seeds
	vte3 and *vte4* (Arabidopsis)	7Sα	8-fold increase in α-tocopherol in seeds
	vte4 (*Perilla frutescens)*	Vicilin	390 nmol/g FW of α-tocopherol content in seeds (10.4-fold)
			52 nmol/g FW of β-tocopherol content in seed (14.9-fold)

Biotechnology and Nutritional Improvement of Crops. Table 7 (Continued)

Species	Genes (source)	Promoters	Tocochromanol levels in transgenic plants
Arabidopsis (*Arabidopsis thaliana*)	*vte4* (Arabidopsis)	DC3	328 μg/g α-tocopherol in seeds (86-fold)
	hppd (Arabidopsis)	CaMV 35S, DC3	37% and 28% increase of tocopherol levels in leaf and seed respectively
	hpt1 (Arabidopsis)	CaMV 35S	4.4-fold increase in total leaf tocopherol content (mainly α-tocopherols)
	hpt1 (Arabidopsis)	Napin	2-fold increase of tocopherols in seed

Source: Data updated from [111]



Biotechnology and Nutritional Improvement of Crops. Figure 7
Synthesis of ascorbic acid in plants [96]. Abbreviations: Gal, galactose; GalL, galactonolactone; GDP guanidine diphophate; Gul, gulose; GulL, gulonolactone; Man, mannose; P, phosphate; UDP, uridine diphosphate

Since at least three separate metabolic pathways are involved in the biosynthesis of ascorbic acid in plants in addition to the recycling of oxidation products, three different strategies have been used to enhance ascorbate levels: overexpression of enzymes involved in ascorbate biosynthesis, overexpression of recycling enzymes (such as DHAR) to enhance regeneration, and antisense suppression of ascorbate oxidase (Table 8). Enhancing ascorbate regeneration has been the most successful approach [122]. The expression of wheat DHAR increased ascorbate levels by twofold in transgenic corn seed and fourfold in

Biotechnology and Nutritional Improvement of Crops. Table 8 Transgenic plants with enhanced ascorbate levels [122]

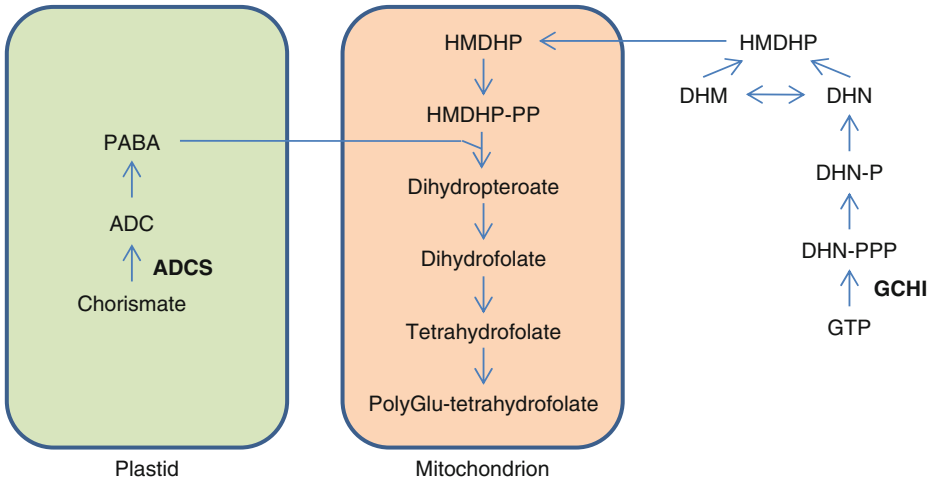
Species	Genes (source)	Promoters	Ascorbate levels in transgenic plants
Corn (*Zea mays*)	*dhar* (wheat; *Triticum aestivum*)	Corn Ubi-1 (constitutive)	~150 nmol/g FW in kernel (1.9-fold)
	dhar (rice; *Oryza sativa*)	Barley (*Hordeum vulgare*) D-hordein	607.2 nmol/g FW (6.1-fold)
Tomato (*Solanum lycopersicum*)	*mMDH*	CaMV 35S (constitutive)	(5–6-fold)
Lettuce (*Lactuca sativa*)	*GLOase* (rat; *Rattus norvegicus*)	CaMV 35S	430–580 nmol/g FW (4–7-fold)
Tobacco (*Nicotiana tabacum*)	*GLOase* (rat)	CaMV 35S	480 nmol/g FW (7-fold)
	dhar (wheat)	CaMV 35S	2,800 nmol/g FW (2.4-fold)
	GalLDH (tobacco)	CaMV 35S	(1.5–2-fold)
	GMP (*Malpighia glabra*)	MgGMP	800–1,000 nmol/g FW (2-fold)
	GGT (GDP-L-galactose D- mannose-1-phosphate guanyltransferase) (*Actinidia chinensis)*	CaMV 35S	1,000 μg/g FW (3-fold)
	PMM (*M. glabra*)	MgGMP	700 μg/g FW (2-fold)
Arabidopsis (*Arabidopsis thaliana*)	*GalUR* (strawberry; *Fragaria* × *ananassa*)	CaMV 35S	~600 nmol/g (3-fold)
	miox4 (Arabidopsis)	CaMV 35S	~500 nmol/g FW (2–3-fold)
	GGT (GDP-L-galactose guanyltransferase) (*Actinidia eriantha*)	CaMV 35S	8.6–12-fold
	GME (GDP-L-mannose-3′,5′-epimerase) (*A. eriantha*)	CaMV 35S	

Source: Data updated from [122]

transgenic tobacco leaves, with concomitant reductions in the levels of DHA [123], whereas the expression of rice DHAR in corn increased the level of ascorbate in the seeds by more than sixfold [96].

Jain and Nessler [124] achieved a sevenfold increase in ascorbate levels by expressing L-gulono γ-lactone oxidase (GLOase) in tobacco and lettuce (the lack of this enzyme accounts for the inability of primates to synthesize their own ascorbic acid). Transgenic tobacco plants expressing phosphomannomutase (PMM) or GDP-D-mannose pyrophosphorylase (GMP) also accumulated double the normal levels of ascorbate [125].

Plants of the genus *Actinidia* are particularly rich in ascorbic acid, so genes from the ascorbate biosynthesis pathway in *Actinidia* species have been expressed in model plants to ascertain if this can be used to boost ascorbate levels. Transgenic tobacco plants expressing *Actinidia chinensis* GDP-L-galactose guanyltransferase contained threefold the normal levels of ascorbate in the leaves [126] whereas Arabidopsis plants expressing the equivalent enzyme from *Actinidia eriantha* contained 4.2-fold more ascorbate than normal. Expressing this enzyme in concert with GDP-L-mannose-3′,5′-epimerase achieved a 12-fold increase in ascorbate revealing an important bottleneck in the L-galactose pathway [127].



Biotechnology and Nutritional Improvement of Crops. Figure 8
Synthesis of folate in plants [96]. Abbreviations: aminodeoxychorismate (ADC), GTP cyclohydrolase I (GCHI), ADC synthase (ADCS), DHN, dihydroneopterin; -P/-PP/-PPP, mono/di/triphosphate; DHM, dihydromonopterin; HMDHP, hydroxymethyldihydropterin

Biotechnology and Nutritional Improvement of Crops. Table 9 Transgenic plants with enhanced folate levels [172]

Species	Genes (source)	Promoters	Folate levels in transgenic plants
Tomato (*Solanum lycopersicum*)	*gtpchI* (mammalian)	Tomato E8	0.8–2.3 nmol/g FW (2-fold)
	gtpchI (mammalian) and *adcs* (Arabidopsis)	Tomato E8	8.4 μg/g DW (25-fold)
Corn (*Zea mays*)	*folE* (*Escherichia coli*)	Barley (*Hordeum vulgare*) D-hordein	1.94 μg/g DW (2-fold)
Arabidopsis (*Arabidopsis thaliana*)	*folE* (*E. coli*)	CaMV 35S	2.27–4.70 nmol/g FW (2–4-fold)
Rice (*Oryza sativa*)	*gtpchI* (Arabidopsis)	Rice endosperm-specific globulin (glb-1)	38.3 nmol/g (100-fold)
	adcs (Arabidopsis)		
Lettuce (*Lactuca sativa*)	*gtpchI* (chicken; *Gallus gallus*)	CaMV 35S	1.9 μg/g (5.4-fold)

Source: Data updated from [172]

Folate Folic acid is a tripartite molecule combining pterin, p-aminobenzoate (PABA), and one or more glutamate moieties. The three parts of the molecule are produced separately in different subcellular compartments (plastids, cytosol, and mitochondria) and then joined together. Pterin is formed from guanosine triphosphate (GTP) in the cytosol and PABA is formed from chorismate in the plastids. These moieties are then transported to the mitochondria, where they condense to form dihydropteroate and are converted to polyglutamates (Fig. 8). Metabolic engineering can be used to increase folate levels in plants but the compartmentalization of the pathway adds a degree of complexity (Table 9).

Initial strategies involving modulation of individual branches of the pathway have been moderately successful. For example, expressing GTP cyclohydrolase 1 (GCH1) enhances the cytosolic (pterin) branch of the pathway and increases pterin levels, which ensures that the other two branches become rate limiting. In tomato, this approach doubled the normal levels of folate in the fruit [128]. Similar results were achieved by enhancing the PABA branch of the pathway by overexpressing aminodeoxychorismate synthase (ADCS) [129]. Each of these experiments revealed the limitations of the other pathway branches, but by crossing the two transgenic lines, the enhanced pathways were combined resulting in complementation between them and the creation of transgenic tomato fruits containing 25-fold the normal levels of folate [129]. The strategy has been replicated in other crops with similarly impressive results: the expression of Arabidopsis GCH1 and ADCS in rice endosperm, e.g., increased folate levels by up to 100-fold [130].

Pantothenate Although most work in the area of vitamin enhancement in plants has focused on vitamins A, B_9 (folate), C, and E, there have been a small number of studies addressing other vitamins. Fouad and Rathinasabapathi [131] expressed the *E. coli panD* gene encoding L-aspartate-α-decarboxylase (ADC) in tobacco and increased the level of pantothenate (vitamin B_5) in the leaves by up to 4.1-fold. Similarly, Chakauya et al. [132] expressed the *E. coli panB* gene encoding ketopantoate hydroxymethyltransferase (KPHMT) in canola, increasing levels of pantothenate in leaves, flowers, siliques, and seeds by 1.5–2.5-fold compared with the wild type.

Increasing Mineral Levels

In contrast to the situation with vitamins and essential amino acids and fatty acids, the mineral content of plants cannot be increased by metabolic engineering because minerals are not synthesized de novo by plants; instead they are taken up from the environment. Therefore, increasing the mineral density of plants using biotechnology involves a different set of strategies, focusing on the introduction of genes that improve the efficiency of mineral extraction from the soil (through improved mobilization and/or improved uptake), improve the efficiency of transport from the roots to storage organs such as seeds and fruits, and increase the capacity of storage organs to store minerals in a form that is available in the diet and not toxic to the plant [23, 32]. In addition, these strategies can be supplemented with those seeking to reduce the abundance of antinutritional compounds such as phytate, which inhibits mineral absorption in the gut, and/or to increase the abundance of nutritional enhancer compounds such as inulin, which facilitate mineral absorption by slowing down the progress of food through the gut (Table 10).

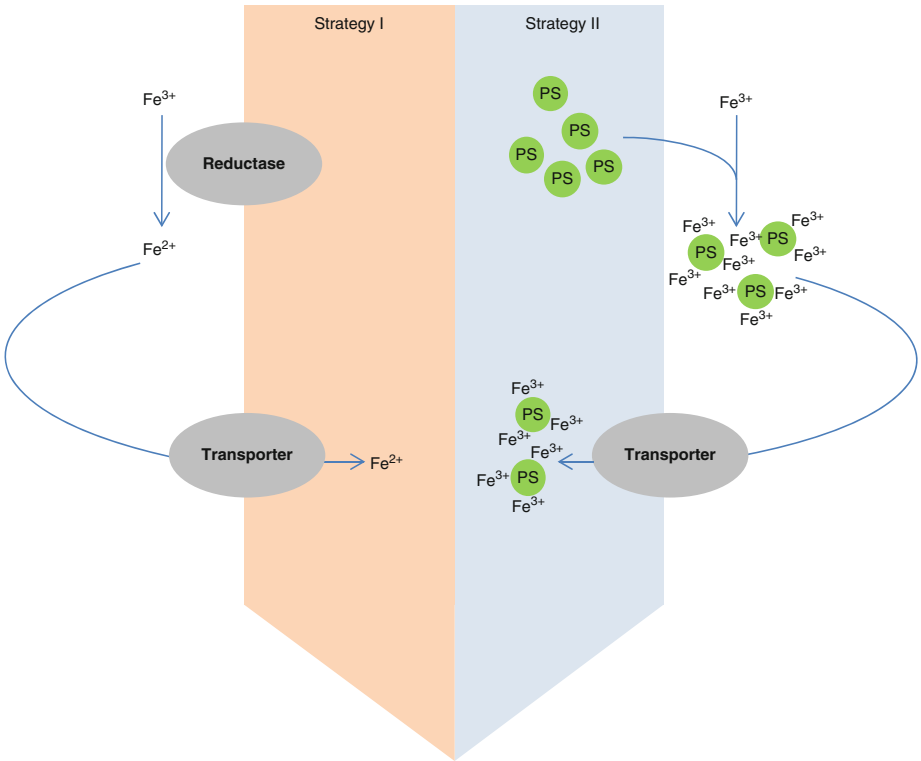
Biotechnology and Nutritional Improvement of Crops.

Table 10 Some examples of mineral nutritional enhancers and antinutrients in plant foods [37]

Nutritional enhancers	Major dietary source
β-Carotene (pro-vitamin A)	Green and orange vegetables
Certain amino acids (cysteine, lysine, etc.)	Animal meats
Certain organic acids (ascorbic acid, citrate, etc.)	Fresh fruit and vegetables
Hemoglobin	Animal meats
Inulin	Chicory, garlic, onion, wheat, artichoke
Long-chain fatty acids	Human breast milk
Antinutrients	**Major dietary source**
Phytic acid (phytate)	Whole legume seeds and cereal grains
Goitrogens	*Brassicas* and *Alliums*
Hemagglutinins	Most legumes and wheat
Oxalic acid (oxalate)	Different vegetables (spinaches, beet, linseed, oca)
Tannins and other polyphenolics	Tea, coffee, beans, sorghum

Source: Adapted from [37]

Mineral Uptake and Transport The efficiency with which minerals are taken up from the soil depends on their accessibility, which in turn reflects their solubility



Biotechnology and Nutritional Improvement of Crops. Figure 9
Strategies for iron acquisition by plants [134]. Strategy I, as used by non-graminaceous plants, involves the acquisition of Fe^{2+} after reduction of Fe^{3+} in the rhizosphere by secreted reductases. Strategy II, as used by graminaceous plants, involves the secretion of phytosiderophores (PS) which chelate Fe^{3+}, allowing absorption of the chelated complexes

and interactions with soil particles. Iron is present in the soil mainly as Fe^{3+}, an insoluble form with the tendency to bind strongly to inert particles. Therefore, plants have evolved two distinct strategies to facilitate iron absorption (Fig. 9). Strategy I is used by non-graminaceous plants and involves acidifying the rhizosphere through the secretion of protons, reducing Fe^{3+} to Fe^{2+} using reductases and then absorbing the soluble Fe^{2+} using specific transporters. Strategy II is used by graminaceous plants and involves the direct acquisition of Fe^{3+} by secreting chelating agents (phytosiderophores) into the rhizosphere, followed by adsorption of the chelated iron complexes. Specific root transporters are then required for the uptake of the soluble Fe^{3+}-phytosiderophore complexes [133]. Zinc is present in soil as Zn^{2+} and can be directly absorbed in this form. Many of the genes required for zinc uptake, sequestration, and redistribution in plants have been identified [44] and it is thought that zinc-hyperaccumulating plants sequester large amounts of zinc in the vacuole, a strategy that confers zinc tolerance. However, Zn^{2+} can also form chelates with phytosiderophores, and the Zn^{2+}-phytosiderophore complexes share the same channels as those used for iron chelates. Therefore, some strategies that have been developed to improve the uptake of iron also improve the uptake of zinc as a beneficial by-product, and vice versa, although this depends on the genes involved [134]. Other minerals are taken up through specific transporters, e.g., plants take up selenium from the soil in the form of selenate (via sulfate transporters), selenite (via phosphate transporters), or as organic compounds (selenoproteins), and they take up calcium via specific Ca^{2+}/ H^{+} antiporters (CAX). Once inside the plant, minerals may also share transport mechanisms – e.g. Fe^{2+}, Zn^{2+}, and Ca^{2+} may all form complexes with nicotianamine for transport through the phloem to sink tissues.

Biotechnology and Nutritional Improvement of Crops. Table 11 Transgenic plants with enhanced mineral levels [23]

Species	Genes (source)	Promoters	Mineral content in seeds (and increase relative to wild type)
Iron			
Corn (*Zea mays*)	*ferritin* (soybean; *Glycine max*) + *phytase (Aspergillus niger)*	Rice glutelin-1	38 μg/g DW (2-fold)
Rice (*Oryza sativa*)	*ferritin* (soybean),	Rice gluB-1	38.1 μg/g DW (3-fold)
		Corn Ubi-1	No increase in seeds
		Rice GluB-1	34.7 μg/g DW (4.4-fold)
		Rice Glb-1	27 μg/g DW (3-fold)
	ferritin (pea; *Pisum sativum*),	CaMV-35S	31.3 μg/g DW (4.82-fold)
	ferritin (common bean; *Phaseolus vulgaris*) + *phytase (Aspergillus fumigatus)*	Rice glutelin-1	22.07 μg/g DW (2-fold)
	nas1 (Arabidopsis; *Arabidopsis thaliana*) + *ferritin* (common bean)	CaMV35S + rice globulin	7 μg/g DW (6.3-fold) (in polished rice)
Tobacco (*Nicotiana tabacum*)	*nas1* (barley; *Hordeum vulgare*)	CaMV35S	(2.3-fold in leaves)
	nas1 (Arabidopsis)	CaMV35S	(1.5-fold in leaves)
Calcium			
Carrot (*Daucus carota*)	sCAX 1 (Arabidopsis)	CaMV35S	3.9 mg/g DW (1.6-fold) (in carrot)
Lettuce (*Lactuca sativa*)	sCAX 1 (Arabidopsis)	Cell division cycle (cdc2a)	18.9 mg/g DW
Potato (*Solanum tuberosum*)	sCAX 1 (Arabidopsis)	CaMV35S	1.7 mg/g DW (3-fold) (in tuber)
	CAX2B chimeric (Arabidopsis)	CaMV35S	2.5 mg/g DW (3-fold) (in tuber)
Rice	sCAX 1 (Arabidopsis)	CaMV35S	not determined
Tomato (*Solanum lycopersicum*)	CAX 4 (Arabidopsis)	CaMV35S	1.8 mg/g DW (in fruit)

Source: Data updated from [23]

In order to improve iron uptake from the soil, transgenic plants have been created expressing heterologous iron transporters, reductases, and enzymes involved in phytosiderophore biosynthesis (Table 11). For strategy I plants, such approaches have usually involved the expression of iron transport proteins, whereas for strategy II plants, iron accumulation can be enhanced by the production of higher levels of phytosiderophores, with the anticipated collateral effects on zinc absorption [23]. In rice, e.g., iron and zinc uptake was improved by the expression of the barley *naat-A* and *naat-B* genes encoding nicotianamine aminotransferases, which are involved in phytosiderophore biosynthesis [135]. Similarly, the overexpression of barley nicotianamine synthase in tobacco doubled the iron and zinc concentrations in leaves [136]. Transgenic barley expressing the Arabidopsis ZIP1 iron/zinc transporter, despite having smaller seeds, accumulated twice the amount of zinc and iron as wild-type plants [137]. In contrast, constitutive overexpression of barley ZIP7 increased the zinc content of the grain by 50%, but had negligible effect on the iron content regardless of the abundance of zinc in the soil, showing that iron and zinc are not always

co-absorbed [138]. Interestingly, expression of the Arabidopsis putative zinc transporter MTP1 in barley led to a dramatic short-term increase in the amount of zinc stored in the roots under high zinc loads, but no difference in the amount of zinc in the seeds over the long term [139].

Several different approaches can be envisaged to enhance the uptake and storage of selenium, based on the multiple inorganic and organic sources of selenium in the soil and the manner in which they are interconverted [140]. ATP-sulfurylase (APS) is rate limiting for selenate reduction and accumulation in most plants, so the overexpression of this enzyme can increase the uptake of selenate [141]. Selenocysteine methyltransferase (SMT) converts selenocysteine into selenium-methylselenocysteine (MetSeCys), a nontoxic form found at high levels in the selenium-hyperaccumulator, *Astragalus bisulcatus*, so the overexpression of this enzyme would be a suitable approach for increasing the accumulation of organic selenium.

Pilon-Smits et al. [141] expressed Arabidopsis APS in Indian mustard, resulting in a threefold increase in selenium levels in leaves and greater tolerance for high selenate levels in the soil. The levels of organic selenium also increased because of the increased capacity for selenate reduction [141, 142]. Indian mustard plants overexpressing both APS and SMT accumulated 10-fold more MetSeCys than normal, reflecting the increased uptake of selenate and its conversion to MetSeCys as well as the reduction of selenite [143]. More recently, McKenzie et al. [144] expressed *Brassica oleracea* APS and *Astragalus bisulcatus* SMT in tobacco plants supplied with selenate. The expression of SMT increased total selenium levels up to 4-fold, and whereas the expression of APS did not increased total selenium level, when combined with the SMT, a greater proportion of selenium was converted into MetSeCys.

The calcium content of crops has been enhanced by expressing specific Ca^{2+}/ H^{+} antiporters located in the vacuolar membrane (Table 11). For example, the Arabidopsis cation exchanger 1 antiporter (sCAX1) [145], enhances the level of bioavailable calcium when expressed in transgenic potato tubers and carrot taproots [33, 146–148]. The same protein has been expressed in transgenic lettuce, which accumulated higher levels of calcium than wild-type leaves without impacting on flavor or crispness [149]. Kim et al. [150] constructed a chimeric Arabidopsis gene (*CAX2B*) by combining a truncated N-terminal portion of *CAX2* with the "B" domain from *CAX1*. Transgenic potatoes expressing this recombinant construct accumulated calcium without affecting the levels of related cations such as Mn^{2+}. Transgenic tomatoes expressing the Arabidopsis *CAX4* gene also accumulated higher levels of calcium than wild-type fruits [150].

Mineral Storage in Sink Tissues In cereal grains, minerals are predominantly stored in the bran (embryo and aleurone layer) rather than the endosperm, which means that much of the nutritional value of cereals is lost during polishing [151]. Encouraging plants to absorb more minerals from the soil is therefore not sufficient to increase the nutritional value of *food*. In addition to improving mineral uptake, cereals must be engineered to accumulate minerals in the endosperm.

Minerals in plants are found both as free ions in solution and as complexes with dedicated proteins that have evolved for the specific function of mineral transport and storage (protecting the plants against both mineral deficiency and overload). Ferritin, e.g., is a 450-kDa protein consisting of 24 subunits that form a shell, enabling the storage of up to 4,500 Fe^{3+} ions as crystallites with hydroxide and phosphate. Recombinant soybean ferritin has been expressed in the endosperm of several cereals allowing the accumulation of iron in the endosperm [152–155] and pea ferritin has also been constitutively expressed in rice [156]. In the best cases, the level of bioavailable iron exceeded 35 mg kg^{-1}.

Combined Strategies to Increase Mineral Density in Cereal Grains Although increasing the uptake of iron and the ability of plants to accumulate iron both work as individual strategies to improve the mineral content of cereal crops, there are limitations when each strategy is applied alone. Increasing the uptake of iron without considering its distribution will result in the hyperaccumulation of iron in vegetative tissues as well as the seeds, eventually resulting in overload and toxicity. Conversely, the overexpression of ferritin without compensating for the increased iron storage capacity results in the sequestration of free iron needed by plant cells for normal physiological functions, inhibiting

photosynthesis and causing chlorosis even if plenty of iron is available in the soil [154]. These issues can be overcome by combining the strategies, i.e., increasing both uptake and storage capacity in the same plant. For example, the combined expression of Arabidopsis nicotianamine synthase and soybean ferritin in rice resulted in rice grains with 6.3-fold more iron in the polished endosperm than wild-type plants (and also elevated levels of zinc), but no adverse effects [157].

Strategies to Ensure Stored Minerals Are Bioavailable Increasing the levels of minerals in plants does not necessarily increase their bioavailability, i.e., the proportion of the mineral that can be absorbed in the human gut [23]. In some cases, bioavailability depends on the chemical form in which a mineral is presented. For example, selenomethionine is a more bioavailable form of selenium than any inorganic source [18], and heme iron is more bioavailable than nonheme iron [158]. For cereals in particular, bioavailability reflects the presence of antinutritional compounds that inhibit absorption, such as phytate and oxalic acid, which chelate divalent cations (Table 10). It has been shown that high levels of such compounds can reduce mineral bioavailability to the extent of causing marginal deficiency diseases even if absolute mineral levels are adequate [159]. This can easily be overcome with a varied diet, but is particularly challenging in developing country settings where a monotonous diet of staple cereals in commonplace.

Strategies to tackle the presence of antinutritional compounds include conventional breeding to reduce the level of phytate in cereals, and biotechnology-based approaches to either reduce phytate levels or increase the levels of nutritional enhancers that counteract the effect of phytate. For example, natural variation in levels of the nutritional enhancer inulin has been investigated to increase the bioavailability of zinc [160]. Mutagenesis and conventional breeding have been used to generate low-phytate corn, barley, rice, and soybean [161, 162], which have 66%, 95%, 64%, and 80% less phytate, respectively, than corresponding wild-type lines, as well as beans with a 90% reduction of the normal levels of phytate but with no adverse effects on plant growth and development [163]. A *Medicago truncatula* mutant has also been bred with low levels of oxalic acid and therefore 22.87% higher calcium bioavailability [164]. Phytate levels have also been reduced in transgenic cereals by expressing a recombinant fungal enzyme (phytase) that degrades the compound. This strategy has been used to increase iron bioavailability in wheat (86% reduction in phytate [165, 166]) and corn (23% reduction in phytate [167]). The combined expression of ferritin and phytase was used in rice [168] and corn [169] (95% reduction of phytate) to increase iron levels and bioavailability in simulated digestion/absorption trials.

Stacking Nutritional Enhancement Traits The vast majority of transgenic plants engineered for nutritional enhancement have been created with the specific intention of tackling one particular nutrient (or class thereof). In many cases, this has involved the transfer of a single gene. In others, discussed above, two transgenic lines have been crossed to combine enhancements in an additive or synergistic manner, e.g., enhancing iron uptake and accumulation, or enhancing two branches of a metabolic pathway to increase the levels of folate. An alternative approach is supertransformation (the transformation of transgenic plants with additional transgenes), which has been used to produce VLC-PUFAs in Arabidopsis [88]. Both methods have two major drawbacks – the long and labor-intensive development process involving several breeding generations, and the fact that the different transgenes are unlinked, leading to segregation in subsequent generations [170]. Cotransformation refers to the simultaneous introduction of two or more transgenes, and its major advantages are that plants carrying multiple transgenes are produced in one generation and all the transgenes are likely to integrate at the same locus, thus preventing segregation. Cotransformation has been used to introduce up to seven transgenes simultaneously for the purpose of nutritional enhancement, in this case, the enhancement of carotenoid synthesis in canola [99], but even in this case, the aim was to modulate the levels of a single nutrient, β-carotene.

It is clear that, regardless of the success of experiments involving individual nutrients, the deployment of a transgenic plant line enhanced for a single nutrient will only serve to shift the problem of nutrient deficiency onto a different compound. In order to

address the challenge of micronutrient deficiency in a global manner, the next objective must be to enhance staple crops for all essential nutrients simultaneously. Zhu et al. [95] reported a unique and surprisingly straightforward approach to this challenge based on combinatorial nuclear transformation in corn. They chose the carotenoid biosynthetic pathway and transformed corn with a collection of five transgenes encoding the enzymes phytoene synthase, phytoene desaturase, lycopene β-cyclase, β-carotene hydroxylase, and β-carotene ketolase. Unlike other transformation experiments where the aim is to achieve a defined outcome, here the aim was to generate maximum diversity, i.e., a library of transformants expressing different combinations of transgenes. Transgenic plants expressing different enzyme combinations and showing distinct metabolic phenotypes were generated, allowing the identification and complementation of rate-limiting steps in the pathway. Individual transgenic lines were identified with extraordinarily high levels of β-carotene (as discussed earlier) and other carotenoids, providing the mechanism to generate plants with high levels of different nutrients in the same experiment. The same group expanded this principle by breeding selected transgenic lines with selected non-transgenic cultivars to generate lines with unprecedented levels of zeaxanthin [34] and by repeating the process with additional transgenes to identify plants simultaneously enhanced for multiple vitamins. Using four transgenes encoding enzymes from three metabolic pathways (corn PSY and bacterial CrtI for carotenoid synthesis, rice DHAR for ascorbate synthesis, and *E. coli* GCH1 for folate synthesis), Naqvi et al. [96] generated transgenic corn lines with a 407-fold increase in β-carotene (57 μg/g DW), a 6.1-fold increase in ascorbate (106.94 μg/g DW), and a 2-fold increase in folate (200 μg/g DW) compared to the non-transformed control plants.

Future Directions

Biotechnology has the potential to address some of the major elements of food insecurity both by increasing the availability of food and making that food more nutritious. The nutritional properties of plants can be improved by increasing the levels of essential amino acids, PUFAs, and vitamins, and by favoring the accumulation of minerals in a bioavailable form. Golden Rice and Multivitamin Corn are key developments in the history of nutritional enhancement, providing models for the development of crops that will particularly benefit subsistence farmers in developing countries. It is imperative that the focus shifts away from single gene strategies and single nutrients and toward the introduction of multiple genes that simultaneously enhance multiple pathways, leading ultimately toward the creation of transgenic crops that are in every sense of the word nutritionally complete.

Bibliography

Primary Literature

1. FAO (2006) The state of food insecurity in the world 2006. FAO, Rome
2. Christou P, Twyman RM (2004) The potential of genetically enhanced plants to address food insecurity. Nutr Res Rev 17:23–42
3. Ramessar K, Naqvi S, Dashevskaya S, Peremarti A, Yuan D, Gomez-Galera D, Velez SMR, Farre G, Sabalza M, Miralpeix B, Twyman RM, Zhu C, Bassie L, Capell T, Christou P (2009) The contribution of plant biotechnology to food security in the 21st century. In: Amsel L, Hirsch L (eds) Food science and security. Nova Science, New York
4. FAO/WHO (2002) Human vitamin and mineral requirements. Report of a joint FAO/WHO expert consultation. FAO/WHO Press, Rome/Geneva
5. FAO/WHO/UNU (2007) Protein and amino acid requirements in human nutrition. Report of a joint WHO/FAO/UNU Expert Consultation. FAO/WHO Press, Rome/Geneva
6. Burr GO, Burr MM, Miller E (1930) On the fatty acids essential in nutrition. III. J Biol Chem 86:1–9
7. Fürst P, Stehle P (2004) What are the essential elements needed for the determination of amino acid requirements in humans? J Nutr 134:1558S–1565S
8. Harrison EH (2005) Mechanisms of digestion and absorption of dietary vitamin A. Annu Rev Nutr 25:87–103
9. UNICEF (2006) Vitamin A deficiency. http://www.childinfo.org/areas/vitamina/
10. Scott JM, Kirke P, Molloy A, Daly L, Weir D (1994) The role of folate in the prevention of neural tube defects. Proc Nutr Soc 53:631–636
11. Basu TK, Dickerson JWT (1996) Vitamin C (ascorbic acid). In: Basu TK, Dickerson JWT (eds) Vitamins in health and disease. CAB International, Oxford, UK, pp 125–147
12. WHO (2003) Battling iron deficiency anemia: the challenge. WHO Press, Geneva
13. Schümann K (2001) Safety aspects of iron in food. Ann Nutr Metab 45:91–101

14. Maret W, Sandstead HH (2006) Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 20:3–18
15. WHO (2004) Iodine status worldwide: WHO global database on iodine deficiency. WHO Press, Geneva
16. Lyons GH, Stangoulis JCR, Graham RD (2004) Exploiting micronutrient interaction to optimize biofortification programs: The case for inclusion of selenium and iodine in the HarvestPlus Program. Nutr Rev 62:247–252
17. Lyons GH, Lewis J, Lorimer MF, Holloway RE, Brace DM, Stangoulis JCR, Graham RD (2004) High-selenium wheat: agronomic biofortification strategies to improve human nutrition. Food Agric Environ 2:171–178
18. Combs GF (2001) Selenium in global food system. Br J Nutr 85:517–547
19. Shrimpton R, Schultink W (2002) Can supplements help meet the micronutrient needs of the developing world? Proc Nutr Soc 61:223–229
20. WHO/WFP/UNICEF (2007) Preventing and controlling micronutrient deficiencies in populations affected by an emergency. Joint statement by the World Health Organization, the World Food Programme and the United Nations Children's Fund. WHO Press, Geneva
21. Allen LH, Gillespie SR (2001) What works? A review of the efficacy and effectiveness of nutrition interventions. ACC/SCN/Asian Development Bank, Geneva/Manila
22. Black RE (2003) Zinc deficiency, infectious disease and mortality in the developing world. J Nutr 133: 1485S–1489S
23. Gomez-Galera S, Rojas E, Sudhakar D, Zhu C, Pelacho AM, Capell T, Christou P (2010) Critical evaluation of strategies for mineral fortification of staple food crops. Transgenic Res 19:165–180
24. Underwood BA, Smitasiri S (1999) Micronutrient malnutrition: policies and programs for control and their implications. Annu Rev Nutr 19:303–324
25. UNICEF (2008) Sustainable elimination of iodine deficiency. UNICEF, New York
26. Sadighi J, Mohammad K, Shikholeslam R, Amirkhani MA, Torabi P, Salehi F, Abdolahi Z (2009) Anaemia control: lessons from the flour fortification programme. Public Health 123:794–799
27. Frossard E, Bucher M, Mächler F, Mozafar A, Hurrell R (2000) Potential for increasing the content and bioavailability of Fe, Zn and Ca in plants for human nutrition. J Sci Food Agric 80:861–879
28. Li Y, Diosady LL, Jankowski S (2008) Effect of iron compounds on the storage stability of multiple-fortified Ultra Rice®. Int J Food Sci Technol 43:423–429
29. Zimmermann MB, Wegmueller R, Zeder C, Chaouki N, Biebinger R, Hurrell RF, Windhab E (2004) Triple fortification of salt with microcapsules of iodine, iron, and vitamin A. J Clin Nutr 80:1283–1290
30. IZINCG (2007) Zinc fortification (technical brief 4). http://www.izincg.org/index.php
31. Nga TT, Winichagoon P, Dijkhuizen MA, Khan NC, Wasantwisut E, Furr H, Wieringa FT (2009) Decreased prevalence of anemia and improved micronutrient status and effectiveness of deworming in rural Vietnamese school children. J Nutr 139:1013–1021
32. Zhu C, Naqvi S, Gomez-Galera S, Pelacho AM, Capell T, Christou P (2007) Transgenic strategies for the nutritional enhancement of plants. Trends Plant Sci 12: 548–555
33. Jeong J, Guerinot ML (2008) Biofortified and bioavailable: The gold standard for plant-based diets. Proc Natl Acad Sci USA 105:1777–1778
34. Naqvi S, Zhu C, Farre G, Sandmann G, Capell T, Christou P (2010) Synergistic metabolism in hybrid corn reveals bottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid zeaxanthin. Plant Biotechnol J. doi:10.1111/j.1467-7652.2010.00554.x.
35. Stein AJ, Meenakshi JV, Qaim M, Nestel P, Sachdev HPS, Bhutta ZA (2008) Potential impacts of iron biofortification in India. Soc Sci Med 66:1797–1808
36. Cakmak I (2008) Enrichment of cereal grains with zinc: agronomic or genetic biofortification? Plant Soil 302:1–17
37. Welch RM, Graham RD (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. J Exp Bot 55:353–364
38. White PJ, Broadley MR (2005) Biofortifying crops with essential mineral elements. Trends Plant Sci 10:586–593
39. Wong JC, Lambert RJ, Wurtzel ET, Rocheford TR (2004) QTL and candidate genes phytoene synthase and zeta-carotene desaturase associated with the accumulation of carotenoids in maize. Theor Appl Genet 108:349–359
40. Santos CA, Simon PW (2002) QTL analyses reveal clustered loci for accumulation of major provitamin A carotenes and lycopene in carrot roots. Mol Genet Genomics 268: 122–129
41. Harjes CE, Rocheford TR, Bai L, Brutnell TP, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R, Williams M, Wurtzel ET, Yan J, Buckler ES (2008) Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319:330–333
42. Yan J, Kandianis CB, Harjes CE, Bai L, Kim EH, Yang X, Skinner DJ, Fu Z, Mitchell S, Li Q, Fernandez MG, Zaharieva M, Babu R, Fu Y, Palacios N, Li J, Dellapenna D, Brutnell T, Buckler ES, Warburton ML, Rocheford T (2010) Rare genetic variation at *Zea mays crtRB1* increases beta-carotene in maize grain. Nat Genet 42:322–327
43. Graham RD, Welch RM, Bouis HE (2001) Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. Adv Agron 70:77–142
44. White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol 182:49–84

45. Prasanna BM, Vasal SK, Singh NN (2001) Quality protein maize. Curr Sci 81:1308–1319
46. Zarkadas C, Hamilton R, Yu Z, Choi V, Khanizadeh S, Rose N, Pattison P (2000) Assessment of the protein quality of 15 new northern adapted cultivars of quality protein maize using amino acid analysis. J Agric Food Chem 48:5351–5361
47. Segal G, Song R, Messing J (2003) A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. Genetics 165:387–397
48. Huang S, Frizzi A, Florida C, Kriger D, Luethy M (2006) High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD α-zeins. Plant Mol Biol 61:525–535
49. Sindhu AS, Zheng ZW, Murai N (1997) The pea seed storage protein legumin was synthesized, processed and accumulated stably in transgenic rice endosperm. Plant Sci 130:189–196
50. Stöger E, Parker M, Christou P, Casey R (2001) Pea legumin overexpressed in wheat endosperm assembles into an ordered paracrystalline matrix. Plant Physiol 125:1732–1742
51. Jung R, Carl F (2000) Transgenic corn with an improved amino acid composition. In: Müntz K, Ulrich W (eds) The 8th international symposium on plant seeds. Institute of Plant Genetics and Crop Plant Research, Gatersleben
52. Yu J, Peng P, Zhang X, Zhao Q, Zhu D, Sun X, Liu J, Ao G (2004) Seed-specific expression of a lysine rich protein *sb401* gene significantly increases both lysine and total protein content in maize seeds. Mol Breed 14:1–7
53. Yang S, Moran D, Jia H, Bicar E, Lee M, Scott M (2002) Expression of a synthetic porcine α-lactoalbumin gene in the kernels of transgenic maize. Transgenic Res 11:11–20
54. Bicar E, Woodman-Clikeman W, Sangtong V, Peterson J, Yang S, Lee M, Scott M (2008) Transgenic maize endosperm containing a milk protein has improved amino acid balance. Transgenic Res 17:59–71
55. Wu XR, Kenzior A, Willmot D, Scanlon S, Chen Z, Topin A, He SH, Acevedo A, Folk WR (2007) Altered expression of plant lysyl tRNA synthetase promotes tRNA misacylation and translation recoding of lysine. Plant J 50:627–663
56. Muntz K, Christov V, Saalbach G, Saalbach I, Waddell D, Pickardt T, Schieder O, Wustenhagen T (1998) Genetic engineering for high methionine grain legumes. Nahrung 42:125–127
57. Chakraborty S, Chakraborty N, Datta A (2000) Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from *Amaranthus hypochondriacus*. Proc Natl Acad Sci USA 97:3724–3729
58. Chiaiese P, Ohkama-Ohtsu N, Molvig L, Godfree R, Dove H, Hocart C, Fujiwara T, Higgins TJ, Tabe LM (2004) Sulphur and nitrogen nutrition influence the response of chickpea seeds to an added, transgenic sink for organic sulphur. J Exp Bot 55:1889–1901
59. Pastorello EA, Pompei C, Pravettoni V, Brenna O, Farioli L, Trambaioli C, Conti A (2001) Lipid transfer proteins and 2S albumins as allergens. Allergy 56:45–47
60. Rascón-Cruz Q, Sinagawa-García S, Osuna-Castro JA, Bohorova N, Paredes-Lopez O (2004) Accumulation, assembly, and digestibility of amarantin expressed in transgenic tropical maize. Theor Appl Genet 108:335–342
61. Tamás C, Kisgyorgy BN, Rakszegi M, Wilkinson MD, Yang MS, Láng L, Tamás L, Bedo Z (2009) Transgenic approach to improve wheat (*Triticum aestivum* L.) nutritional quality. Plant Cell Rep 28:1085–1094
62. Zhang P, Jaynes JM, Potrykus I, Gruissem W, Puonti-Kaerlas J (2003) Transfer and expression of an artificial storage protein (*asp1*) gene in cassava (*Manihot esculenta* Crantz). Transgenic Res 12:243–250
63. Galili G, Höfgen R (2002) Metabolic engineering of amino acids and storage proteins in plants. Metab Eng 4:3–11
64. Frizzi A, Huang S, Gilbertson LA, Armstrong TA, Luethy MH, Malvar TM (2008) Modifying lysine biosynthesis and catabolism in corn with a single bifunctional expression/silencing transgene cassette. Plant Biotechnol J 6:13–21
65. Falco SC, Guida T, Locke M, Mauvais J, Sandres C, Ward RT, Webber P (1995) Transgenic canola and soybean seeds with increased lysine. Biotechnol (NY) 13:577–582
66. Mazur B, Krebbers E, Tingey S (1999) Gene discovery and product development for grain quality traits. Science 285:372–375
67. Karchi H, Shaul O, Galili G (1994) Lysine synthesis and catabolism are coordinately regulated during tobacco seed development. Proc Natl Acad Sci USA 91:2577–2581
68. Karchi H, Miron D, Ben-Yaacob S, Galili G (1995) The lysine-dependent stimulation of lysine catabolism in tobacco seeds requires calcium and protein phosphorylation. Plant Cell 7:1963–1970
69. Zhu X, Galili G (2003) Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also transregulates the metabolism of other amino acids in Arabidopsis seeds. Plant Cell 15:845–853
70. Huang S, Kruger DE, Frizzi A, D'Ordine RL, Florida CA, Adams WR, Brown WE, Luethy MH (2005) High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation. Plant Biotechnol J 3:555–569
71. Houmard NM, Mainville JL, Bonin CP, Huang S, Luethy MH, Malvar TM (2007) High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. Plant Biotechnol J 5:605–614
72. Hacham Y, Avraham T, Amir R (2002) The N-terminal region of Arabidopsis cystathionine gamma-synthase plays an important regulatory role in methionine metabolism. Plant Physiol 128:454–462
73. Hacham Y, Schuster G, Amir R (2006) An in vivo internal deletion in the N-terminus region of Arabidopsis cystathionine gamma-synthase results in CGS expression that is insensitive to methionine. Plant J 45:955–967
74. Hacham Y, Matityahu I, Schuster G, Amir R (2008) Overexpression of mutated forms of aspartate kinase and cystathionine gamma-synthase in tobacco leaves resulted in the high accumulation of methionine and threonine. Plant J 54:260–271

75. Cho HJ, Brotherton JE, Song HS, Widholm JM (2000) Increasing tryptophan synthesis in a forage legume *Astragalus sinicus* by expressing the tobacco feedback-insensitive anthranilate synthase (*ASA2*) gene. Plant Physiol 123:1069–1076
76. Wakasa K, Hasegawa H, Nemoto H, Matsuda F, Miyazawa H, Tozawa Y, Morino K, Komatsu A, Yamada T, Terakawa T, Miyagawa H (2006) High-level tryptophan accumulation in seeds of transgenic rice and its limited effects on agronomic traits and seed metabolite profile. J Exp Bot 57: 3069–3078
77. Yamada T, Tozawa Y, Hasegawa H, Terakawa T, Ohkawa Y, Wakasa K (2004) Use of a feedback-insensitive a subunit of anthranilate synthase as a selectable marker for transformation of rice and potato. Mol Breed 14:363–373
78. Ishimoto M, Rahman SM, Hanafy MS, Khalafalla MM, El-Shemy HA, Nakamoto Y, Kita Y, Takanashi K, Matsuda F, Murano Y, Funadashi T, Miyagawa H, Wakasa K (2010) Evaluation of amino acid content and nutritional quality of transgenic soybean seeds with high-level tryptophan accumulation. Mol Breed 25:313–326
79. Inaba Y, Brotherton JE, Ulanov A, Widholm JM (2007) Expression of a feedback insensitive anthranilate synthase gene from tobacco increases free tryptophan in soybean plants. Plant Cell Rep 26:1763–1771
80. Tabe L, Wirtz M, Molvig L, Droux M, Hell R (2009) Overexpression of serine acetlytransferase produced large increases in O-acetylserine and free cysteine in developing seeds of a grain legume. J Exp Bot 61:721–733
81. Goyens P, Spilker M, Zock P, Katan M, Mensink R (2005) Compartmental modeling to quantify γ-linolenic acid conversion after longer term intake of multiple tracer boluses. J Lipid Res 46:1474–1483
82. Napier JA (2007) The production of unusual fatty acids in transgenic plants. Annu Rev Plant Biol 58:295–319
83. Robert SS (2005) Production of eicosapentaenoic and docosahexaenoic acid-containing oils in transgenic land plants for human and aquaculture nutrition. Mar Biotechnol 8:103–109
84. Truksa M, Vrinten P, Qiu X (2009) Metabolic engineering of plants for polyunsaturated fatty acid production. Mol Breed 23:1–11
85. Wu G, Truksa M, Datla N, Vrinten P, Bauer J, Zank T, Cirpus P, Heinz E, Qiu X (2005) Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants. Nat Biotechnol 23:1013–1017
86. Kajikawa M, Matsui D, Ochiai M, Tanak Y, Kita Y, Ishimoto M, Kohzu Y, Shoji S, Yamato KT, Ohyama K, Fukuzawa H, Kohchi T (2008) Production of arachidonic and eicosapentaenoic acids in plants using bryophyte fatty acid Δ6-desaturase, Δ6-elongase and Δ5-desaturase genes. Biosci Biotechnol Biochem 72:435–444
87. Kinney AJ, Cahoon EB, Damude HG, Hitz WD, Kolar CW, Liu Z-B (2004) Production of very long chain polyunsaturated fatty acids in oilseed plants. WO 2004/071467 A2
88. Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, Napier JA, Stobart AK, Lazarus CM (2004) Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. Nat Biotechnol 22:739–745
89. Eisenreich W, Schwarz M, Cartavrade A, Arigoni D, Zenk MH, Bacher A (1998) The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. Chem Biol 5:221–233
90. Zhu C, Naqvi S, Capell T, Christou P (2009) Metabolic engineering of ketocarotenoid biosynthesis in higher plants. Arch Biochem Biophys 483:182–190
91. Enfissi E, Fraser P, Lois L, Boronat A, Schuch W, Bramley P (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol J 3:17–27
92. Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305
93. Paine J, Shipton C, Chaggar S, Howells R, Kennedy M, Vernon G, Wright S, Hinchliffe E, Adams J, Silverstone A, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23:482–487
94. Aluru M, Xu Y, Guo R, Wang Z, Li S, White W, Wang K, Rodermel S (2008) Generation of transgenic maize with enhanced provitamin A content. J Exp Bot 59:3551–3562
95. Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in corn. Proc Natl Acad Sci USA 105:18232–18237
96. Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Conesa D, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106:7762–7767
97. Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412
98. Ravanello M, Dangyang K, Alvarez J, Huang B, Shewmaker C (2003) Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. Metab Eng 5:255–263
99. Fujisawa M, Takita E, Harada H, Sakurai N, Suzuki H, Ohyama K, Shibata D, Misawa N (2009) Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. J Exp Bot 60:1319–1332
100. Diretto G, Welsch R, Tavazza MF, Pizzichini D, Beyer P, Giuliano G (2007) Silencing of beta-carotene hydroxylase increases total carotenoid and beta-carotene levels in potato tubers. BMC Plant Biol 7:11
101. Diretto G, Al-Babili S, Tavazza R, Papacchioli V, Beyer P, Giuliano G (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. PLoS ONE 2:e350

102. Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413–419
103. D'Ambrosio C, Giorio G, Marino I, Merendino A, Petrozza A, Salfi L, Stigliani A, Cellini F (2004) Virtually complete conversion of lycopene into beta-carotene in fruits of tomato plants transformed with the tomato *lycopene beta-cyclase* (t*lcy-b*) cDNA. Plant Sci 166:207–214
104. Dharmapuri S, Rosati C, Pallara P, Aquilani R, Bouvier F, Camara B, Giuliano G (2002) Metabolic engineering of xanthophylls content in tomato fruits. FEBS Lett 519:30–34
105. Apel W, Bock R (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene to provitamin A conversion. Plant Physiol 151:59–66
106. Wurbs D, Ruf S, Bock R (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. Plant J 49:276–288
107. Davuluri GR, van Tuinen A, Fraser PD, Manfredonia A, Newman R, Burgess D, Brummell DA, King SR, Palys J, Uhlig J, Bramley PM, Pennings HM, Bowler C (2005) Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. Nat Biotechnol 23:890–895
108. Li L, Paolillo DJ, Parthasarathy MV, Dimuzio EM, Garvin DF (2001) A novel gene mutation that confers abnormal patterns of beta-carotene accumulation in cauliflower (*Brassica oleracea* var. botrytis). Plant J 26:59–67
109. Lu S, Eck J, Zhou X, Lopez A, O'Halloran D, Cosman K, Conlin B, Paolillo D, Garvin D, Vrebalov J, Kochian L, Küpper H, Earle E, Cao J, Lia L (2006) The cauliflower *Or* gene encodes a DNAJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. Plant Cell 18:3594–3605
110. Lopez AB, Eck JV, Conlin BJ, Paolillo DJ, O'neill J, Li L (2008) Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. J Exp Bot 59:213–223
111. DellaPenna D, Pogson B (2006) Vitamin synthesis in plants: tocopherols and carotenoids. Annu Rev Plant Biol 57: 711–773
112. Collakova E, DellaPenna D (2003) The role of homogentisate phytyltransferase and other tocopherol pathway enzymes in the regulation of tocopherol synthesis during abiotic stress. Plant Physiol 133:930–940
113. Tsegaye Y, Shintani D, DellaPenna D (2002) Overexpression of the enzyme p-hydroxyphenylpyruvate dioxygenase in Arabidopsis and its relation to tocopherol biosynthesis. Plant Physiol Biochem 40:913–920
114. Falk J, Brosch M, Schäfer A, Braun S, Krupinska K (2005) Characterization of transplastomic tobacco plants with a plastid localized barley 4-hydroxyphenylpyruvate dioxygenase. J Plant Physiol 162:738–742
115. Cho EA, Lee CA, Kim YA, Baek SH, Reyes BG, Yun SJ (2005) Expression of γ-tocopherol methyltransferase transgene improves tocopherol composition in lettuce (*Latuca sativa* L.). Mol Cells 19:16–22
116. Raclaru M, Gruber J, Kumar R, Sadre R, Luhs W, Zarhloul K, Friedt W, Frentzen M, Weier D (2006) Increase of the tocochromanol content in transgenic *Brassica napus* seeds by overexpression of key enzymes involved in prenylquinone biosynthesis. Mol Breed 18:93–107
117. Rippert P, Scimemi C, Dubald M, Matringe M (2004) Engineering plant shikimate pathway for production of tocotrienol and improving herbicide resistance. Plant Physiol 134:92–100
118. Karunanandaa B, Qi Q, Haoa M, Baszis S, Jensen P, Wong Y, Jiang J, Venkatramesh M, Gruys K, Moshiri F, Beittenmiller D, Weiss J, Valentin H (2005) Metabolically engineered oilseed crops with enhanced seed tocopherol. Metab Eng 7:384–400
119. Van Eenennaam A, Lincoln K, Durrett T, Valentin H, Shewmaker C, Thorne G, Jiang J, Baszis S, Levering C, Aasen E, Hao M, Stein J, Norris S, Last R (2003) Engineering vitamin E content: from Arabidopsis mutant to soy oil. Plant Cell 15:3007–3019
120. Tavva V, Kim Y, Kagan I, Dinkins R, Kim K, Collins G (2007) Increased α-tocopherol content in soybean seed overexpressing the *Perilla frutescens* γ-tocopherol methyltransferase gene. Plant Cell Rep 26:61–70
121. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365–369
122. Ishikawa T, Dowdle J, Smirnoff N (2006) Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. Physiol Plant 126:343–355
123. Chen Z, Young T, Ling J, Chang S, Gallie D (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc Natl Acad Sci USA 100:3525–3530
124. Jain AK, Nessler CL (2000) Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. Mol Breed 6:73–78
125. Badejo A, Eltelib H, Fukunaga K, Fujikawa Y, Esaka M (2009) Increase in ascorbate content of transgenic tobacco plants overexpressing the acerola (*Malpighia glabra*) phosphomannomutase gene. Plant Cell Physiol 50:423–428
126. Laing WA, Wright MA, Cooney J, Bulley SM (2007) The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. Proc Natl Acad Sci USA 104:9534–9539
127. Bulley SM, Rassam M, Hoser D, Otto W, Schünemann N, Wright M, MacRae E, Gleave A, Laing W (2009) Gene expression studies in kiwifruit and gene over-expression in Arabidopsis indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. J Exp Bot 60:765–778
128. Díaz de la Garza RI, Quinlivan EP, Klaus SM, Basset GJ, Gregory JF III, Hanson AD (2004) Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. Proc Natl Acad Sci USA 101:13720–13725
129. Díaz de la Garza R, Quinlivan EP, Klaus SM, Basset GJ, Gregory JF, Hanson AD (2007) Folate biofortification in

tomatoes by engineering the pteridine branch of folate synthesis. Proc Natl Acad Sci USA 104:4218–4222

130. Storozhenko S, De Brouwer V, Volckaert M, Navarrete O, Blancquaert D, Zhang GF, Lambert W, Van Der Straeten D (2007) Folate fortification of rice by metabolic engineering. Nat Biotechnol 25:1277–1279
131. Fouad W, Rathinasabapathi B (2006) Expression of bacterial L-aspartate-α-decarboxilase in tobacco increases β-alanine and pantothenate levels and improves thermotolerance. Plant Mol Biol 60:495–505
132. Chakauya E, Coxon K, Wei M, MacDonald M, Barsby T, Abell C, Smith A (2008) Towards engineering increased pantothenate (vitamin B5) levels in palnts. Plant Mol Biol 68:493–503
133. Ishimaru Y, Suzuki M, Tsukamoto T, Suzuki K, Nakazono M, Kobayashi T, Wada Y, Watanabe S, Matsuhashi S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2006) Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. Plant J 45:335–346
134. Ghandilyan A, Vreugdenhil D, Aarts MGM (2006) Progress in the genetic understanding of plant iron and zinc nutrition. Physiol Plant 126:407–417
135. Takahashi M, Nakanishi H, Kawasaki S, Nishizawa N, Mori S (2001) Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. Nat Biotechnol 19:466–469
136. Takahashi M, Terada Y, Nakai I, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK (2003) Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. Plant Cell 15:1263–1280
137. Ramesh SA, Choimes S, Schachtman DP (2004) Over-expression of an Arabidopsis zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content. Plant Mol Biol 54:373–385
138. Tiong J, Genc Y, McDonald GK, Langridge P, Huang CY (2009) Over-expressing a barley ZIP gene doubles grain zinc content in barley (*Hordeum vulgare*). In: Proceedings of the international plant nutrition colloquium XVI. UC Davis, California
139. Van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JA, Hooykaas PJ (1999) Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiol 119:1047–1055
140. Sors TG, Ellis DR, Salt DE (2005) Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynth Res 86:373–389
141. Pilon-Smits EAH, Hwang S, Lytle CM, Zhu Y, Tai JC, Bravo RC, Chen Y, Leustek T, Terry N (1999) Overexpression of ATP sulfurylase in Indian mustard leads to increased selenate uptake, reduction and tolerance. Plant Physiol 119:123–132
142. Terry N, Zayed AM, de Souza MP, Tarun AS (2000) Selenium in higher plants. Annu Rev Plant Physiol Plant Mol Biol 51:401–432
143. LeDuc DJ, AbdelSamie M, Móntes-Bayon M, Wu CP, Reisinger SJ, Terry N (2006) Overexpressing both ATP sulfurylase and selenocysteine methyltransferase enhances selenium phytoremediation traits in Indian mustard. Environ Pollut 144:70–76
144. McKenzie MJ, Hunter DA, Pathirana R, Watson LM, Joyce NI, Matich AJ, Rowan DD, Brummell DA (2009) Accumulation of an organic anticancer selenium compound in a transgenic Solanaceous species shows wider applicability of the selenocysteine methyltransferase transgene from selenium hyperaccumulators. Transgenic Res 18:407–424
145. Mei H, Zhao J, Pittman JK, Lachmansingh J, Park S, Hirschi KD (2007) In planta regulation of the Arabidopsis Ca^{2+}/H^{+} antiporter CAX1. J Exp Bot 58:3419–3427
146. Park S, Kim CK, Pike LM, Smith RH, Hirschi KD (2004) Increased calcium in carrots by expression of an Arabidopsis H^{+}/Ca^{2+} transporter. Mol Breed 14:275–282
147. Park S, Kang TS, Kim CK, Han JS, Kim S, Smith RH, Pike LM, Hirschi KD (2005) Genetic manipulation for enhancing calcium content in potato tuber. J Agric Food Chem 53:5598–5603
148. Connolly EL (2008) Raising the bar for biofortification: enhanced levels of bioavailable calcium in carrots. Trends Biotechnol 26:401–403
149. Park S, Elless MP, Park J, Jenkins A, Lim W, Chambers E IV, Hirschi KD (2009) Sensory analysis of calcium-biofortified lettuce. Plant Biotechnol J 7:106–117
150. Kim CK, Han JS, Lee HS, Oh JY, Shigaki T, Park SH, Hirschi K (2006) Expression of an *Arabidopsis CAX2* variant in potato tubers increases calcium levels with no accumulation of manganese. Plant Cell Rep 25:1226–1232
151. Sellappan K, Datta K, Parkhi V, Datta SK (2009) Rice caryopsis structure in relation to distribution of micronutrients (iron, zinc, beta-carotene) of rice cultivars including transgenic indica rice. Plant Sci 177:557–562
152. Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seeds by the soybean ferritin gene. Nat Biotechnol 17:282–286
153. Drakakaki G, Christou P, Stöger E (2000) Constitutive expression of soybean ferritin cDNA in transgenic wheat and rice results in increased iron levels in vegetative tissues but not in seeds. Transgenic Res 9:445–452
154. Qu LQ, Yoshihara T, Ooyama A, Goto F, Takaiwa F (2005) Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. Planta 222:225–233
155. Vasconcelos M, Datta K, Oliva N, Khalekuzzaman M, Torrizo L, Krishnan S, Oliveira M, Goto F, Datta SK (2003) Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. Plant Sci 164:371–378
156. Hong-Xia Y, Mei L, Ze-Jian G, Qing-Yao S, Xiao-Hui X, Jin-Song B (2008) Evaluation and application of two high-iron transgenic rice lines expressing a pea ferritin gene. Rice Sci 15:51–56
157. Wirth J, Poletti S, Aeschlimann B, Yakandawal N, Drosse B, Osorio S, Tohge T, Fernie AR, Günther D, Gruissem W, Sautter C (2009) Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. Plant Biotechnol J 7:631–644

158. Theil EC, Briat JF (2004) Plant ferritin and non-heme iron nutrition in humans, HarvestPlus technical monographs 1. International Food Policy Research Institute/International Center for Tropical Agriculture (CIAT), Washington, DC/California
159. Karunaratne AM, Amerasinghe PH, Sadagopa Ramanujam VM, Sandstead HH, Perera PAJ (2008) Zinc, iron and phytic acid levels of some popular foods consumed by rural children in Sri Lanka. J Food Compos Anal 21:481–488
160. Welch RM, Graham RD (2005) Agriculture: the real nexus for enhancing bioavailable micronutrients in food crops. J Trace Elem Med Biol 18:299–307
161. Raboy V (2002) Progress in breeding low phytate crops. J Nutr 132:503S–505S
162. Liu QL, Xu XH, Ren XL, Fu HW, Wu DX, Shu QY (2007) Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.). Theor Appl Genet 114:803–814
163. Campion B, Sparvoli F, Doria E, Tagliabue G, Galasso I, Fileppi M, Bollini R, Nielsen E (2009) Isolation and characterization of an *lpa* (low phytic acid) mutant in common bean (*Phaseolus vulgaris* L.). Theor Appl Genet 118:1211–1221
164. Morris J, Nakata PA, McConn M, Brock A, Hirschi KD (2007) Increased calcium bioavailability in mice fed genetically engineered plants lacking calcium oxalate. Plant Mol Biol 64:613–618
165. Brinch-Pedersen H, Hatzack F, Sørensen LD, Holm PB (2003) Concerted action of endogenous and heterologous phytase on phytic acid degradation in seed of transgenic wheat (*Triticum aestivum* L.). Transgenic Res 12:649–659
166. Brinch-Pedersen H, Hatzack F, Stöger E, Arcalis E, Pontopidan K, Holm PB (2006) Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): deposition pattern, thermostability, and phytate hydroslysis. J Agric Food Chem 54:4624–4632
167. Chen R, Xue G, Chen P, Yao B, Yang W, Ma Q, Fan Y, Zhao Z, Tarczynski MC, Shi J (2008) Transgenic maize plants expressing a fungal phytase gene. Transgenic Res 17:633–643
168. Lucca P, Hurrell R, Potrykus I (2002) Fighting iron deficiency anemia with iron-rich rice. J Am Coll Nutr 21:184S–190S
169. Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, Christou P, Stöger E (2005) Endosperm specific co-expression of recombinant soybean ferritin and *Aspergillus* phytase in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 59:869–880
170. Halpin C, Barakate A, Askari BM, Abbott JC, Ryan MD (2001) Enabling technologies for manipulating multiple genes on complex pathways. Plant Mol Biol 47:295–310
171. Farré G, Sanahuja G, Naqvi D, Bai C, Capell T, Zhu C, Christou P (2010) Travel advice on the road to carotenoids in plants. Plant Sci 179:28–48
172. Bekaert S, Storozhenko S, Mehrshahi P, Bennett MJ, Lambert W, Gregory JF III, Schubert K, Hugenholtz J, Van Der Straeten D, Hanson AD (2008) Folate biofortification in food plants. Trends Plant Sci 13:28–35

Books and Reviews

Lemaux PG (2008) Genetically engineered plants and foods: a scientist's analysis of the issues (Part I). Annu Rev Plant Biol 59:771–812

Lemaux PG (2009) Genetically engineered plants and foods: a scientist's analysis of the issues (part II). Annu Rev Plant Biol 60:511–559

Mène-Saffrané L, DellaPenna D (2009) Biosynthesis, regulation and functions of tocochromanols in plants. Plant Physiol Biochem 48:301–309

Rosati C, Diretto G, Giuliano G (2009) Biosynthesis and engineering of carotenoids and apocarotenoids in plants: state of the art and future prospects. Biotechnol Genet Eng Rev 26:139–162

Breeding in Beef Cattle

Matthew L. Spangler
Department of Animal Science, University of Nebraska, Lincoln, NE, USA

Article Outline

Glossary

Beef Improvement Federation (BIF) accuracy A metric of accuracy that is more conservative than true accuracy. It is linearly related to prediction error variance.

Bio-economic index A collection of EPD that are relevant to a breeding objective whereby each EPD is multiplied by an economic weight.

Composite A crossbred animal. Generally thought of in terms of a pedigreed seedstock animal that is a cross of two or more breeds.

Expected progeny difference Equivalent to half of a breeding value.

Molecular breeding value The sum of marker effects multiplied by the number of copies of a given marker.

Definition of the Subject

The beef cattle industry represents a diverse and unique sector of animal agriculture with varying breeds, production climates, and marketing objectives. The industry is not vertically integrated and thus breeding and selection decisions are controlled by individual farmers and ranchers. In the United States, the average herd size is less than 30 cows. However, there exists sound tools from which to make genetic selection decisions to ultimately make genetic change and improve profitability. These tools, some of which have been utilized for decades, are available to all beef cattle producers. The utilization of current genetic selection tools can aid in the profitability and ultimately the sustainability of beef enterprises.

Introduction

The beef cattle industry is comprised of seedstock and commercial producers. In general, genetic improvement, or accumulation of breeding value, occurs in the seedstock sector and flows to the commercial industry via the purchase of bulls and/or semen. Less than 10% of producers in the United States utilize artificial insemination (AI). Selection decisions can be made based on a plethora of information, but the most informative are breeding values or expected progeny differences (EPDs) in the US and economic index values. There are several EPD and index values across multiple breeds.

There have also been an increasing number of commercially available genomic tests made available for multiple traits. Some of these have undergone independent validation, while others have not. This technology holds the promise to increase the rate of genetic progress and to allow for selection on those traits that are expensive or challenging to measure routinely. However, there still exist several caveats to solve before this is completely brought to fruition.

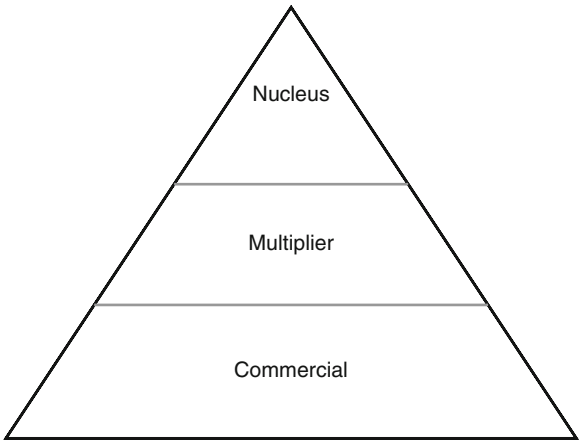
Sustainability from a beef perspective will depend on economics and consumer perception. Genetic selection will need to focus on tools that aim solely at the genetic component of phenotypes and tools that apply economic parameters need to be quantified such that a return on investment can be determined. Consumer demand will ultimately be dictated by price and product quality suggesting that efficient genetic selection is needed and that selection tools need to evolve to improve end-product quality.

Structure of the Beef Industry

In other species, such as swine and poultry, the breeding pyramid is much more clearly defined than in

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Breeding in Beef Cattle. Figure 1
The breeding pyramid representing the three broad segments of the beef industry

beef cattle given formalized breeding companies. However, it still exists in beef cattle (Fig. 1) as some purebred producers have a much larger influence over the gene pool of a breed compared to others. At the nucleus level, animals (particularly sires) are produced for use at the multiplier level although some nucleus animals are sold directly to commercial herds. The nucleus herds are the drivers of genetic change. The multiplier herds, as the name implies, multiply genes from the nucleus populations to produce animals for use in the commercial sector. It is possible, and does occur, for animals from multipliers to enter into nucleus herds.

There are four main pathways from which herds can influence the rate and direction of genetic change: producing sires of sires, sires of dams, dams of sires, and dams of dams. The most influential is sires of sires. As an example, Marquez et al. [1] characterized the population structure of the Red Angus breed and found that indeed it could be divided into nucleus and multiplier herds and that only 30% of the total herds produced sires of sires.

Basic Principles of Genetic Improvement

In order to make genetic improvement the target trait (s), observable or measureable characteristics of an animal, must display variation. The goal of genetic improvement is to improve the phenotype, observed category or measured level of performance for a trait, by improving the genotype or genetic makeup of an animal to best fit the system and to make the best product for consumers. The phenotype (P) of an animal is comprised of both genetic (G) and nongenetic factors such as the environment (E) such that

$$P = G + E$$

These two primary factors, G and E, can be further divided into additive (A), dominance (D), and epistatic (I) genetic effects and both permanent (E_P) and temporary (E_T) environmental effects such that

$$P = G_A + G_D + G_I + E_P + E_T$$

Temporary genetic effects can be thought of as differences in feeding regime or climate and are generally accounted for by contemporary groups whereas PE are long lasting changes that influence future performance (i.e., the loss of one-quarter of the udder). Contemporary groups are considered to be a group of animals that have been subjected to the same environmental effects or managed alike such as being born in the same year, season, and herd, being of the same sex, and fed the same diet.

Trait Types

There are two broad categories of traits, simply inherited and polygenic traits. Simple traits are expressed qualitatively, controlled by only one or two genes, and are typically influenced little by environment. In contrast, polygenic traits are controlled by many genes, are greatly influenced by the environment, and are quantitative in nature (i.e., they are expressed numerically or on a continuous scale).

Simple Traits

In beef cattle, the phenotype of horns is controlled by dominance wherein the horned allele is recessive. If a heterozygous male is mated to a heterozygous female the resulting calf has a 25% chance of being

horned and a 75% chance of being polled (absence of horns). This is illustrated in the Punnett Square below where P denotes the polled allele, and p represents the horned allele.

	P	p
P	PP	Pp
p	pP	pp

Black and red coat color is controlled in the same manner where black is dominant to red such that homozygous (BB) and heterozygous (Bb) animals are black and only homozygous animals for the recessive allele (bb) are red.

Similarly, many genetic defects in cattle are also recessive in nature. A list of known genetic defects in beef cattle can be found in Table 1. Use the example of Neuropathic Hydrocephalus (NH), a recessive lethal mutation found in Angus cattle. If a heterozygous bull (Nn) is mated to a homozygous normal female (NN) then the resulting offspring would be 100% normal (i.e., not afflicted) with 50% of them being homozygous and the other 50% heterozygous.

	N	N
N	NN	NN
n	nN	nN

Coat color in shorthorn cattle is controlled by co-dominance. There are three possible phenotypes that can result from the pairing of two alleles, red (R) and white (W). When two R or two W alleles pair together the resulting animals is either red (RR) or white (WW), respectively. However, the heterozygous animals (RW or WR) or an intermediate color, a mixture of both red and white called "roan." The example below illustrates the genotypes and phenotypes that would be expected from the mating of a roan bull to a roan cow. In this example there is a 25% chance the calves will be red, 50% chance they will be roan, and 25% chance the will be white.

	R	W
R	RR	RW
W	WR	WW

Sex-influenced traits have different expressions in different sexes given the same genotype. A classic example of a sex-influenced trait in beef cattle is the presence

Breeding in Beef Cattle. Table 1 Genetic defects currently monitored by US breed associations

Genetic abnormality	Primary breed(s) of incidence	Lethal or nonlethal	Mode of inheritance	DNA test available
Alpha (α)-mannosidosis	Red Angus	Lethal	Simple recessive	Yes
Arthrogryposis multiplex (AM)	Angus and derivatives	Lethal	Simple recessive	Yes
Beta (β)-mannosidosis	Salers	Lethal	Simple recessive	Yes
Fawn calf syndrome (FCS)	Angus	Nonlethal	Simple recessive	Yes
Neuropathic hydrocephalus (NH)	Angus	Lethal	Simple recessive	Yes
Hypotrichosis (hairless calf)	Hereford	Nonlethal	Simple recessive	No
Idiopathic epilepsy	Hereford	Nonlethal	Simple recessive	Yes
Osteopetrosis	Angus and Red Angus	Lethal	Simple recessive	Yes
Protoporphyria	Limousin	Nonlethal	Simple recessive	Yes
Pulmonary hypoplasia and anasarca (PHA)	Maine-Anjou and Shorthorn	Lethal	Simple recessive	Yes
Tibial hemimelia (TH)	Shorthorn and Maine-Anjou	Lethal	Simple recessive	Yes

or absence of scurs. Here the scured allele (Sc) would appear to be dominant in males but recessive in females.

Males	Females
SS = no scurs	SS = no scurs
SSc = scurs	SSc = no scurs
ScSc = scurs	ScSc = scurs

Polygenic Traits

The number of gametes that an individual can produce is equal to 2^n where n is the number of heterozygous loci. The number of possible genotypes that can be produced from any one mating is equal to $3^n \times 2^m$ where n is the number of loci where both parents are heterozygous and m is the number of loci where only one parent is heterozygous. From this, it is obvious that from a single mating there can arise numerous possible genotypes.

	Individual heterozygous at every loci	Both individual heterozygous
Number of genes	Number of gametes	Number of genotypes
1	2 (2^1)	3 (3^1)
2	4 (2^2)	9 (3^2)
10	1,024 (2^{10})	54,049 (3^{10})

Basics of an EPD

A breeding value (BV) is cumulative additive value of an animal. A transmitting ability (TA) is the average of gametes an individual passes to offspring.

$$TA = 1/2BV$$

In beef cattle, a TA is referred to as an expected progeny difference (EPD).

EPDs allow for the comparison of animals within a breed for their genetic potential as parents for a given trait. EPDs have existed in the beef industry for decades and their use has produced intended genetic change in many traits.

Many traits (e.g., weaning weight, yearling weight (YW), ultrasound measurements, etc.) must be recorded within certain age windows (ranges when it is acceptable to measure animals). Animals measured outside of defined age windows will not have their own record incorporated into an EPD calculation. This allows for a fair comparison of animals. Specific age windows can be found on the corresponding breed association Web site. Records are then adjusted to a constant endpoint, most generally age (Table 2).

Breeding in Beef Cattle. Table 2 Beef Improvement Federation (BIF) standard adjustment factors for birth and weaning weight

AOD	Birth weight	Male	Female
2	+8	+60	+54
3	+5	+40	+36
4	+2	+20	+18
5–10	0	0	0
11 and older	+3	+20	+18

Too often, seedstock producers and bull buyers focus on actual weights and ultrasound data when selecting sires. Expected progeny differences provide a measure by which animals within a breed can be compared to one another for their genetic potential as parents for specific traits. EPDs incorporate multiple sources of information, including full pedigree, an animal's own record, and progeny information. As additional sources of information become available, the accuracy of the EPD value increases. Prior to a National Cattle Evaluation (NCE), animals are given interim EPDs. During a genetic evaluation, all pedigree information would be included.

Pedigree estimate:

$$\text{Sire EPD} = 0.20 \quad \text{Dam EPD} = 0.10$$

$$\searrow \qquad \swarrow$$

$$\text{Progeny EPD} = \left(\frac{0.20 + 0.10}{2}\right) = 0.15$$

Pedigree estimate + animal record:

$$EPD_I = (0.5 * \text{EPD}_S) + (0.5 * \text{EPD}_D) + (0.5 * \phi)$$

Where EPD_I is the EPD for some individual I, EDP_S is the EPD for the sire of animal I, EPD_D is the EPD for the dam of animal I, and ϕ is the Mendelian Sampling

effect. The phenomena of Mendelian sampling arises due to the fact that each parent passes a sample half of its alleles to its offspring and every allele has an equal likelihood of being passed on. This effect can be quantified using contemporary group deviations and is a measure of how much better or worse an animal is compared to the average of his parents. One could envision a scenario where an animal could receive only the most desirable alleles from both parents resulting in a favorably large Mendelian sampling effect or the exact opposite which could result in an unfavorably large sampling effect. Perhaps the best example is a set of flush mates. Although all of them have the same pedigree estimate, they differ considerably in terms of performance and consequently their EPD, once they have a record, differ due to Mendelian sampling.

When using EPD it is important to understand that the role of EPD is to provide a measure of comparison within a breed. To compare animals across breeds, estimates from the US Meat Animal Research Center (MARC) can aid in determining differences between EPD of different breeds (Table 3). These across breed adjustment factors, adjusted to an Angus basis, are updated annually and can be found at http://www.beefimprovement.org/proceedings.html.

Example:

If a Hereford bull has a birth weight EPD of 1.5 and a Simmental bull has a birth weight EPD of 1.0 these adjustment factors can be used to approximate what these two bulls birth weight EPD would be on an Angus basis so that they can be compared. On an Angus basis, the Hereford bull would have a birth weight EPD of 4.2 (1.5 + 2.7) and the Simmental bull would have a birth weight EPD of 6.4 (1.0 + 5.4). Therefore, it can be expected in this scenario that the Hereford bull would sire calves that are 2.2 lbs. lighter at birth. This table can be an effective tool to determine differences in weight and milk potential between major US breeds. This information can be used to help determine which breed(s) are better suited to different environments. For instance, in a low input environment, breeds with a negative adjustment factor for milk might be more desirable.

Breeding in Beef Cattle. Table 3 2008 Adjustment factors for comparison of expected progeny difference (EPD) across various breeds

Breed	Birth weight	Weaning weight	Yearling weight	Milk
Angus	0.0	0.0	0.0	0.0
Charolais	9.6	39.0	47.3	2.9
Gelbvieh	4.4	5.0	−22.4	7.0
Hereford	2.7	−2.9	−12.8	−15.3
Limousin	4.0	−3.8	−27.8	−11.9
Red Angus	2.8	−5.2	0.9	−3.9
Simmental	5.4	23.3	16.9	13.9

Source: Adapted from Kuehn et al. [2]. More breeds and more traits are available in the full results from the US Meat Animal Research Center

Breed Average and Percentile Ranks

Table 4 illustrates a percentile rank table. These will be different for every breed and will change yearly with the addition of new animals with performance information recorded. The 50th percentile represents breed average. If an animal is in the top 1% for a given trait then it can be said that 99 animals in a hundred are "worse" for that trait. Conversely, if an animal is in the 95th percentile then it can be said that 94 in 100 animals will be better than him/her for that trait. Knowledge of percentile table gives you an idea of how an individual ranks within a breed for a specific trait or index. However, it may not be beneficial to choose extreme animals. For instance, even though a sire might be in the top 1% of

Breeding in Beef Cattle. Table 4 Percentile rank

Top %	CED	BW	WW	YW	Milk
1	14	−2.5	67	117	34
5	11	−.9	59	105	30
10	10	−.1	55	99	28
20	9	.7	50	92	25
50	6	2.2	42	79	20
75	3	3.4	36	69	16
95	-2	5.2	26	50	10

CED calving ease direct, *BW* birth weight, *WW* weaning weight, *YW* yearling weight, *Milk* maternal milk (maternal component of weaning weight)

the breed for Milk, his Milk value may be too extreme for your production environment.

EPD Definitions

	BULL A	BULL B
Calving ease direct	10	6
Birth weight	+2.0	+3.5
Weaning weight direct	20	+22
Yearling weight	+40	+52
Yearling height	.3	.6
Milk	+3	−2
Maternal weaning weight	+13	+9
Gestation length	−.1	+1.1
Calving ease maternal	4	6
Mature daughter height	+.5	+1.0
Mature daughter weight	0	+30
Scrotal circumference	+.1	−.45
Heifer pregnancy	6	9
Carcass weight	+2.0	+20
Percent retail cuts	0	+.2
Marbling	0	−.3
Ribeye area	+.06	+1.6
Fat thickness	−.01	−.09
Tenderness	−.1	.1
Days to finish	15	10
Stayability	10	6
Maintenance energy	0	10
Docility	6	2

Calving ease direct – Bull A should have 4% more unassisted births from first-calf heifers than Bull B. While birth weight is an indicator of calving ease, it does not tell the whole story. Calving ease is an economically relevant trait. Producers should not use both birth weight and calving ease EPD together since the birth weight EPD is already used in the calculation of calving ease.

Birth weight – Bull B's calves would be on the average 1.5 lb heavier at birth. Normally, producers should select bulls for use on heifers that are at or less than the breed average for birth weight. Keep in mind that when crossing breeds, heterosis or hybrid vigor can increase birth weights 10–15% over a straight-bred average.

Weaning weight direct – Calves from Bull B should average 2 lb more on adjusted weaning weights because of additional growth. Because of the low accuracy associated with yearling bulls, the amount of emphasis placed on such a small difference should be limited. These EPDs are virtually the same even if the accuracies were high.

Yearling weight – Bull B's calves should average 12 lb heavier at 1 year of age.

Yearling height – Bull B's calves should be 0.3 in. taller on average at a year of age compared to the offspring of Bull A. Height measurements are taken at the hip. Height (the actual measurement and not the EPD), along with age, is used to calculate frame score.

Milk – Daughters from Bull A should produce calves that are 5 lb (the difference between +3 and −2) heavier at weaning. This is not a measure of pounds of milk but rather weaning weight due to milk production. This 5 lb, unlike the weaning weight figure attributed to growth from the bull, is the result of differences in the daughters' milk production and mothering ability. Excessively high milk levels in low input environments should be discriminated against due to increased nutrient requirements of cows.

Total maternal (maternal weaning weight) – Daughters from Bull A will produce calves that are 4 lb heavier at weaning because of their combined genetics for growth and milk. This is a calculated figure of one-half the bull's weaning weight direct EPD plus his milk EPD. For example, Bull A has a maternal weaning weight value of 13 which is equal to half of his weaning weight direct EPD (20/2 = 10) plus his milk EPD [3].

Gestation length – Calves from Bull A should have a 1-day shorter gestation.

Calving ease maternal – Bull B's daughters should calve as first-calf heifers with 2% more unassisted births (6–4) than the daughters of Bull A.

Mature height – Bull B's daughters should be.5 in. taller at maturity.

Mature weight – Bull B's daughters should be 30 lb heavier when mature.

Scrotal circumference – Bull calves from Bull A should have.55 cm larger adjusted scrotal circumferences. Scrotal circumference is an indicator of the age of maturity of a bull's daughters. Bulls with larger scrotal circumference should have daughters that reach puberty earlier.

Heifer pregnancy – Daughters of Bull B are 3% more likely to become pregnant as heifers.

Carcass weight – Bull B should produce calves that have 18 lb more adjusted carcass weight.

Percent retail product – The calves from Bull B should yield 0.2% more closely trimmed, boneless retail cuts from the round, loin, rib, and chuck. Some breeds may report a Yield Grade (YG) EPD. The same factors (back fat, ribeye area, and carcass weight) would be included but a lower YG is more desirable as opposed to percent retail product where a higher value is more desirable. In either percent, retail product or YG fat thickness contributes the most to these two calculations. Consequently, selecting for decreased YG or increased percent retail product will lead to leaner animals so caution should be used to avoid extremely lean replacement females.

Marbling – Calves from Bull A should have a marbling (MARB) score of >0.3. Marbling scores range from 1.0 which is devoid of marbling and a utility quality grade to 10.9 which is abundant marbling and a prime + quality grade. For example, if calves sired by Bull B had a marbling score of 5.0 then calves sired by Bull A are expected to have a marbling score of 5.3. Ultrasound EPDs were calculated for a number of breeds for traits of ribeye area (REA), fat, and intramuscular fat (IMF), which is correlated to marbling, but now the majority of breeds use these ultrasound measurements in the calculation of carcass EPDs. Therefore, instead of seeing both an IMF EPD and a marbling EPD you just see the marbling EPD but it has ultrasound measurements included in the calculation.

Ribeye area – At a given end point, calves from Bull B should have ribeye areas that are 1.54 square inches larger than Bull A's calves.

Fat Thickness – At a given end point, calves from Bull A should be.08 in. fatter when measured at the 12th rib. This would be less desirable on a carcass animal, but extremely lean females going back into a cowherd may also be undesirable.

Tenderness – Calves sired by Bull A should produce meat that is more tender than that of calves sired by Bull B by 0.2 lbs. of shear force. Tenderness is measured by Warner Bratzler Shear Force (WBSF) that is reported in the pounds of force required to cut through a one inch thick piece of meat. A lower value is more desirable.

Days to finish – Calves sired by Bull B should spend 5 fewer days on feed to reach a constant fat endpoint.

Stayability – A measure of reproductive longevity. Daughters of Bull A are 4% more likely to stay productive in the herd to age 6.

Maintenance energy – The Red Angus Association of America calculates a Maintenance Energy (ME) Expected Progeny Difference (EPD) that indicates differences in the Mcal/month needed for maintenance due to mature size (corrected for body condition score) and milking ability (The Rancher's Guide to EPD available at www.redangus.org). A much simpler way to think of it is that a bull with a ME EPD of +10 compared to one that is +0 will produce daughters that will require approximately 11 more pounds of average quality forage per month (assuming average quality forage =.86 Mcal/lb.).

Docility – Bull A should sire 4% more calves that have a temperament in the most docile score than Bull B. The actual measurement of docility is recorded either at weaning or at yearling (depending on the breed association) and is categorized as the animals' behavior as they enter, are restrained in, and exit the chute.

Beef Improvement Federation (BIF) temperament scoring system.

1. Docile – Mild disposition – gentle and easily handled. Stands and moves slowly during processing, undisturbed, settled, and somewhat dull and does not pull on the headgate when in the chute – exits the chute calmly.
2. Restless – Quieter than average but slightly restless, might be stubborn during processing, might try to back from the chute, pulls back on the headgate, some tail flicking, exits the chute promptly.
3. Nervous – Typical temperament – manageable but nervous and impatient with a moderate amount of struggling, movement, and tail flicking as well as repeated pushing and pulling on the headgate – exits the chute briskly.

4. Flighty – Wild, jumpy and out-of-control, quivers and struggles violently, might bellow and froth at the mouth, continuous tail flicking, defecates and urinates during processing, frantically runs the fence line and might jump when penned individually, exhibits long flight distance, and exits the chute nervously.
5. Aggressive – Similar to Score 4 but with added aggressive behavior, fearful, extreme agitation, continuous movement that might include jumping and bellowing while in the chute, exits the chute frantically, and might exhibit attack behavior when handled alone.
6. Very Aggressive – Extremely aggressive temperament. Thrashes about or attacks wildly when confined in small, tight places. Pronounced attack behavior.

Breeding in Beef Cattle. Table 5 Approximate number of progeny needed to reach accuracy levels (true (r) and the Beef Improvement Federation standard) for three heritabilities (h^2)

Accuracy		Heritability levels		
R	BIF	h^2 (0.1)	H^2 (0.3)	h^2 (0.5)
0.1	0.01	1	1	1
0.2	0.02	2	1	1
0.3	0.05	4	2	1
0.4	0.08	8	3	2
0.5	0.13	13	5	3
0.6	0.2	22	7	4
0.7	0.29	38	12	7
0.8	0.4	70	22	13
0.9	0.56	167	53	30
0.999	0.99	3,800	1,225	700

Accuracy

The uncertainty surrounding early predictions of genetic merit are a result of Mendelian sampling. Every animal is passed a random sample of alleles from each parent, half coming from the dam and half from the sire. An estimate of the average effect of what was passed from parent(s) to offspring in the form of pedigree estimates can be calculated, but the certainty of how correct this estimate is (i.e., the accuracy) is low. As more information is collected, such as an individual's own record and data from progeny, accuracy increases. For lowly heritable traits, like measures of reproduction, it can take a considerable number of offspring to reach high BIF accuracy levels, given that the BIF scale is more conservative than true accuracy (r) as illustrated in Table 5. To calculate r in the context of progeny test sires the following equation can be used where n is the number of progeny:

$$r = \sqrt{\frac{nh^2}{4 + (n-1)h^2}}$$

To convert BIF accuracy to true accuracy (r) the following equation can be used:

$$r = \sqrt{1 - (1 - \text{BIF})^2}$$

Theoretically, accuracy is the correlation between an animal's predicted EPD and its true EPD. However, it is much simpler to think of it as a measure of risk indicating how much an animal's EPD may change with the inclusion of additional information. Accuracy is not an indication of how variable a particular sire's calves will be but rather how the estimate of an animal's EPD is likely to change. As the accuracy value increases, the amount by which an EPD is likely to change decreases. Possible change values differ for every trait and for every breed (e.g., an Angus bull with an accuracy of 0.30 may have a different possible change value than a Limousin bull with the same accuracy value for the same trait). Possible change tables are available on breed association Web sites.

Example: Possible Change in the Marbling EPD of a Limousin Sire

Assume that a young Limousin sire has a marbling EPD of 0.10 with an accuracy of 0.20. Using the values in Table 6, it is expected that 2/3 of the time his true EPD would fall within +/− 0.20 (one standard deviation) of his printed EPD or between −0.10 and 0.30. If there is 2 in 3 chance that the true EPD is between −0.10 and 0.30 then there must be 1 in 6 chance that his true EPD is below −0.10 and a 1 in 6 chance that his true EPD is above 0.30.

Breeding in Beef Cattle. Table 6 Accuracy related to possible change in Limousin cattle

BIF accuracy	Ribeye area EPD possible change	Marbling score EPD possible change
0.0	0.46	0.24
0.1	0.41	0.22
0.2	0.37	0.20
0.3	0.32	0.17
0.4	0.28	0.14
0.5	0.23	0.12
0.6	0.18	0.10
0.7	0.14	0.07
0.8	0.09	0.05
0.9	0.05	0.02

Source: www.nalf.org

Multiple-Trait Selection

Through genetic correlations, selection for one trait can influence other traits. This knowledge is useful when practicing multiple-trait selection. There are three primary methods of multiple-trait selection: tandem selection, independent culling levels (ICLs), and selection indexes.

Tandem selection. Tandem selection is the process of placing selection pressure on one trait and once the desired level of the trait under selection has been reached. Then selection for a new trait would begin. This is the simplest form of multiple-trait selection and the most inefficient. The two (or more) traits involved are selected for independently such that progress made in one trait could be eroded once selection for another trait becomes the focus of the breeding scheme. This is the poorest way to select for multiple traits.

Independent culling levels. Independent culling levels (ICLs) describe the process where threshold criteria for multiple traits are set and any animal not meeting all criteria (threshold levels for all traits) are excluded as candidates for selection. Although this ensures a certain level of superiority across multiple traits it may cull a particular animal that is just below the threshold level for one trait.

Economic Index

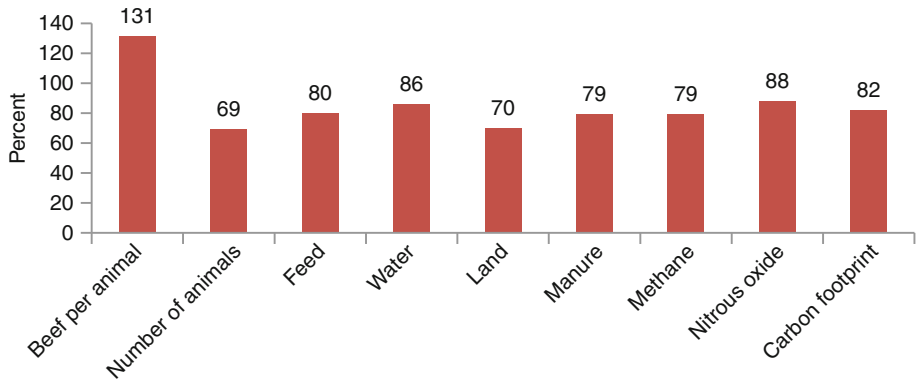
An economic index is a collection of EPDs that are weighted by their economic value such that traits with a greater impact on a production goal have a larger economic weight associated with them. Assume that there is an index centered on improving profit potential of terminal animals sold on a grid. In this particular index, the traits of economic importance might be yearling weight (YW), ribeye area (REA), marbling (MARB), and external fat (BF). Below is an example of how this index might look.

$$I = EPD_{YW} \times a_{YW} + EPD_{REA} \times a_{REA} + EPD_{MARB} \times a_{MARB} + EPD_{BF} \times a_{BF}$$

Where I is the index value, EPD_{YW} is the animal's EPD for yearling weight, and a_{YW} is the economic weight associated with trait yearling weight. The same approach is used for the other traits in this index, MARB and BF, where the animal's EPDs for these traits are multiplied by an economic weight. The economic weights are derived by computer simulation work to determine the economic value of changing particular traits.

Several breeds publish economic indexes including Angus, Hereford, Gelbvieh, Charolais, Simmental, and Limousin. Each index has an intended purpose and is catered toward particular breeding objectives.

An addition the breeding objective of beef cattle in the future will likely be some measure of environmental impact, such as green house gas (GHG) emissions. There are conflicting estimates of how much beef cattle production contributes to worldwide GHG production, but there are convincing estimates that suggest that per unit of product, beef cattle production is more environmentally friendly now that at any time point in the past despite decades of artificial selection to increase output. Figure 2 illustrates that in 30 years, from 1977 to 2007, beef production has increased while the number of cows has decreased. During the same three decades, the resources needed for beef production (land, water, feed) decreased along with gas emissions and the carbon footprint [3].



Breeding in Beef Cattle. Figure 2
Changes in metrics of beef production from 1997 to 2007 in the United States

Breed Selection

Correct breed selection is the critical first step to initializing a crossbreeding system. Choosing a breed(s) is dependent upon:

1. Production and marketing goals
2. Production environment
3. Available feed and labor resource

Choosing a breed that is best suited to your production environment is dependent on several factors including the availability of feed, and level of stress (temperature, amount of moisture, etc.). Table 7 outlines the biological type of cattle that are best suited for particular levels of feed resources and stress.

The decision of whether or not to utilize a particular strategic system of crossbreeding depends upon individual production goals. In order to take advantage of breed complementarity, breeds must be paired such that they excel in different areas that are critical to the overall production goal(s). For instance, if the goal were to sell calves at weaning then it would make sense to use a breed, most likely Continental, which will maximize direct weaning weights. However, this purebred system will maximize outputs, but may require large inputs as well. With that in mind, perhaps it would make more sense from an economic perspective to use a British breed as the genetic base for all dams and use a continental breed for sires thus minimizing the input costs from the female side yet still capturing added growth in the calves due to the direct growth provided by the sire breed. This would be a very simple example of a crossbreeding system.

Matching Biological Type to Environment

Benefits of heterosis make it important for producers to have a crossbreeding program. It is just as important that the producer match the type of crossbred cow to the environment and management system. This can be viewed as the foundation of a crossbreeding program. If the biological type does not match the resources, the system will fail, regardless how perfect the end product may be.

Cow weight is probably easier to understand than milk production, but research has shown that cows with the genetic propensity to milk heavily require more nutrients year round, not just when they are milking. The National Research Council (NRC) data shows that a cow producing 25 lbs. of milk at peak lactation requires 10% more feed energy than a cow producing 15 lbs. of milk at peak lactation. To see a 10% difference in feed energy with regards to mature weight it would require moving from a 1,000 lb. cow to a 1,200 lb. cow, or a change of 200 lbs. of body weight. There are breed differences in lactation yields so breed selection is critical when matching genetics to your environment. These breed differences can be found in literature from research at the Meat Animal Research Center (MARC). Moderating mature cow size and selecting for an optimal window of milk production is beneficial when it comes to cutting costs regardless of

Breeding in Beef Cattle. Table 7 Matching genetic potential for different traits to production environments

Production environment			Traits				
Feed availability	Stress[a]	Milk	Mature size	Ability to store energy[b]	Resistance to stress[c]	Calving ease	Lean yield
High	Low	M to H[d]	M to H	L to M	M	M to H	H
	High	M	L to H	L to H	H	H	M to H
Medium	Low	M to H	M	M to H	M	M to H	M to H
	High	L to M	M	M to H	H	H	H
Low	Low	L to M	L to M	H	M	M to H	M
	High	L to M	L to M	H	H	H	L to M

Source: Adapted from Gosey [4]

[a]Heat, cold, parasites, disease, mud, altitude, etc.

[b]Ability to store fat and regulate energy requirements with changing (seasonal) availability of feed

[c]Physiological tolerance to heat, cold, internal and external parasites, disease, mud, and other factors

[d]*L* low, *M* medium, *H* High

your production environment. However, in limited feed environments females with high maintenance energy requirements may also have difficulty maintaining an acceptable body condition score and rebreeding. Nugent et al. [5] determined that with limited nutrient availability, breeds with a high genetic potential for milk production had longer anestrous periods which lead to lower conception rates during a fixed breeding season. Other researchers have concluded that selection for increased milk production past an adequate threshold is not economically or biologically efficient [6]. It can be challenging to determine if cows within a herd have too much milking potential. Other than knowledge of the genetics of sires used in the past, it is important to note the body condition score of females. Extremely thin females fed the same as those with an acceptable body condition score may produce too much milk for their environment. If a producer is constantly supplementing females to maintain body condition to ensure they will successfully breed back, this might be an indication that bulls you buy in the future should have a more moderate value of milk genetics.

Another critical aspect of fitting genetics to the production environment is regional adaptation. If natural selection is allowed to play a large role in selecting the parents for the next generation, indigenous breeds should be well suited to their production environment. An example of an indigenous breed that has thrived in number is the Nelore breed in Brazil. The Nelore breed makes up roughly 65% of the world's bovine population and is well suited to tropical conditions. With adaptation in mind, indigenous breeds can be used as the base and exotic (foreign) breeds used in strategic crossbreeding systems to change target traits such as growth or carcass attributes. A case study [7] in Zimbabwe compared indigenous breeds of Afrikaner (a breed developed in South Africa), Tuli, Nkone, and Mashona, to breeds developed outside of the African Continent such as Brahman, Charolais, Hereford, Simmental, Aberdeen Angus, and Sussex. The results showed that indigenous breeds excelled in fitness traits (fertility and survivability) while the foreign breeds excelled in growth, feed conversion, and carcass attributes.

Crossbreeding

Heterosis

Too often heterosis (hybrid vigor) is thought to be the exclusive goal of crossbreeding. Heterosis is nothing more than an unexpected and often beneficial deviation from the average of the two parental lines. Hybrid vigor can also be thought of as the

"anti-inbreeding." Inbreeding increases uniformity by increasing homozygosity but also creates "inbreeding depression" or a general decrease in survival and reproductive traits that can be caused by a decrease in heterozygosity. Percent heterosis can be calculated as:

$$\%\,\text{Heterosis} = \left[\frac{(\text{crossbred average} - \text{straightbred average})}{\text{straight bredaverage}}\right] \text{x}100$$

A simple example would be the percent heterosis realized in the average weaning weight from breeding a herd of Breed A cows to a group of Breed B bulls. Let 525 lb be the average weaning weight of the F1 calves, 450 lb be the average weaning weight of the Breed A population, and 550 lb be the average weaning weight of the sire's population.

The pounds of heterosis would be:

$$\text{Pounds of heterosis} = 525 - \left[\frac{(450 + 550)}{2}\right] = 25\ \textit{pounds}$$

and the percent of heterosis would be:

$$\%\ \text{heterosis} = \frac{25}{[(450 + 550)/2]} = .05\ \text{or}\ 5\%$$

The amount of heterosis that is realized for a particular trait is inversely related to the heritability of the trait. This is logical since traits that are lowly heritable have a small additive component (proportionally speaking) and crossbreeding takes advantage of dominance and epistatic effects. With that in mind, traits of low heritability (e.g., reproductive traits) generally benefit from heterosis the most (Table 8). They generally have a heritability of less than 10% and can be improved through the adequate use of crossbreeding systems. End-product traits on the other hand that benefit from heritability in the moderate to high range benefit less from heterosis. Another benefit of crossbreeding may be a decrease in methane production per unit of beef produced. In a comparison of the methane production per ton of live weight weaned from a 16,000 ha farm in Australia, changing from Shorthorns (mean cow weight 422 kg) to composite breed cattle in (mean cow weight 507 kg), reduced methane production per ton of weight weaned by 31%. This was largely due to higher weaning rates of composite breed females (80 vs 55%) [8].

Breeding in Beef Cattle. Table 8 Individual heterosis: advantage of the crossbred calf

Trait	Observed improvement	% Heterosis
Calving rate	3.2	4.4
Survival to weaning	1.4	1.9
Birth weight	1.7	2.4
Weaning weight	16.3	3.9
ADG	0.08	2.6
Yearling weight	29.1	3.8

Source: Adapted from Cundiff and Gregory [9]

There are three main types of heterosis:

1. Individual
2. Maternal
3. Paternal

Retained Heterosis Unfortunately there exists a popular misconception that heterosis exists only in the first generation of crossbreds (F1). Heterosis is *retained* in the breeding of crossbred animals and is related to the probability of alleles from different parental lines joining together. For instance, if two F1 animals are mated, heterosis in the corresponding offspring is called *retained heterosis* and is equal to the following:

$$\text{Heterosis retained} = 1 - [(P_{S1}P_{D1}) + \ldots + (P_{Sn}P_{Dn})]$$

Where P_{S1} is the proportion of the sire from breed 1 and P_{D1} is the proportion of the time from breed 1 and n is equal to the total number of breed involved.

Maternal Heterosis The offspring of an F1 female will benefit from maternal heterosis (Table 9), thought of as realized heterosis of milk production.

Paternal Heterosis Fewer examples of paternal heterosis exist and consequently crossbred sires have often been ignored. The benefit of crossbred or composite

Breeding in Beef Cattle. Table 9 Maternal heterosis: Advantage of the crossbred cow

Trait	Observed improvement	% Heterosis
Calving rate	3.5	3.7
Survival to weaning	0.8	1.5
Birth weight	1.6	1.8
Weaning weight	18.0	3.9
Longevity	1.36	16.2
Cow lifetime production		
No. Calves	0.97	17.0
Cumulative weaning weight, lb.	600	25.3

Source: Adapted from Cundiff and Gregory [9]

sires lies in their ability to inject heterosis and breed complementarity into a herd with greater ease than the rotational crossbreeding systems described above.

Composites

Some of the first such animals are the American Breeds, or Brahman Derivatives. Perhaps there is no greater example of breed complementarity (breeding animals to adapt to a specific environment) and consequently of heterosis.

The American Gelbvieh Association, North American Limousin Foundation, and American Simmental Association are three breed associations that have implemented new programs to introduce composites such as the Balancer, Lim-Flex, and SimAngus, respectively. There is no doubt that some of these programs have met with opposition from within the respective associations due to an ignorant stance that purebred animals are superior. I greatly admire those who have pushed, pulled, and prodded these programs through.

Crossbred females have proven to be very profitable and well accepted from a commercial standpoint. The use of crossbred bulls has not been accepted so easily. Some common fears have been the perception of larger amounts of variation within composite populations and the lower accuracy of EPDs of composite animals. In a study of three composite lines at MARC and their parental purebreds, there were no statistical significant differences in the coefficient of variation for reproduction, production, or carcass traits measured (Table 10).

Breeding in Beef Cattle. Table 10 Coefficients of variation for purebred versus composite steers

Trait	Purebreds	Composites
Birth weight	0.12	0.13
Wean weight	0.10	0.11
Carcass weight	0.08	0.09
Retail product %	0.04	0.06
Marbling	0.27	0.29
Shear force	0.22	0.21

Source: Adapted from [10]

Breed Complementarity

In addition to heterosis, crossbreeding programs can increase production efficiency by using additive (highly heritable) traits through *complementarity*. Differing breeds can be matched to complement traits, more closely fitting the genetic goals for biological type.

Complementarity is fully exploited under a system when crossbred cows of lower frame size, and optimum milk are crossed with a large framed terminal breed, noted for rapid growth and carcass leanness in order to produce market animals [11].

Simulation work by Lamb et al. [12] shows that generally English breeds are more biologically efficient, while Continental breeds are more economically efficient. The English X Continental cross was the most biologically and economically efficient. In some environments, heat and disease tolerance of the Brahman (*Bos Indicus*) may be required to complement the carcass traits, disposition, and age at puberty of an English breed [13].

Crossbreeding Systems

There are many other crossbreeding systems that vary significantly in terms of difficulty. Items that

can influence the success of a crossbreeding system include:

1. Number of cows in the herd
2. Number of available breeding pastures
3. Labor and management
4. Production and marketing system
5. Availability of high-quality bulls of the various breeds

Terminal Cross All offspring from a terminal cross are sold. The sire used is usually from a breed noted for high growth, and desirable carcass end product. Under this system, genetic differences between maternal and terminal breeds can be exploited through complementarity. Individual heterosis is improved an additional 5% from this type of cross [11]. If female replacements are not generated, little emphasis needs to be placed upon milking genetics of the sires used. By purchasing crossbred F1 females, maximum maternal heterosis can also be achieved.

Two-Breed Terminal In this simple situation, cows of breed A are bred to bulls of breed B and all offspring are sold. In this system, the offspring are F1s and will benefit from 100% of the possible individual heterosis.

Three-Breed Terminal In this situation, cows that are F1 females comprised of ½ breed A and ½ breed B are mated to bulls of breed C for the production of terminal offspring. In this system, calves not only benefit from 100% of the possible individual heterosis, but maternal heterosis is realized as well. In general, the females should be a cross of two maternal breeds that emphasize efficiency and milking ability while the sire breed should inject growth.

Rotational Systems The three-breed rotation is similar to the two-breed rotation. A third breed is added to this system, thus a third breeding pasture is needed, unless AI is utilized. Management of this system is patterned after the two-breed rotation. If the maximum amount of heterosis is 23.3% (14.8% + 8.5%) then the expected level of heterosis is higher with a three-breed than two-breed rotation, 86% versus 67% [14].

Two-Breed Rotation A two-breed rotation is a simple crossbreeding system requiring two breeds and two breeding pastures. The two-breed rotational crossbreeding system is initiated by breeding cows of breed A to bulls of breed B. The resulting progeny (A*B) chosen as replacement females would then be mated to bulls of breed A for the duration of their lifetime. The service sire is the opposite breed of the female's own sire. These progeny are then one-quarter breed A and three-quarters breed B. Since these animals were sired by breed B bulls, they are mated to breed A bulls. Each succeeding generation of replacement females is mated to the opposite breed of their sire. Initially only one breed of sire is required. Following the second year of mating, two breeds of sire are required. After several generations, the amount of retained heterosis stabilizes at about 67% of the maximum heterosis, resulting in an expected 16% increase in the pounds of calf weaning weight per cow exposed above the average of the parent breeds [14]. In this system, a minimum of two breeding pastures and 50 cow units are required.

Three-Breed Rotation A three-breed rotational system achieves a higher level of retained heterosis than a two-breed rotational crossbreeding system does. After several generations, the amount of retained heterosis stabilizes at about 86% of the maximum heterosis, resulting in an expected 20% increase in the pounds of calf weaning weight per cow exposed above the average of the parent breeds [14]. Like the two-breed system, distinct groups of cows are formed and mated to bulls of the breed that represents the smallest fraction of the cows breed makeup. A cow will only be mated to a single breed of bull for her lifetime.

A minimum of three breeding pastures is required for a three-breed rotational system. Replacement females must be identified by breed of sire to ensure proper matings. The minimum herd size is approximately 75 cows with each one-third being serviced by one bull of each breed. Scaling of herd size should be done in approximately 75 cow units to make the best use of service sires, assuming one bull per 25 cows. Replacement females are mated to herd bulls in this system, so extra caution is merited in sire selection for calving ease to minimize calving difficulty. The progeny produced from these matings that do not conform to the breed type of the herd should all be marketed.

Breeding in Beef Cattle. Table 11 Summary of crossbreeding systems by advantage and other factors

Type of system		Advantage[a]	Retained heterosis[b]	Minimum no. of breeding pastures	Minimum herd size	No. of breeds
2-breed rotation	A*B rotation	16	67	2	50	2
3-breed rotation	A*B*C rotation	20	86	3	75	3
Terminal cross with straightbred females[c]	T*A	8.5	0	1	Any	2
Terminal cross with purchased F1 females	T*(A*B)	24	100	1	Any	3
Rotating unrelated F1 bulls	A*B x A*B	12	50	1	Any	2
	A*B x A*C	16	67	1	Any	3
	A*B x C*D	19	83	1	Any	4

Source: Adapted from Ritchie et al. [14]
[a]Measured in percentage increase in lb of calf weaned per cow exposed
[b]Relative to F1 with 100% heterosis
[c]Cundiff and Gregory [15]

Breeds used in rotational systems should be of similar biological type to avoid large swings in progeny phenotype due to changes in breed composition. The breeds included have similar genetic potential for calving ease, mature weight and frame size, and lactation potential to prevent excessive variation in nutrient and management requirements of the herd.

When choosing a crossbreeding system it is critical to consider more than the amount of heterosis retained. After all, each system requires different levels of input (pastures, etc.) and differing levels of difficulty. Table 11 describes the advantages and requirements for the above mentioned crossbreeding systems.

Molecular Information

Molecular Information: Paternity and Simply Inherited Traits

Molecular-based tools are another source of information that has received considerable attention by producers throughout the beef industry and by both the academic community and the private sector. These tools initially came in the form of candidate genes but have now grown to the inclusion of multiple markers called Single Nucleotide Polymorphisms (SNPs). The use of molecular information has grown from simple applications such as identifying animals that are carriers of the red allele to identifying animals that are carriers of lethal genetic defects, to paternity assignment, and a growing number of diagnostic tests for a suite of complex traits ranging from reproduction to carcass.

Genotyping to determine parentage allows for a sire to be correctly linked to a corresponding calf. The identification of an animal's sire via DNA marker technology can be advantageous in multi-sire breeding pastures, or for ascertaining if a calf is the product of an artificial insemination (AI) mating or a clean-up bull. This promotes knowledgeable culling and breeding decisions by determining which sire(s) are contributing the most (or least) to a particular breeding objective. In the case of commercial ranch settings, for example, it may be beneficial to determine the sire that is responsible for calving difficulties.

Because paternity identification is a process of excluding potential sires on the basis of their genotype, it is important that DNA from all possible sires be included in paternity tests. It will be more difficult to definitively make paternity assignments on closely related bulls in a multiple-sire breeding pasture, given they are likely to share a similar genotype. Although microsatellites have typically been the marker of choice for paternity analysis, the use of SNP markers is becoming more common for a number of reasons including

their abundance, high potential for automation, low genotyping error rates, and ease of standardization between laboratories.

Although identifying carriers of genetic defects is a rather simple application of DNA technology, it is an important tool when making mating decisions. It is known that afflicted animals can only arise if two carrier animals are mated. In this scenario, there exists a 25% chance that the corresponding calf will have the defect. Unfortunately, this added information has been used as the primary selection tool whereby carrier animals are automatically discarded. If a producer potentially has carrier females, then carrier bulls should be avoided. However, if this is not the case, then it could be beneficial to use the best available bull, regardless of his status as a carrier. As an industry, there is the ability to make informed decisions based on science concerning this issue and not throw away animals that are superior across the remainder of their genome because they have a flaw that can be effectively managed around.

Molecular Information: Complex Traits

Several advancements in molecular technology have occurred with regard to complex traits (i.e., production, carcass, and reproduction traits). Including the number of markers included in a given panel, reporting styles of the results, and the number of traits for which a diagnostic test exists. Recently, this information is included in the Angus National Cattle Evaluation (NCE) for the first time.

The promise of the inclusion of marker information into EPD calculations holds three primary benefits:

1. Increased accuracy for young animals (i.e., yearling bulls), which is particularly beneficial when selecting on traits that are measured late in life (e.g., stayability)
2. Shortened generation intervals
3. EPD values for novel traits (i.e., efficiency, end-product healthfulness, disease susceptibility) that may have, at best, sparse collection of phenotypes

However, the magnitude of these benefits will depend on the proportion of variation explained by a given marker panel. At present, the best objective source of information regarding this is the National Beef Cattle Evaluation Consortium (NBCEC) Web site (www.nbcec.org). Pertinent information from this Web site includes population, trait, regression coefficient (b), and the p-value (p). The population defines what breed(s) were used to validate the test. If the test was validated in *Bos taurus* animals then it is possible that the test will not explain the same proportion of variation in *Bos indicus* animals. The trait defines what the test was validated for. If it is a metric of efficiency like residual feed intake (RFI) then it will explain how the trait is defined. Generally, a p-value of less than 0.05 suggests that the test is a statistically significant predictor of differences in phenotypes. The regression coefficient is equal to the regression of phenotypes for the trait of interest on the molecular score. It explains the units of change in the phenotype that would be expected for a one-unit change in the molecular score (i.e., MBV). Ideally, these b values should be 1. For example, if two animals have molecular scores for RFI of −1.5 and 1.0, respectfully, the difference between those scores is 2.5. Normally it is expected that, on average, these two animals' phenotypes would differ by 2.5 lb of RFI. However, if the regression coefficient is 0.4 then their phenotypes are expected to differ by 1 lb (2.5*0.4).

Without the seamless integration of this technology into EPD calculations, the industry is faced with two disjoined pieces of information: traditional EPD and marker panel results. In this scenario, it is impossible to directly compare EPD to marker panel results even if the results come in the form of molecular breeding values (MBVs). This is because the molecular scores only explain a portion of the additive genetic variation. Further, some of the marker panel results have a metric of accuracy associated with them. At the current time, this metric is not comparable to the Beef Improvement Federation accuracy value associated with EPD simply due to differences in the way they are computed. While it is logical that the accuracy value of a MBV should be related to the proportion of additive genetic variation explained by the test there is not a standardized metric that is being used. Thallman et al. [16] analyzed different methods of calculating this proportion for MBV in light of the fact that there is not a standardized method and recommended the use

of the square of the additive genetic correlation between the MBV and the trait of interest.

In contrast to the thought process of DNA marker panel results being a separate and disjoined piece of information, these test results should be thought of as a potentially useful indicator that is correlated to the trait of interest. As such, the MBV can be included in NCE as a correlated trait following methods of Kachman [17]. Other methods have been proposed including using large (50,000 +) SNP panels to form a genomic relationship matrix that could allow for known relationships between animals based on genotypes across SNP loci. Combining these sources of information, molecular tools and traditional EPDs, has the potential to allow for the benefits of increased accuracy and increased rate of genetic change as discussed earlier.

MacNeil et al. [18] utilized Angus field data to look at the potential benefits of including both ultrasound records and MBVs for marbling as correlated traits in the evaluation of carcass marbling score. MacNeil and colleagues used a 114 SNP marker panel that was developed using 445 Angus animals and calculated to have a genetic correlation (r) of 0.37 with marbling (i.e., the test explained $(0.37)^2 = 0.137$, or 13.7%, of the additive genetic variation). For animals with no ultrasound record or progeny data, the marker information improved the BIF accuracy of the Angus marbling EPD from 0.07 to 0.13. Assuming a heritability of 0.3 for marbling, a BIF accuracy of 0.13 is equivalent to having approximately 5 progeny carcass records on a young animal or an ultrasound record on the individual itself. In this particular study, both ultrasound records and MBV were found to be beneficial indicators of carcass marbling. The genetic correlation between MBV and ultrasound was found to be 0.80. Some breeds have begun to integrate this technology and it is likely that more will do so in the future.

Considerations

Current marker panels are likely to work best in the populations where discovery occurred, but will potentially decrease in predictive power as the target population becomes more genetically distant from the discovery population [19]. Below is an example of scenarios where the discovery population is close to the target population and progresses to more distant populations.

Discovery	Target	
Angus	Angus	Closest relationship
Angus	Charolais	
Angus	*Bos indicus*	Most distant relationship

Marker panels are likely to become larger in the future with the possibility of whole genome selection (WGS). Currently, genome selection in beef cattle is in its infancy. Although preliminary data from the dairy industry look promising [20], the structure of the beef industry offers unique challenges. It is not known how well this approach will work in beef cattle with its diversity of breeds, diverse sector-specific selection goals, and less extensive phenotype and data collection resources.

Future Directions

Undoubtedly, molecular discoveries and their seamless integration into existing infrastructures and genetic selection tools will aid in the increased rate of genetic change and hopefully profitability of producers. These molecular tools will be in the form of larger (770 K) SNP panels and genome sequences. At the same time, a suite of reduced marker panels will be commercialized for use on progeny while the larger panels are used for herd bulls. These molecular tools will be integrated into breeding value estimations allowing for higher accuracy values on younger animals and allowing for genetic predictions of traits that have not yet been exploited such as the nutrient content of beef, feed efficiency, and susceptibility to disease. Effort will also be placed on precision mating, or specific combining ability, when developing composite animals, aided by molecular technology. One final area of effort includes the robustness of marker effects across breeds and environments, and how to accommodate these differences in national cattle evaluations. All of this has the potential to allow for selection on total profitability and not just production, ultimately aiding in the sustainability of the beef industry worldwide.

Bibliography

Primary Literature

1. Marquez GC, Speidel SE, Enns RM, Garrick DJ (2010) Genetic diversity and population structure of American Red Angus cattle. J Anim Sci 88:59–68
2. Keuhn LA, Van Vleck LD, Thallman RM, Cundiff LV (2008) Across-breed EPD tables for the year 2008 adjusted to breed differences for birth year of 2006. In: Proceedings of beef improvement federation annual meeting. Calgary, Alberta, June 30–July 3, pp 53–77
3. Capper JL (2010) Comparing the environmental impact of the US beef industry in 1977 to 2007. J Anim Sci 88:(Suppl 2)
4. Gosey J (1994) Composites: a beef cattle breeding alternative. In: Proceedings of beef improvement federation annual meeting. West Des Moines, 1–4 June 1994, p 93
5. Nugent RA III, Jenkins TG, Roberts AJ, Klindt J (1993) Relationship of post-partum interval in mature beef cows with nutritional environment, biological type and serum IGF-1 concentrations. Anim Prod 56:193–200
6. Oijen M, Montano-Bermudez M, Nielsen MK (1993) Economical and biological efficiencies of beef cattle differing in level of milk production. J Anim Sci 71:44–50
7. Mpoful N (2002) Comparison of indigenous and foreign cattle for beef production at Matopos Research Station in Zimbabwe. In: Proceedings of the 7th world congress on genetics applied to livestock production, Montpellier, France
8. Bently D, Hegarty R, Alford A (2008) Managing livestock enterprises in Australia's extensive rangelands for greenhouse gas and environment outcomes: a pastoral company perspective. Aust J Exp Agric 48:60–64
9. Cundiff LV, Gregory KE (1999) What is systematic crossbreeding? In: Proceedings of NCBA Cattleman's College, Charlotte, Feb 1999
10. Cundiff LV, Gregory KE, Koch RM (1994) Breeding cattle for improved product consistency. Presented 1994 national cattlemen's convention and trade show, Reno, 26–29 Jan 1994
11. Cundiff LV (1981) Evaluation of maternal breeds. In: Proceedings of the beef cow symposium VII, Rapid City, 7–9 Dec 1981
12. Lamb MA, Tess MW, Robinson OW (1992) Evaluation of mating systems involving five breeds for integrated beef production systems: I. Cow-calf segment. II. Feedlot segment. III. Integrated system. J Anim Sci 70:689
13. Winder JA, Rankin BJ, Bailey CC (1992) Maternal performance of Hereford, Brangus, and reciprocal crossbred cows under semidesert conditions. J Anim Sci 70:1032
14. Ritchie H, Banks D, Buskirk D, Cowley J (1999) Crossbreeding systems for beef cattle. Michigan State University Extension Bulletin E-2701
15. Gregory KE, Cundiff LV (1980) Crossbreeding in beef cattle: evaluation of systems. J Anim Sci 51:1224–1242
16. Thallman RM, Hanford KJ, Quaas RL, Kachman SD, Templeman RJ, Fernando RL, Keuhn LA, Pollak EJ (2009) Estimation of the proportion of genetic variation accounted for by DNA tests. In: Proceedings of the 41st beef improvement federation, Sacramento
17. Stephen K (2008) Incorporation of marker scores into national cattle evaluations. In: Proceedings of the 9th genetic prediction workshop, Kansas City, pp 88–91
18. MacNeil MD, Nkrumah JD, Woodward BW, Northcutt SL (2010) Genetic evaluation of Angus cattle for carcass marbling using ultrasound and genomic indicators. J Anim Sci 88:517–522
19. de Roos APW, Hayes BJ, Spelman RJ, Goddard ME (2008) Linkage disequilibrium and persistence of phase in Holstein-Fresian, Jersey, and Angus cattle. Genetics 179:1503–1512
20. VanRaden PM, Van Tassell CP, Wiggans GR, Sonstegard TS, Schnabel RD, Taylor JF, Schenkel FS (2009) Invited review: reliability of genomic predictions for North American Holstein dairy bulls. J Dairy Sci 92:16–24

Books and Reviews

Beef Improvement Federation (2002) Guidelines for uniform beef improvement programs. Beef Improvement Federation, Athens, Georgia

National beef cattle evaluation consortium (2010) Sire selection manual. National beef cattle evaluation consortium, Lexington, Kentucky

Breeding in Developing Countries and Tropics

SWP Cloete
Department of Animal Sciences, University of Stellenbosch, Matieland, South Africa
Institute for Animal Production, Elsenburg, South Africa

Article Outline

Glossary

Fitness A measure of the ability of an organism to survive and reproduce in a particular environment.

Genetic resource Genetic material of plants or animals of value as a resource for future generations of humanity.

Genomics The study of the genomes of organisms, including intensive efforts to determine the entire DNA sequence of organisms as well as fine-scale genetic mapping.

Phenotype The appearance of an organism based on an interactive combination of genetic traits and environmental factors.

Selection Choosing organisms with desirable genetic characteristics for propagation from candidates available.

Sustainability The capacity to endure, that is, the ability of biological systems to remain diverse and productive over time.

Definition of the Subject and Its Importance

Species breeding has led to marked genetic improvement of production by livestock in the developed world over the past decades. Selection has been so successful that the emphasis in the breeding objective has been changed to welfare, behavior, and other consumer-orientated objectives. The same level of progress has not been achieved in the developing world. Current and projected future population growth requires that food production from farm animals in these regions be improved markedly from the present inadequate levels. Limitations in the institutional infrastructure and the unique challenges of livestock production in the emerging world and the tropics were discussed in detail. This led to recommendations on how these challenges can be overcome and how "new" issues like global warming and animal welfare should be handled in a sustainable manner. Focus should be on the phenotyping of individuals for production and particularly for traits associated with fitness. Data should be linked to pedigree information where possible, to generate a broad-based institutional environment where the genetic improvement of animals in the developing countries can be strived for. Samples for the acquisition of DNA should be obtained from phenotyped individuals, for possible later genomic studies. Continued further learning and the accrual of information as well as collaboration to mutual benefit across institutional and national boundaries are needed to enable this. Sustainable livestock improvement in the developing world could accrue if these prerequisites are met.

Introduction

It is fair to say that the past decades have been marked by rapid change. Mankind had to adapt to immense technological progress, rapid urbanization, instability on monetary markets, as well as becoming part of the global village. The changes listed above are by no means exhaustive and many other challenges to the current generation can also be noted.

Yet it is important to note that there is still immense poverty in parts of the world, despite groundbreaking scientific breakthroughs as well as rapid progress in communication systems allowing easy access to the information that are generated elsewhere in the global

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

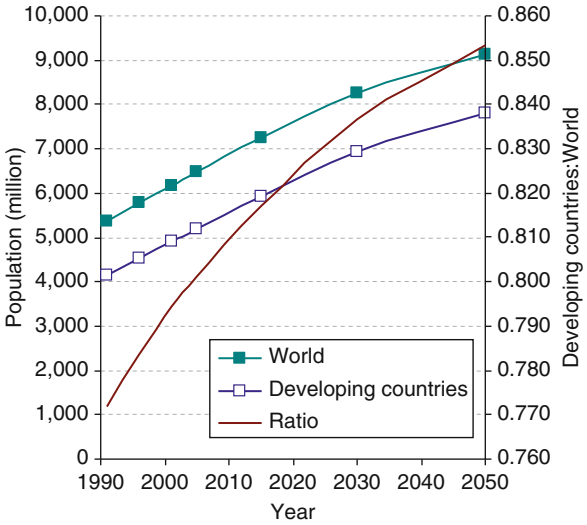
Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

village. While the developed world appears to be going from strength to strength in the acquisition and application of knowledge, the developing countries are lagging behind. In the Millennium Development Goals Report [1], Kofi Annan (the then secretary general of the United Nations) stated: "We will not enjoy development without security, we will not enjoy security without development, and we will not allow either without respect for human rights. Unless all these causes are advanced, none will succeed."

As a point of departure it has to be observed that the fundamental human rights of millions of people in the developing world are closely interwoven with livestock and the meat, milk, or fiber they produce [2–5]. The livestock industry is inextricably linked with the eradication of extreme poverty and hunger (Goal 1) and the ensuring of environmental sustainability (Goal 7) [1]. Improved and sustainable levels of animal production could not only contribute to improved household food security, but also to an increased household income in the developing world. This could lead to spinoffs indirectly benefitting other millennium development goals, such as the achievement of universal primary education (Goal 2), the reduction of infant mortality (Goal 4), and an improved maternal health (Goal 5). The benefits of including meat in diets of vulnerable groups are becoming evident now [6, 7].

During an exercise where livestock and poverty in the developing world were mapped by Thornton et al. [4] it became evident that large areas of the developing countries are only fit for rangeland-based livestock production. Considerable other areas are also utilized by livestock in combination with cropping in rainfed or irrigated mixed production systems. Ruminant livestock strongly depends on crop residues and the intercrops planted in the ley-farming systems which are preferred at present [8].

Against this background, it is reasonable to assess the contribution of livestock breeding to the sustainability of livestock production systems. This topic is addressed by providing background information leading to thoughts on global food security, information pertaining to farm animal genetic resources and livestock products contributing to food security, and external and internal challenges, the advent of the technological age and challenges stemming from it. These topics culminate in proposed future directions to place sustainable farm animal breeding in the developing world and the tropics on a solid foundation, before some concluding remarks.



Breeding in Developing Countries and Tropics. Figure 1 The population growth of the developing countries relative to the entire human population of the world from 1990–1992 to 2004–2006, and extrapolated beyond that to 2050. The human population in the developing countries is also expressed as a ratio of the world population (Source: FAO [9])

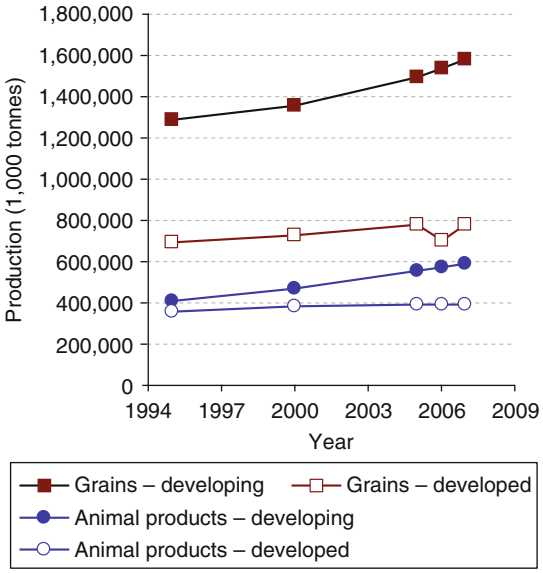
Setting the Scene

World Population

The world population grew steadily from 1990–1992 to 2004–2006 (Fig. 1). Extrapolation beyond the data to 2050 suggests sustained population growth from 5,358 million people in 1990–1992 to 9,136 million people in 2050, an increase of 71%. In the case of the developing world, this increase amounts to 89%, from 4,135 million people in 1990–1992 to 7,797 million people in 2050. Expressed as a ratio, 0.77 of people were living in the developing world in 1990–1992, compared to an estimated 0.85 in 2050. The impact of this change of population structure on the available resources in terms of plant and animal production is evident.

Global Food Production

Given the increase in the global population and the even faster population growth in the developing



Breeding in Developing Countries and Tropics. Figure 2
The production of grains and animal products (meat, milk, and eggs) in countries of the developing world and the developed world (Source: FAO [10])

Breeding in Developing Countries and Tropics. Table 1
Per capita production of grain and animal products (meat, milk, and eggs) in developing and developed countries during 1995, 2000, and 2005

Product type and regions	Year		
	1995	2000	2005
Grains			
Developing regions	281.9	275.4	286.0
Developed regions	563.1	582.8	611.3
Livestock products			
Developing regions	90.4	95.0	107.4
Developed regions	293.4	306.5	308.2

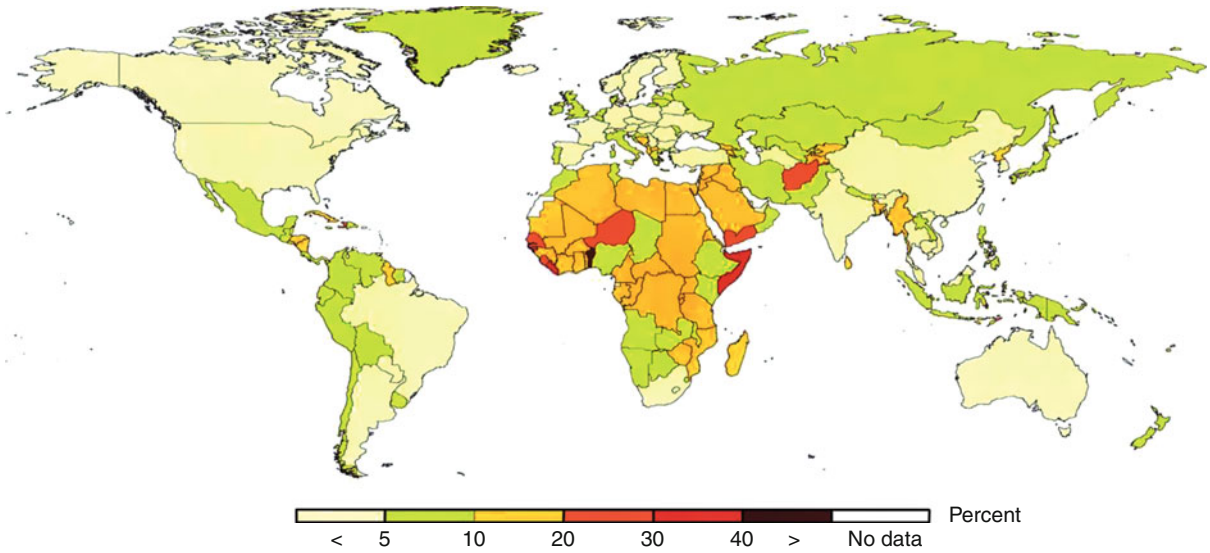
Source: FAO [10].

countries, it is imperative to have a look at global food production. The production of grain in the developing world has increased from ~1.3 million kilotons in 1995 to just below 1.6 million kilotons in 2007 (Fig. 2). Expressed relative to 1995 production, this increase amounted to 23%. Grain production in the developed world amounted to just below 0.69 million kilotons in 1995 and 0.78 million kilotons in 2007, an increase of about 12%. Animal products (meat, milk, and eggs) in the developing world increased from 0.41 million kilotons in 1995 to 0.59 million kilotons in 2007, an increase of 44%. Animal products in the developed world, in contrast, remained fairly stable at between 0.36 and 0.40 million kilotons over the entire period. There thus seems to be an effort on the part of the developing world to compensate for the faster population growth by a higher food production. However, this increase has to be seen in relation to the relative size of the respective populations in the developing and developed countries. Grain production per head of the developed world increased by 8.5% from 563 kg/head in 1995 to 611 kg/head in 2005 (Table 1). Grain production per head in the developing world, in contrast, remained relatively stable at a substantially lower level of between 275 (during 2000) and 286 (during 2005) kg/head (a difference of 4.0%). The output of animal products accordingly ranged from 293 to 308 kg/head in the developed world, compared to between 90 and 107 kg/head in the developing world.

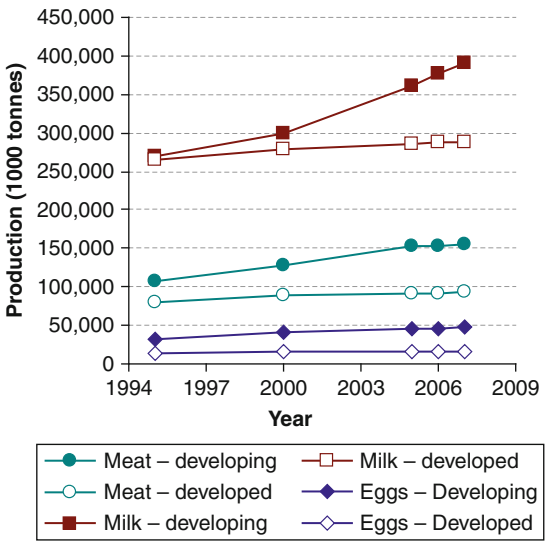
The lower per capita production of food in the developing world results in the need for imports to contribute to the food supply of the local population. The global importation of food in relation to total imports is provided in Fig. 3. It is evident that food imports exceed 5% of overall imports in many countries in the developing world. Africa and the Middle East are particularly dependent upon imports of food to contribute to local food security, with food imports exceeding 10% of total imports in most countries. On the upper end of the scale, food imports contribute more than 30% to total imports in some countries in these regions.

Products of an Animal Origin

Table 1 suggests substantial differences between the developing world and the developed world in terms of per capita animal production. As this entry deals with animal products, this topic needs to be elucidated further. In terms of quantity, milk was the most important animal product, followed by meat and eggs (Fig. 4).



Breeding in Developing Countries and Tropics. Figure 3
The share of food imports in relation to total imports on a global basis (Source: FAO [10])

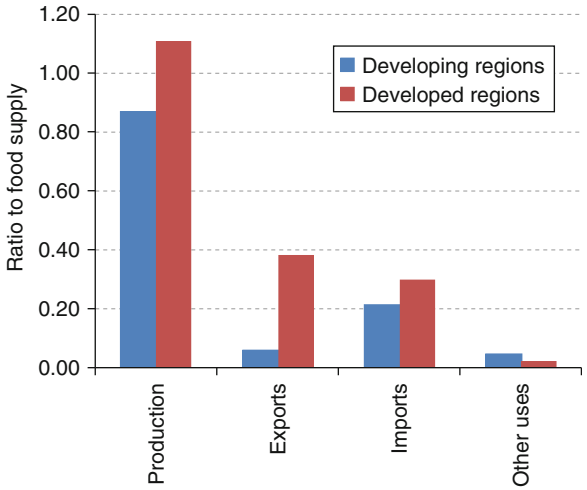


Breeding in Developing Countries and Tropics. Figure 4
Relative quantities of the most important livestock products in developing and developed countries (Source: FAO [10])

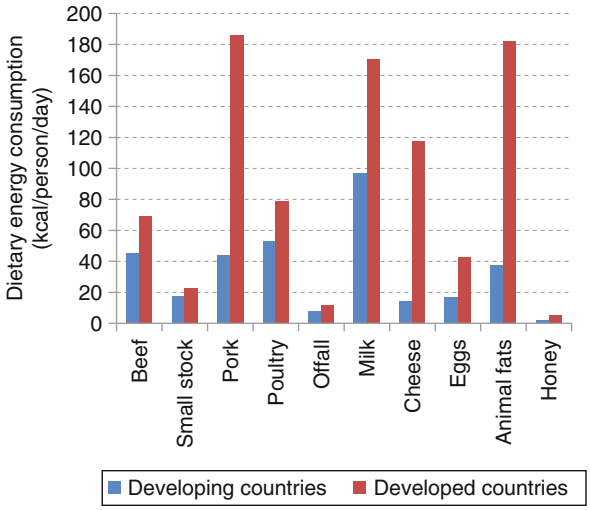
The same ranking applied both in developing and developed countries. It is evident that the production of all livestock products increased substantially over the past decade in the developing world (Fig. 4). Production in the developed world, on the other hand, remained stagnant or increased by smaller increments. These trends are set to continue in the future [11]. However, it needs to be considered that the population of the developing world exceeded 80% of the global population during 2005. It is thus evident that the somewhat higher animal production in these regions is canceled by the larger population density, hence the almost threefold lower per capita output of animal products in Table 1.

Against this background, local meat production is balanced against overall food supply for this commodity in developing countries and developed countries in Fig. 5. It is seen that there is a clear deficit in local production in the developing countries, as opposed to an oversupply in the developed countries. The deficit in own production in the developing countries is negated by imports, while only a small proportion of meat is exported. Developed countries are more likely to export meat, but also import relatively more meat than developing countries. The proportion of meat affected by stock changes and other purposes (mostly cultural) is relatively small, but appears to be somewhat higher in developing countries.

Table 1 suggests a substantially lower per capita production of animal products in developing countries



Breeding in Developing Countries and Tropics. Figure 5
A food balance for animal meats for the developing world and the developed world relative to the overall food supply in kiloton (Source: FAO [10])



Breeding in Developing Countries and Tropics. Figure 6
Per capita energy consumption of the top ten animal products in the developing world and in the developed world (Source: FAO [10])

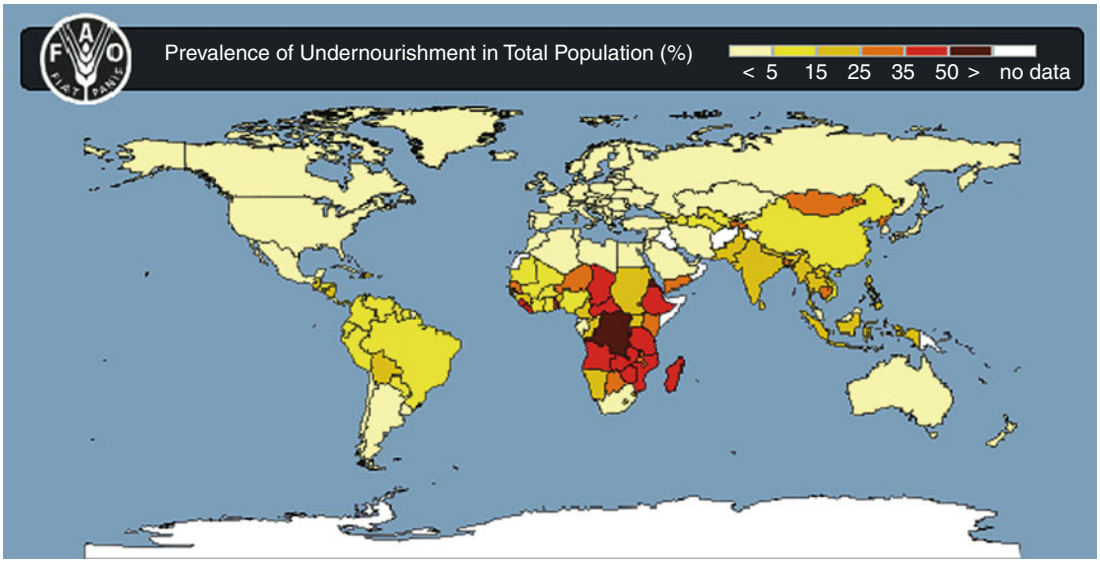
relative to developed countries. In Fig. 6, this tendency is quantified for the top ten animal products globally. Per capita energy consumption from small stock (sheep and goat) meat in the developing regions amounted to 79% of that in the developed world. Other products where per capita energy consumption of the developing world exceeded 50% of the corresponding figure in the developed world are poultry (67%), offal (67%), beef (65%), and milk (57%). Inhabitants of the developing world consumed energy from eggs (39%), honey (38%), pork (24%), animal fats (21%), and cheese (12%) at much lower quantities than their counterparts in the developed world.

Global Food Security

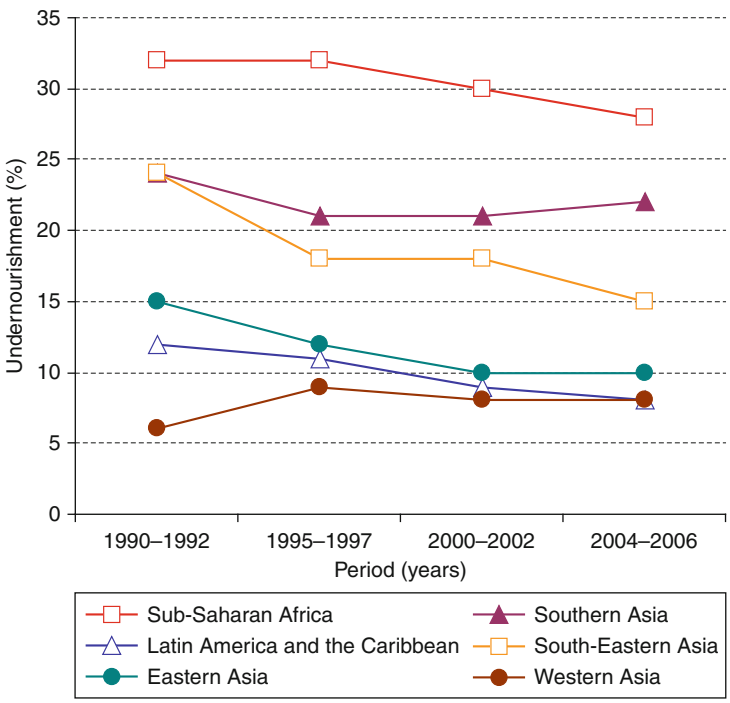
It is evident from Fig. 6 that inhabitants of the developing world consume substantially less energy from animal products than their counterparts in the developed world. It is thus important to consider relative food security of both groupings in view of these results. It is estimated that ~854 million people in the world lack adequate nourishment for a healthy and productive lifestyle, a figure that remained fairly stable over the last two decades [9]. The number of people dependent on one or other form of food aid for a livelihood is estimated at ~73 million worldwide. It is notable that the most insecure areas in terms of the provision of food are in developing countries in Asia and the Pacific, Latin America and the Caribbean, and Near East and Sub-Saharan Africa. Figure 7 depicts the frequency of undernourishment in different areas of the world as a percentage of the total population. It is evident that people in the central part of Africa are particularly at risk from undernourishment, but that variable levels of undernourishment also occurs in South and Central America, in South and central Asia, and in Oceania.

The percentage of undernourished people worldwide was estimated at 16% in 1990–1992, 14% in 1995–1997, 14% in 2002–2004, and at 13% in 2004–2006 [10]. It is notable that less than 5% of the total population suffered from undernourishment in the developed world. Less than 5% of the population of the developing states in Northern Africa correspondingly suffers from undernourishment. The occurrence of undernourishment is compared for other regions in the developing world over time from 1990–1992 to 2004–2006 in Fig. 8.

It is notable that most regions reported a slight decline in food insecurity, but that it still remains at



Breeding in Developing Countries and Tropics. Figure 7
The prevalence of undernourished people as a percentage of the total population in different countries throughout the world (Source: FAO [9])



Breeding in Developing Countries and Tropics. Figure 8
The frequency of undernourishment in regions within the developing world over time from 1990–1992 to 2004–2006 (Source: FAO [9])

unacceptably high levels. Sub-Saharan Africa and Southern Asia were the regions where the population was most at risk during recent years (Fig. 8).

So far the discussion has centered around trends in the global human population, livestock products, as well as the issue of food security. The animal resources at the disposal of the global community for the alleviation of food insecurity need to be considered next.

Animal Resources

Domestication

Present-day production systems depend on Livestock species domesticated in the distant past. Fifteen out of 148 non-carnivorous species weighing more than 45 kg were domesticated, while only 10 out of an estimated 10,000 avian species were domesticated [12]. The time lapses since domestication of mammal livestock (in ascending order) is estimated at 10,000 years for goats, 8,500 years for sheep, 7,000–9,500 years for cattle, 6,500 years for horses and the South American camelids, 6,000 years for donkeys, 4,500–5,000 years for camels, 4,500 years for yaks and 4,000–4,500 years for water buffalo [12]. Chickens were domesticated between 5,000 and 7,500 years ago.

Distribution of Livestock Domestication, the resultant controlled breeding and selection, and the dispersal of domesticated livestock by human migration resulted in a wealth of diverse animal genetic resources. These livestock species are widely dispersed and adapted to a wide variety of environments and production systems. The percentages of countries in regions throughout the world that reported the presence of a specific domesticated mammalian species are listed in Table 2.

The wide representation across countries among the mammalian livestock species traditionally used

Breeding in Developing Countries and Tropics. Table 2 The percentage of countries in different regions of the world reporting the presence of specific mammalian livestock species

Mammalian species	Region						
	Africa	Asia	Europe and the Caucasus	Latin America and the Caribbean	Near and Middle East	North America	Southwest Pacific
Cattle	98	96	100	94	75	100	77
Sheep	92	86	100	91	100	100	31
Goats	96	96	93	94	83	100	69
Pigs	70	82	91	91	8	100	92
Horses	46	93	91	64	58	100	23
Donkeys	38	46	36	39	50	50	–
Buffalo	8	57	25	27	25	0	0
Deer	2	25	14	9	0	50	15
Rabbit	38	39	39	48	8	0	0
Dromedary	32	25	2	0	58	0	8
Bactrian camel	0	25	5	0	0	0	0
Yak	0	32	2	0	0	0	0
Guinea pig	8	0	0	15	0	0	0
Alpaca	2	0	0	12	0	0	0
Llama	0	0	0	15	0	0	0
Vicuña	0	0	0	12	0	0	0

Source: Adapted from Rischkowsky et al. [13].

Breeding in Developing Countries and Tropics. Table 3 The percentage of countries in different regions of the world reporting the presence of specific avian livestock species

Avian species	Region						
	Africa	Asia	Europe and the Caucasus	Latin America and the Caribbean	Near and Middle East	North America	Southwest Pacific
Chicken	78	93	86	70	50	100	85
Turkeys	24	43	57	30	17	100	8
Ducks	32	61	50	33	17	0	46
Geese	16	39	61	21	17	50	8
Muscovy ducks	16	39	20	18	17	0	62
Guinea fowl	28	18	11	9	8	0	0
Pigeons	10	21	9	6	17	0	15
Quails	2	39	14	6	0	50	0
Partridges	4	7	7	0	0	0	0
Pheasants	0	7	9	6	0	0	0
Ostriches	12	11	7	0	0	0	8
Emus	2	4	2	3	0	0	0
Cassowary	0	4	2	0	0	0	0
Peacocks	0	0	0	2	0	0	0

Source: Adapted from Rischkowsky et al. [13].

for food production (cattle, sheep, goats, and pigs) is evident from Table 2. The exceptions are sheep that are somewhat underrepresented in the Southwest Pacific at 31%, and pigs that are underrepresented in the Near and Middle East at 8%. Species like buffaloes, rabbits, and deer are important contributors to food security in some regions. Other species (yaks, camels, South American camelids, and guinea pigs) also contribute to regional food security, but are extremely localized in their distribution.

Domestic chickens are the most important avian livestock species by far (Table 3). Livestock species like turkeys, ducks, geese, Muscovy ducks, pigeons, quails, and guinea fowl also contribute in most regions of the world, but are nowhere close to the role played by domestic chickens. Other species (partridges, pheasants, peacocks, and the ratite species) also contribute to regional food security, but are fairly to extremely localized in their distribution.

At ~1.35 billion head, cattle have to be considered as the most important mammalian livestock species in the World (Table 4). Cattle are closely followed by sheep (1.08 billion head), pigs (0.96 billion head), and goats (0.81 billion head). The only other species exceeding half a billion head are rabbits at 0.54 billion head. Regions housing more than 10% of the world cattle population are Asia (32%), Latin America and the Caribbean (28%), Africa (14%), and Europe and the Caucasus (11%). Sheep are mostly found in Asia (36%), Europe and the Caucasus (18%), and Africa (16%). Asia (62%) and Europe and the Caucasus (20%) house the majority of the global pig population, while the most important goat-rearing regions are Asia (62%) and Africa (22%). Rabbits are mostly found in Asia (74%) and Europe and the Caucasus (24%). Most of the world's camels are found in Africa (40%), the Near and Middle East (38%), and Asia (20%). Table 4 confirms the status of buffaloes and South American camelids as truly regional livestock species.

Domestic chickens are by far the most important avian livestock species, with a more than tenfold

Breeding in Developing Countries and Tropics. Table 4 The world's population of the most important mammalian livestock species, with the relative contribution of the different world regions as percentages

Species	Region							World population (billion head)
	Africa (%)	Asia (%)	Europe and the Caucasus (%)	Latin America and the Caribbean (%)	Near and Middle East (%)	North America (%)	Southwest Pacific (%)	
Cattle	14	32	11	28	3	8	3	1.355
Sheep	16	36	18	7	9	1	14	1.081
Pigs	2	62	20	8	0	8	0	0.960
Goats	22	62	4	4	8	0	0	0.808
Rabbits	0	74	24	1	2	0	0	0.537
Buffalo	0	97	0	1	2	0	0	0.174
Horse	6	25	13	44	0	11	1	0.055
Donkeys	27	38	4	20	12	0	0	0.041
Camels	40	20	2	0	38	0	0	0.019
Camelids	0	0	0	100	0	0	0	0.006

Source: Adapted from Rischkowsky et al. [13].

Breeding in Developing Countries and Tropics. Table 5 The world's population of the most important avian livestock species, with the relative contribution of the different world regions as percentages

Species	Region							World population (billion head)
	Africa (%)	Asia (%)	Europe and the Caucasus (%)	Latin America and the Caribbean (%)	Near and Middle East (%)	North America (%)	Southwest Pacific (%)	
Chicken	6	48	14	15	3	13	1	16.740
Duck	1	90	7	2	1	1	0	1.046
Turkey	3	1	43	18	1	33	1	0.280
Geese	1	90	6	0	3	0	0	0.302

Source: Adapted from Rischkowsky et al. [13].

advantage above ducks in the second place (Table 5). Most chickens are found in Asia (48%), while Latin America and the Caribbean (15%), Europe and the Caucasus (14%), and North America (13%) also house more than 10% of the world population.

Turkeys are found in Europe and the Caucasus (43%), North America (33%), and Latin America and the Caribbean (18%). Ducks and geese are almost exclusively found in Asia, where 90% of the world population can be found for both species.

It is also notable that most livestock can be found in the developing countries of Asia, the only exceptions being horses, camels, camelids, and turkeys (Tables 4 and 5). This is not surprising, given the high population density in this region, and the need for food produced by mammalian and avian farm animal genetic resources.

Biodiversity as Reflected by Breed Animal biodiversity is also illustrated by the number of distinct breeds that are identifiable in domestic livestock. The mammalian livestock species with the highest number of distinct breeds is sheep, followed by cattle, horses, pigs, goats, and rabbits (Table 6).

About half of the sheep breeds are found in Europe and the Caucasus, a quarter in Asia and about 12% in Africa. Regions with more than 10% of the cattle breeds are Europe and the Caucasus (31%), Asia (26%), Africa (19%), and Latin America and the Caribbean (14%). Most pig breeds are found in Asia (41%), followed by Europe and the Caucasus (32%), and Latin America and the Caribbean (12%). The regions with the most goat breeds are Asia (35%), Europe and the Caucasus (33%), and Africa (18%). Domestic chickens are the most diverse avian livestock species in terms of breed, with nearly five times as many distinct breeds than ducks in the second place (Table 7). Most chicken breeds are found in Europe and the Caucasus (58%)

Breeding in Developing Countries and Tropics. Table 6 The biodiversity, as reflected by distinct breeds, of the most important mammalian livestock species, with the relative contribution of the different world regions as percentages

Species	Region							Number of breeds
	Africa (%)	Asia (%)	Europe and the Caucasus (%)	Latin America and the Caribbean (%)	Near and Middle East (%)	North America (%)	Southwest Pacific (%)	
Sheep	12	25	48	4	5	3	3	1,129
Cattle	19	26	31	14	4	3	3	990
Horses	7	24	48	11	2	4	4	633
Pigs	9	41	32	12	0	3	2	566
Goats	18	35	33	5	6	1	2	559
Rabbits	7	8	76	7	2	0	0	207
Donkeys	14	28	28	15	11	3	2	150
Buffalo	2	73	9	9	6	0	2	132
Camels	47	24	3	0	24	0	2	97
Camelids	0	0	0	100	0	0	0	13

Source: Adapted from Rischkowsky et al. [13].

Breeding in Developing Countries and Tropics. Table 7 The biodiversity, as reflected by distinct breeds, of the most important mammalian livestock species, with the relative contribution of the different world regions as percentages

Species	Region							World Population (billion head)
	Africa (%)	Asia (%)	Europe and the Caucasus (%)	Latin America and the Caribbean (%)	Near and Middle East (%)	North America (%)	Southwest Pacific (%)	
Chicken	8	22	58	8	2	1	2	1,132
Ducks	9	38	36	11	2	0	0	234
Geese	6	24	65	3	1	0	1	166
Turkeys	13	13	42	13	4	13	2	85

Source: Adapted from Rischkowsky et al. [13].

and Asia (22%). None of the other regions had more than 10% of the chicken breeds of the world. Regions with more than 10% of the world's duck breeds are Asia (38%), Europe and the Caucasus (36%), and Latin America and the Caribbean (11%). Goose breeds mostly originate from Europe and the Caucasus (65%) and Asia (24%). Most turkey breeds are found in Europe and the Caucasus (42%), with Africa, Asia, Latin America and the Caribbean, as well as North America all contributing 13% to the global animal diversity in terms of breeds The contribution of Europe and the Caucasus to biodiversity, as reflected by distinct breeds, is higher than suggested by the contribution of this region to the overall livestock populations (Tables 4 and 5). The only exception in this respect is the turkey, where the contribution of the region to the population size and the number of breeds are roughly equal. It has been suggested that many of the breeds in this region are recognized as separate entities, while being quite similar genetically [13]. In contrast, breed recording and characterization may be constrained by technical and human resources in parts of the developing world, such as Sub-Saharan Africa. This topic will be addressed later.

The Most Important Livestock Products for Ensuring Food Security

From the foregoing, it is possible to rank the domestic livestock species in terms of their importance to human food security, based on their population size and breed diversity. There is little doubt that chickens, cattle, pigs, sheep, and goats need to be regarded as the most important livestock species in this context. More attention will thus be given to these species in the subsequent text. The importance of other regional species also needs to be considered. However, this would involve substantial additional information, which cannot be accommodated within the constraints of the present study.

Meat products are seen as important dietary sources of protein, iron, zinc, and vitamin b12 among others [7]. Meat products were related to increased live weight gains and improved cognitive function in children [14]. Meat thus has to be considered as an extremely important product for both the developed world and the developing world. Milk also provides calcium, phosphorus, vitamin B12, desirable fatty acids, and good-quality protein, and was also reported to have similar benefits to live weight and height of children under conditions where food supply is marginal [14]. The outlook for animal products (meat and dairy products) is subsequently provided per species for the major species listed above.

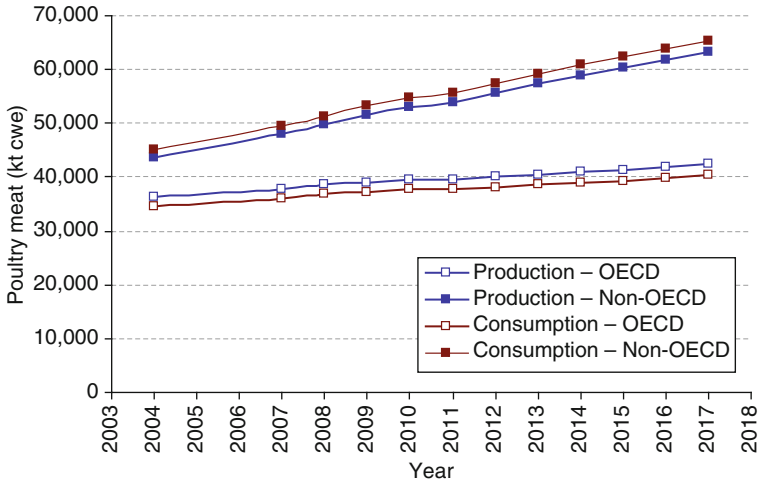
Meat from avian origin in this section is termed as poultry meat. However, with domestic chickens constituting more than 90% of avian livestock in the world (Table 5), it is safe to assume that the trends would also hold true for this species. Data were obtained from the outlook document of the OECD-FAO [11], and the results are provided for OECD-member states (representing the developed world) and non-OECD countries (representing the developing world). After considering production and consumption figures per species, attention is focused upon the per capita consumption of the various species meats.

Poultry

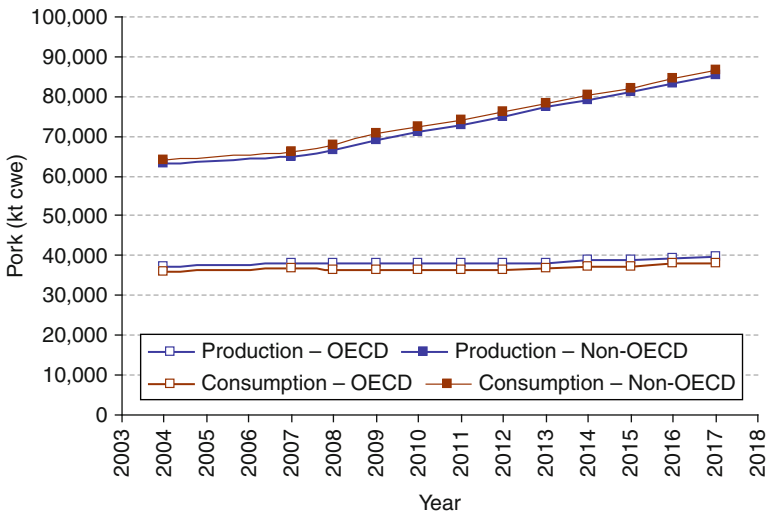
Poultry meat production in the non-OECD countries is forecast to increase steadily by 45% from ~43,600 kt carcass weight equivalent (cwe) in the early 2000s to ~63,300 kt cwe in 2017 (Fig. 9). The corresponding increase in the OECD countries is forecast to be more modest, namely, an increase of 17% from ~26,400 to ~42,400 kt cwe. Poultry consumption, on the other hand, is seen to increase by 17% from ~34,600 to ~40,400 kt cwe in OECD countries, while the concomitant increase in non-OECD countries is expected to amount to 45% (from 45,100 to 65,300 kt cwe). A deficit in non-OECD countries is evident from Fig. 9, which can be partly offset by a surplus in production in the OECD countries.

Pigs

The production of pork is expected to increase from ~63,200 kt cwe in 2004 to ~85,500 kt cwe in 2017 in the non-OECD countries, an increase of 35% (Fig. 10). OECD countries are likely to record a comparatively small increase, from ~37,100 to ~39,800 kt cwe. This increase only amounts to 7%. Consumption in the non-OECD countries is seen to increase by 36% from



Breeding in Developing Countries and Tropics. Figure 9
Poultry meat production and consumption in kiloton carcass weight equivalent (cwe) in OECD and non-OECD countries (Source: OECD-FAO [11])

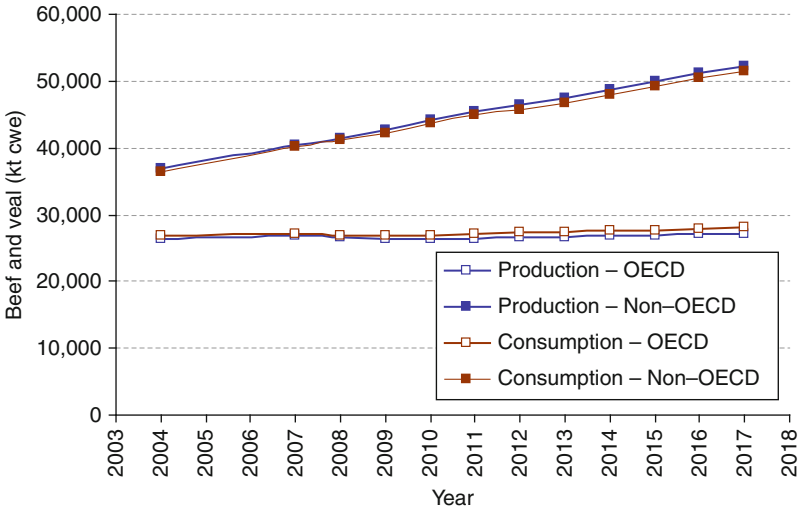


Breeding in Developing Countries and Tropics. Figure 10
Pig meat production and consumption in kiloton carcass weight equivalent (cwe) in OECD and non-OECD countries (Source: OECD-FAO [11])

63,900 to 86,900 kt cwe, while a modest increase of 7% was predicted for OECD countries (~35,800 to ~38,200 kt cwe). As for poultry meat, it was noted that a slight overproduction in OECD countries can once again be of value to counter a deficit in the non-OECD countries.

Cattle

Beef and veal production is expected to increase by 41% in the non-OECD countries, from ~37,000 kt cwe in 2004 to 52,200 kt cwe in 2017 (Fig. 11). In contrast, production in OECD countries is



Breeding in Developing Countries and Tropics. Figure 11
Beef and veal production and consumption in kiloton carcass weight equivalent (cwe) in OECD and non-OECD countries (Source: OECD-FAO [11])

forecast to be relatively stable at between 26,500 and 27,200 kt cwe. As with production, consumption of beef and veal in the non-OECD region is expected to increase by 41%, from 36,500 to 51,500 kt cwe over the period from 2004 to 2017. A slight increase of about 5% is expected in the consumption of beef and veal in the OECD countries (from 26,800 to 28,000 kt cwe). Production and consumption seem to be in closer correspondence in both regions for this commodity.

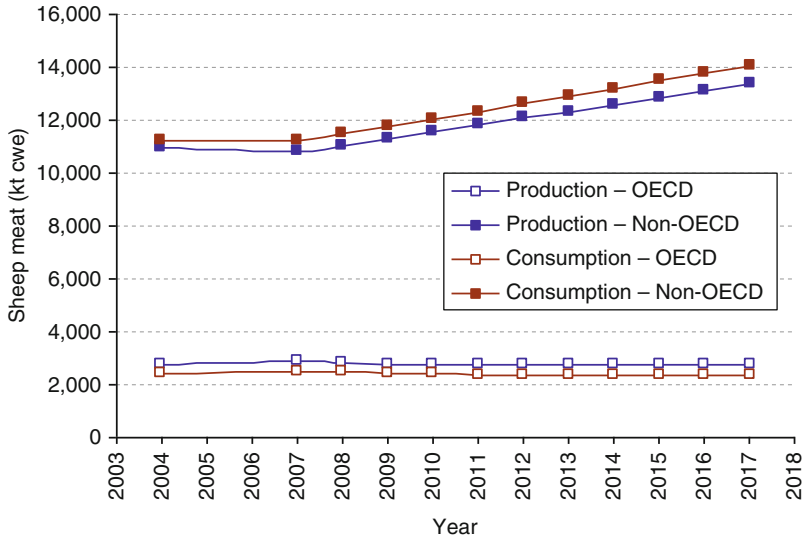
Small Stock

Global production of sheep meat (mutton and lamb) is modest compared to the other types of meat. Production in non-OECD countries is expected to increase by 22% from ~10,900 kt cwe in 2004 to ~13,500 kt cwe in 2017 (Fig. 12). No increase in sheep meat production is forecast for OECD countries, and figures are expected to remain stable at 2,300–2,400 kt cwe over the entire period. Consumption of sheep meat is expected to increase by 25% in non-OECD countries (from ~11,300 to ~14,100 kt cwe), while sheep meat consumption is expected to decline by 3% in OECD countries (from 2,417 to 2,340 kt cwe). As for other meats, surplus production of sheep meat in the OECD countries may play a role in alleviating the deficit expected in non-OECD countries.

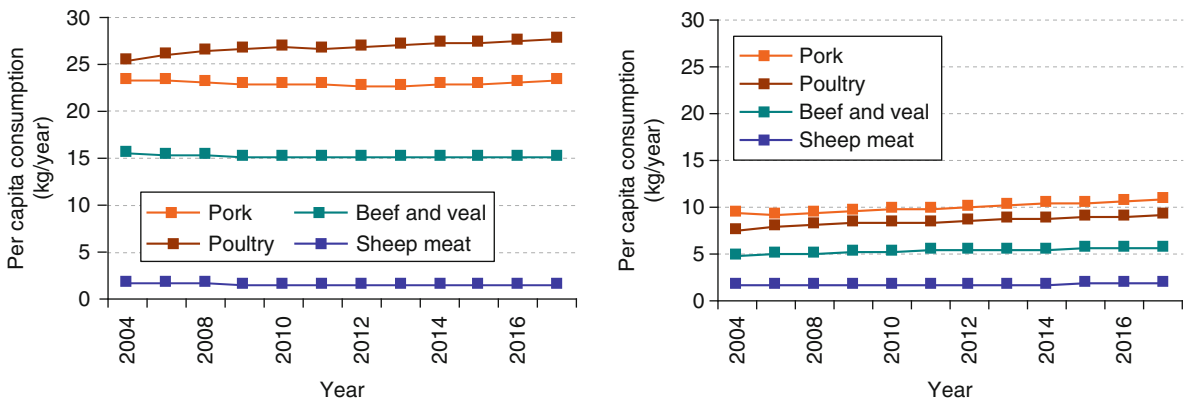
Global goat meat (chevron) production all but doubled from 2.05 million ton in 1985 to 4.05 million ton in 2005 [15]. This trend could mostly be ascribed to a 2.5-fold increase in goat meat production from 1.3 million ton in 1985 to 3.3 million ton in 2005 in Asia. Less spectacular increases from 1985 to 2005 were reported for other countries in the developing world, namely, from 0.53 to 0.86 kt in Africa and from 0.59 to 0.83 kt in South America. In the developed world, Europe led the way with a 33% increase in chevron production from 0.09 to 0.12 kt. In contrast, goat meat production in Oceania declined markedly from 0.12 kt in 1985 to 0.02 kt in 2005 [15].

Per Capita Meat Consumption

Per capita consumption of pork as well as of beef and veal is forecast to remain largely stable up to 2017 in the OECD countries, at respectively ~23 and ~15 kg/person/year (Fig. 13). The per capita consumption of poultry meat is expected to increase by 9% from 25.4 to 27.8 kg/person/year. In contrast, the consumption of sheep meat is predicted to decline by 11% from 1.8 to 1.6 kg/person/year. The ranking of the meat from the various species is consistent over years, the sequence being poultry, pork, beef and veal, and finally sheep meat. The per capita consumption of all meat types, with the exception of sheep meat, is



Breeding in Developing Countries and Tropics. Figure 12
Sheep meat production and consumption in kiloton carcass weight equivalent (cwe) in OECD and non-OECD countries (Source: OECD-FAO [11])



Breeding in Developing Countries and Tropics. Figure 13
Per capita consumption of meat from the various species in the OECD countries (*left*) and in the non-OECD countries (*right*) for the period from 2004 to 2017 (Source: OECD-FAO [11])

forecast to be lower in non-OECD countries (Fig. 13). Per capita consumption of meat from all the different species is predicted to increase over time in the latter grouping. In sequence of the importance of the different types of meat, per capita consumption is expected to rise by 15% for pork (from 9.5 to 10.9 kg/person/year), by 22% for poultry (from 7.6 to 9.3 kg/person/year), by 18% for beef and veal (from 4.9 to 5.8 kg/person/year), and by 5% for sheep meat (from 1.9 to 2.0 kg/person/year).

It is evident from Fig. 13 that the wealthier OECD countries are able to sustain high levels of protein intake from livestock. With the exception of sheep meat, developing countries are lagging behind,

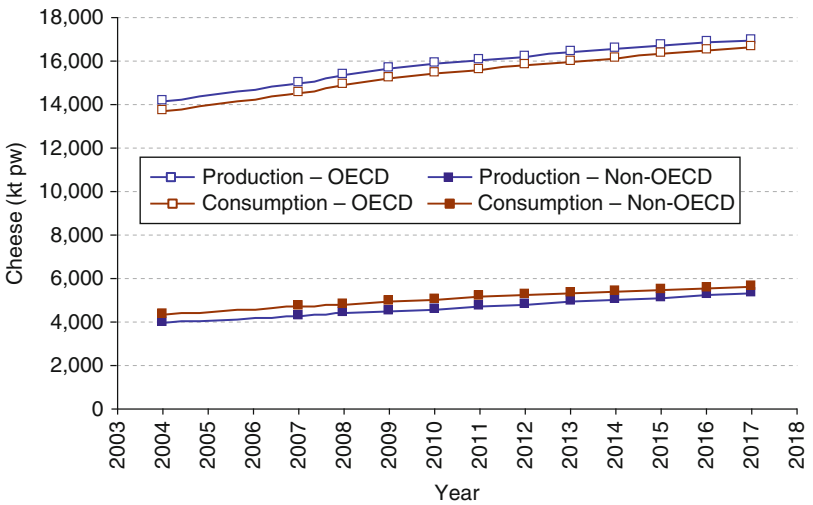
suggesting that there is almost unlimited scope for the improvement of livestock productivity for meat production in these regions. When the relatively small contribution of sheep meat in both regions is considered, it is tempting to discard this industry as an important contributor to food security. However, it is recognized that the sheep industry is able to thrive under marginal conditions where other livestock species find it difficult to adapt. Because of this, expansion in numbers (and thus product quantity) is not always easy to achieve. It is thus important to recognize the importance of this species to local food security, and also to the sustainable utilization of resources in marginal areas [8].

Dairy Products

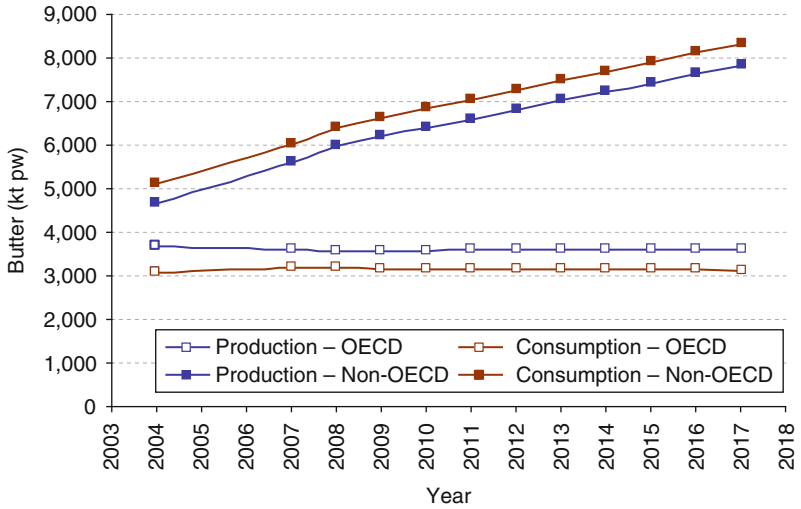
The outlook is for both the OECD countries and non-OECD countries to increase their production and consumption of cheese in the immediate future (Fig. 14). Production is expected to increase from respectively ~14,200 and ~4,000 kt product weight (pw) in 2004 to respectively ~17,000 and ~5,300 kt pw in 2017. When expressed relative to 2004 production, these changes amount to respectively 20% and 35%. Corresponding increases in consumption amount to respectively 21% and 31%, from ~13,700 kt pw in OECD countries and ~4,300 kt pw in non-OECD countries to respectively ~16,600 and ~5,700 kt pw. As for other commodities, the surplus in OECD countries is forecast to be able to assist in alleviating the deficit in non-OECD countries.

World production of goat cheese increased by 28% from 343 kt in 1985 to 438 kt in 2005 [15]. Regional production in the developing world more than tripled from 35 kt in 1985 to 122 kt in 2005, stayed constant at ~4 kt in South America, and declined by 13% from 114 kt in 1985 to 99 kt in 2005. In the developed world, European goat cheese production rose by 36% from 132 kt in 1985 to 180 kt in 2005, while production in northern and central America halved from 34 kt in 1985 to 18 kt in 2005. According to Dubeuf and Boyazoglu [15], selection of goats is limited to milk goats in developed countries. There thus seems ample scope to improve performance at all levels of the dairy goat industry in developing countries.

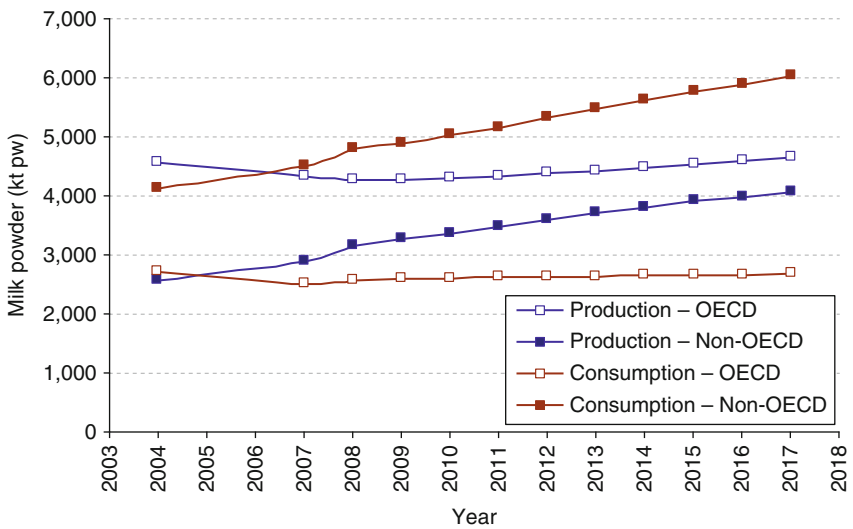
When butter is considered, it is evident that production and consumption are predicted to remain constant at respectively between 3,600 to 3,700 kt pw and 3,100 to 3,200 kt pw in OECD countries (Fig. 15). In the case of the non-OECD countries, production of butter is expected to increase by 68% from ~4,700 kt pw in 2004 to ~7,800 kt pw in 2017.



Breeding in Developing Countries and Tropics. Figure 14
Cheese production and consumption in kiloton product weight (pw) in OECD and non-OECD countries (Source: OECD-FAO [11])



Breeding in Developing Countries and Tropics. Figure 15
Butter production and consumption in kiloton product weight (pw) in OECD and non-OECD countries (Source: OECD-FAO [11])



Breeding in Developing Countries and Tropics. Figure 16
Milk powder production and consumption in kiloton product weight (pw) in OECD and non-OECD countries (Source: OECD-FAO [11])

Consumption is forecast to increase by 61% from ~5,100 to ~8,300 kt pw over the same period.

Milk powder production (the sum of skim milk powder production and whole milk powder production) is projected to increase by 58%, from ~2,560 kt pw in 2004 to 4,060 kt pw in 2017, in non-OECD countries (Fig. 16). Consumption of milk powder is forecast to increase by 46%, from 4,140 to 6,030 kt pw over the same period. Both milk production and milk consumption remained fairly constant in OECD countries over the same period. The deficit between milk powder production and milk powder

consumption in non-OECD countries is quite marked in this case. Fortunately the forecasted surplus of milk powder produced in the OECD countries will go a long way to alleviate this deficit in the non-OECD countries, as have been found for most of the other commodities.

It is evident that the production of butter and milk powder is substantially lower in OECD countries, compared to their non-OECD counterparts. In contrast, cheese production is much higher in OECD countries. This trend is understandable if it is considered that cheese is a prestigious, high-value product.

Challenges

It needs to be considered that livestock production in developing regions is faced with a number of challenges to ensure sustainability [16, 17]. These challenges need to be overcome to ensure sustainable breeding programs. A broad subdivision can be considered as challenges imposed by the environment (or external challenges) and those related to infrastructure and capacity (indicated as internal challenges).

External Challenges

Production under Various Systems It should be considered that the natural and/or cultivated plant resources form the basis for all types of livestock production. The more industrialized livestock sector relies on feedstuffs (concentrates, and roughages for dairy cows) that are in many cases imported from elsewhere for the sustenance of farmed animals. In contrast, natural and cultivated pastures are particularly important for the grazing ruminants that are able to convert low-quality roughage to nutritious protein for human consumption. Owing to a lack of control over pasture conditions in farming systems reliant on natural precipitation, periods of nutrient undersupply may occur. Persistent droughts may occur from time to time, adding to the challenges faced by tropical livestock [18]. Moreover, tropical pastures are known to have a high lignin content, particularly at the end of the growing season [19].

These afore-mentioned factors may act as constraints to efficient livestock production in developing and tropical regions. Low-potential extensive natural rangeland sustains a large portion of the well-developed sheep sector in South Africa [8]. Seasonal and unprecedented longer-term droughts are the rule rather than the exception. According to Mirkena et al. [19], adaptation of farm animals to such periods of feed scarcity can be achieved in a number of ways. Animals may develop a reduced metabolic requirement, or an ability to slow down metabolism. Digestive efficiency could be improved, while an improved utilization of low-quality roughage could be enabled. An alternative strategy could be to deposit adipose body reserves to act as a safeguard against periods of inadequate nutrient supply. As pertaining to a reduction in metabolic requirement, Silanikova [20] demonstrated that desert Bedouin goats have lower energy requirements than suggested by their body size, while they are also able to utilize low-quality roughage with a high fiber content. The latter type of goat also tolerated intake levels of ~50% below ad lib intake, compared to ~20% in Saanen goats. Localized fat reserves are found in a number of species adapted to harsh environments. Tail fat reserves of Horro and Mentz lambs were proportionally more reduced in comparison to other fat depots during periods of food scarcity and resultant loss of body condition [21].

On the other hand, Fig. 13 clearly indicates that an increasingly large portion of per capita meat consumption is expected to be produced by poultry and pigs under more intensive production systems, also referred to as industrial systems [22]. This development leads to some challenges in itself. The intensification of animals on smaller areas is feared to lead to unprecedented spinoffs, like an increase in the transmission of diseases between animals and mankind [23]. This topic is dealt with in the next section, with a number of appropriate examples. However, it is important to note that some doubts exist about the governance of some countries in the developing world to adequately deal with this potential threat [23]. According to Gummow [23], this problem is compounded by a poor immune status in many countries because of diseases like HIV/AIDS. Additionally, child nutrition was found to be inadequate in many developing countries, leading to complications like stunting and retarded growth [6], as can also be inferred from Fig. 8.

Disease It is important to recognize that disease plays an important role in livestock production throughout the world, both in developing and developed regions. There is evidence that local, indigenous cattle developed tolerance to tsetse and trypanosomis challenge in parts of Africa infested by the tsetse fly [19]. A study by Van der Waaij [24] suggested that several quantitative trait loci (QTL) have a role to play in the tolerance of trypanosomis of cattle in the African tropics. Animals imported to these regions are not likely to survive, unless a high level of husbandry and the usage of appropriate chemical treatments are applied. Infestation of farm animals with ticks in developing countries often results in death owing to tick-borne diseases [25]. Local breeds of cattle, sheep, and goats are often tolerant to the challenge provided by such diseases [19]. In this respect, it has been shown that local Small East African goats were less likely to be affected by ticks and abscesses caused by ticks than imported Toggenburg goats in Tanzania [26]. Consistent differences in favor of the local Small East African goats were found for all three species of ticks that were considered in the study.

Analyses conducted on the national database of the Bonsmara breed in the South African National Beef Recording and Improvement Scheme indicated useful genetic variation in tick numbers, particularly when the data were structured to involve environments with a higher level of tick infestation [27]. Genetic variation on the underlying liability scale was also reported for the ability of Merino sheep to resist breech strike by the sheep blowfly, *Lucilia cuprina* [28–30]. The significant genetic variation was supported by distinct line differences between Merino lines divergently selected for their ability to rear multiple offspring [31]. The challenge of animals with breech strike is controversial from an ethical viewpoint, while the transient nature of breech strike makes an adequate natural challenge under all conditions infeasible. Alternatively, it has been suggested that indicator traits should receive some attention. Traits that are considered include breech fold score, dag score, breech cover score, and crutch cover score. A number of studies suggested that all these indicator traits show significant genetic variation [28, 29, 32, 33]. In general, the indicator traits were not unfavorably related to wool and fleece traits. There is also some evidence that dag score [28, 29], breech cover score [29], and breech fold score [30] are genetically related to breech strike. Earlier work also suggested a genetic basis for body strike in sheep, as reviewed by Morris [34]. Selection for combined resistance to body strike and fleece-rot also resulted in genetic gains that accorded with expectations [35]. Infestation with the sheep body louse, *Bovicola ovis*, was accordingly reported to be heritable in a New Zealand study [36]. However, lice infestation was not conclusively associated with susceptibility to gastrointestinal nematodes.

Most species of farmed livestock are also affected by internal parasites. In this respect, marked breed differences in resistance to *Haemonchus contortus* were reported between the indigenous Kenyan Red Maasai sheep compared to the Dorper breed, an import from South Africa. Indigenous Red Maasai sheep had lower parasite burdens (as reflected by fecal worm egg counts), higher packed cell volumes as well as an improved lamb survival compared to Dorpers [37]. A later study reported small breed differences in a more arid environment with an inherently lower parasite challenge [38]. The relative advantage of the Red Maasai became evident in an environment conducive to helminth infestation, as reflected by a threefold advantage in levels of production on Red Maasai sheep compared to Dorpers [38]. Nimbkar et al. [39] reported corresponding differences in crosses of the Garole breed with the Banmur and Deccani breeds in India. These breed differences are supported by ample evidence of genetic variation in fecal worm egg counts as a measure of resistance to gastrointestinal roundworms in sheep [40, 41], as well as realized genetic progress [34, 41, 42]. Selection for lower fecal worm egg counts resulted in lower outputs of larvae on the pasture by reproducing ewes [43] and concomitant economic gains [44].

Genetic parameters and progress in other diseases of sheep were reviewed by Morris [34]. Apart from four mycotoxic diseases, the review also considered bacterial diseases like mastitis, footrot, and pneumonia. All diseases have shown moderate genetic variation, and realized genetic gains were reported for facial eczema and ryegrass staggers. Mastitis and footrot also formed part of the review by Bishop [45], indicating how the epidemiology of transmittable diseases affects options to utilize genetic variation in disease resistance in a number of species.

In dairy cattle, improved modeling and revised trait definitions are being investigated for usage in the genetic improvement of resistance to mastitis on a national scale, as based on readily available somatic cell count data [46, 47]. Similar studies are underway as pertaining to bovine Johne's disease [48] and bovine tuberculosis [49].

Additionally, cognizance should be taken of new diseases of a noninfectious nature. Gummow [23] pointed out that increased industrialization and pollution also impacted on livestock farming, and listed chronic copper and vanadium overdose as an important source of livestock deaths in the Mpumalanga province of South Africa. Other examples of noninfectious conditions listed in the latter review were the impact of environmental contaminants on the life expectancy of humans in Russia as well as air pollution in the United States. These conditions need to be understood better, while pollution needs to be brought down to manageable levels [23].

The previous discussion dealt with the importance of disease, as well as heritable resistance to known pathogens. The complexity of selection for resistance to known pathogens, as reviewed by Bishop [45], is also recognized. The relevance of the above-mentioned to the eventual outcome of the chapter will become clear in the section on future directions.

It is important to note that intensification in the more industrialized livestock species (poultry, pigs, and dairy cows) are likely to be conducive to an increased risk of infection by some diseases [23]. Owing to close contact with mankind, the transmission of some animal diseases to humans (also referred to as zoonoses) places a further dimension on the importance of livestock disease. A number of recent and historic epidemics, resulting in loss of life among animals and humans alike, have been listed by Gummow [23]. The transmission of animal diseases to humans falls beyond the scope of this review, and will not be dealt with in detail. However, it is necessary to refer briefly to recent occurrences of Bovine Spongiform Encephalopathy (better known as BSE), foot and mouth disease, and Avian Influenza to get an indication of the impact of such diseases on mankind. Avian Influenza in particular has led to recent scares, associated with a loss of approximately 200 human lives [22]. An outbreak of Rift Valley Fever during the summer and autumn of 2010 similarly resulted in substantial livestock losses [50], as well as at least 17 human fatalities in South Africa during the past year [51]. This has resulted in the suspension of raw wool exports worth an estimated 1 billion South African Rand (ZAR) to the Chinese market on August 16, 2010 [52]. The impacts of such events on livestock, humans, and the economy should be clear from the foregoing.

Heat Stress and Water Requirements The climate can influence animal production in many ways. The impact of low and unreliable rainfall patterns already received attention previously. One of the other main stressors of livestock in the developing world, and particularly in the tropics, is heat [19]. Heat stress is reflected in increases in a number of physiological responses that result in impaired production and reproduction [53]. Heat stress is aggravated by an increased humidity [19]. According to Marai et al. [53], the following temperature humidity index (THI) can be an indication of the amount of heat stress experienced by animals:

$$\mathrm{THI} = \mathrm{db}^{\circ}\mathrm{C} = [(0.31 - 0.31\,\mathrm{RH})\mathrm{db}^{\circ}\mathrm{C} - 14.4]$$

With $\mathrm{db}^{\circ}\mathrm{C}$ = the dry bulb temperature in °C
RH = the relative humidity (RH%/100w)

Values below 22.2 suggest an absence of heat stress, a value from 22.2 to <23.3 is indicative to moderate heat stress, a value from 23.3 to <25.6 suggests severe heat stress and a value exceeding 25.6 reflects extreme heat stress. It is suggested that adapted animals are able to increase their sweating rate under adverse conditions in terms of heat stress, while they are able to control heart rate and rectal temperature better than their poorly adapted contemporaries.

A Brazilian study compared a number of physiological responses of five naturalized Brazilian cattle breeds (Curraleiro, Crioulo Lageano, Pantaneiro, Junqueira, and Mocho Nacional – the former four breeds from a *Bos taurus ibericus* origin and the latter from a *B taurus ibericus* x *B taurus aquitanicus* origin), as well as the commercially available Holstein (*B taurus taurus*) and Nellore (*B taurus indicus*) breeds [54]. Of the latter two breeds, it was thought that the Holstein would be poorly adapted to heat stress, while the Nellore was thought to be well adapted. Physiological traits measured on the animals are reported in Table 8.

Breeding in Developing Countries and Tropics. Table 8 Means for physiological traits of five naturalized Brazilian cattle breeds, as well as the Holstein and Nellore breeds

Breed	Physiological trait						
	RT	RR	HR	SR	Cort	PCV	TPP
Crioulo Lageano	38.7	33.8	64.4	242	14.8	32.1	7.34
Curraleiro	38.8	31.0	70.7	251	11.6	34.3	7.34
Junqueira	38.7	26.4	69.9	234	14.7	32.7	7.40
Mocho Nacional	38.8	38.8	70.6	259	13.6	35.0	7.23
Pantaneiro	38.8	33.3	69.9	252	15.9	36.8	7.50
Holstein	39.0	32.7	62.6	258	13.8	37.7	7.34
Nellore	38.6	27.7	68.9	225	12.0	33.1	6.99

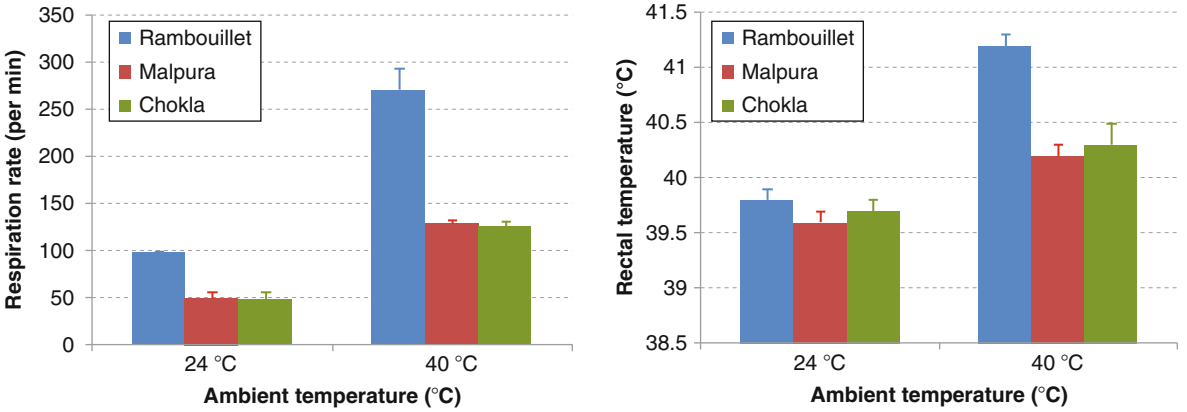
RT Rectal Temperature (°C), *RR* Respiration rate (movements/min), *HR* Heart rate (movements/min), *SR* Sweating rate (g/m/h), *Cort* Serum cortisol concentration (ng/mL), *PCV* Packed cell volume, *TPP* Total plasma protein
Source: Adapted from McManus et al. [54].

The temperatures were not extreme during the duration of the study. Maxima ranged from just above 20°C to just below 30°C, while minima ranged from ~12°C to ~22°C. Humidity ranged from ~50% to 90% in the mornings and from ~25% to ~58% in the afternoons. Although all physiological traits in Table 8 were within normal ranges for cattle, it is notable that Nellore cattle consistently had a lower rectal temperature, respiration rate, sweating rate, serum cortisol concentration, and packed cell volume compared to their Holstein contemporaries. Rectal temperature of Holstein cattle differed by 1.4°C from the morning to the afternoon, whereas all the other breeds differed by less than 1.1°C. The respiration rate of Holstein cattle also differed by 5.7 movements/min from the morning to the afternoon, compared to 0.4 movements/min in the Junqueira breed. Based on the packed cell volume and total plasma protein concentration, it was suggested that Holstein cattle were more dehydrated than the other breeds, and the Nellore breed in particular. The clear breed differences in Table 8 clearly suggest that the Holstein would be less adapted to heat than the Nellore breed, as anticipated. Of the naturalized breeds, the Junqueira seemed to be best adapted. In contrast, adaptation of the Mocho Nacional breed appeared to be the worst among the naturalized breeds.

The impact of heat stress on sheep was reviewed by Marai et al. [53]. Lambs borne by heat-stressed ewes had lower birth weight than those of ewes not subject to heat stress. The impact seems to be brought about by a reduction in embryo cell number and placentome size. Heat stress also depressed lamb growth, with solar radiation together with heat stress aggravating the situation. Exposure of sheep to heat stress conditions leads to impaired feed intake levels, as well as increases in respiration rate, body temperature, and water intake. The digestion of forage is also impaired by the exposure of sheep to heat stress conditions [53].

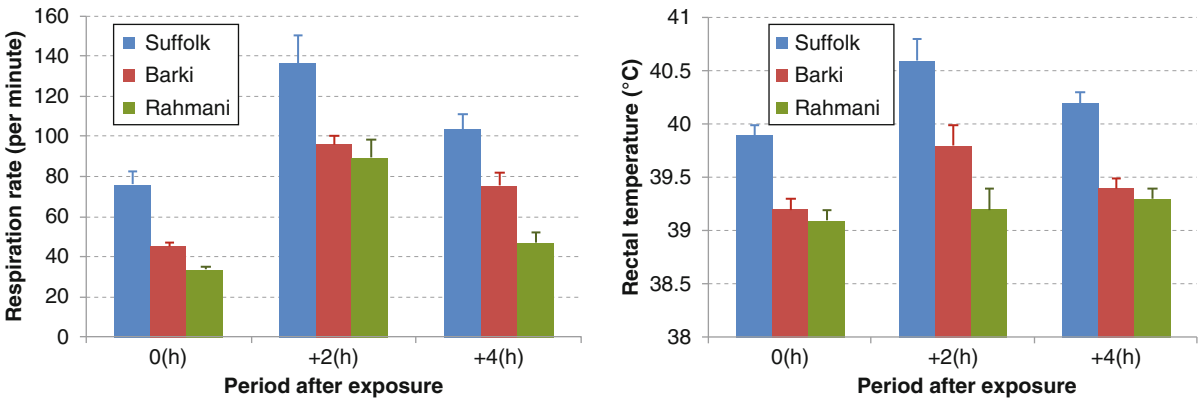
The physiological response of a temperate sheep breed (Rambouillet) was compared to those of two Indian breeds (Malpura and Chokla) at ambient temperatures of 24°C and 40°C in a study by Singh et al. [55]. The better adaptation of the Indian breeds to the ambient temperature of 40°C is evident in Fig. 17, as reflected by reduced respiration rates and rectal temperatures.

Very similar results were reported for Suffolk sheep, as representative of a temperate breed relative to local Egyptian breeds (the Barki and Rahmani) in the study of El-Sheikh et al. [56]. In this study, respiration rate and rectal temperature were studied at exposure to elevated temperatures (0 h), and after 2 and 4 h of exposure to high temperatures (respectively +2 h and +4 h).



Breeding in Developing Countries and Tropics. Figure 17

Means for respiration rate (*left*) and rectal temperature (*right*) of animals from the Rambouillet, Malpura, and Chokla sheep breeds at ambient temperatures of 24°C and 40°C (Adapted from Singh et al. [55])



Breeding in Developing Countries and Tropics. Figure 18

Means for respiration rate (*left*) and rectal temperature (*right*) of animals from the Suffolk, Barki, and Rahmani sheep breeds after 2 and 4 h of exposure to elevated temperatures (adapted from El-Sheik et al. [56])

The better adaptation of the two local breeds compared to the Suffolk is evident from Fig. 18.

The better adaptation of local breeds to conditions of excessive heat is evident from the foregoing. It could therefore be expected that animals from temperate breeds could find it difficult to adapt and thrive under such conditions. Menjo et al. [16] reported that 25% of Holstein-Friesian heifers born from artificial insemination in Kenya were lost to involuntary culling prior to their first calving. It is notable that the survival of heifers bred from semen of Kenyan, South African, and Israeli bulls had an improved survival relative to those bulls originating from Australia, New Zealand, the United States, and Canada. Conditions in terms of ambient temperature in the dairy producing areas of the latter countries are arguably cooler than those experienced in Kenya, while harsher conditions are expected in South Africa and Israel. Unfortunately, the relative milk yields of the heifers that originated from semen from the respective regions were not reported and could, thus, not be considered. Boonkum et al. [57] accordingly reported that days open were increased with an increased proportion of Holstein genes in Thai Holstein and Holstein cross cows.

The heritability of days open was slightly higher for summer calvings compared to late rainy season calvings. Susceptibility to heat stress increased with increasing parities, possibly leading to the observed high erosion rates of cows from first to third parity [58]. Whereas genetic trends for milk yield was favorable from first to third parity, second and third lactation cows became more susceptible to heat stress with time. These effects are expected to be aggravated with global warming. It is reassuring that statistical methods are sufficiently robust to rank animals for heat tolerance for selection purposes. Genomic selection is expected to improve the accuracy of evaluating young animals for selection purposes.

Many developing countries are in water-scarce areas of the world. It is therefore important to consider adaptation of livestock to water scarcity. Mirkena et al. [19] noted that breeds originating from arid countries can withstand water deprivation for longer periods than their counterparts from temperate countries. Camels are extremely well adapted in this sense, being able to withstand water deprivation for 17 days on dry feed [59] and for 30–60 days on green feed [60]. There are also donkey, goat, sheep, and some cattle breeds that are able to go without water for several days [61]. Black Bedouin and Barmer goats in the Middle East may go for 4 days without water [20]. Adapted breeds minimize water losses in urine and feces and are able to produce milk despite low water intakes. Because of the increasing strategic importance of water, production practices for the future should adopt water conservation practices [62].

The adaptation of local tropical breeds to extreme conditions was discussed in this section. The importance of this adaptation to breeding in the developing world will become clear in subsequent sections.

Internal Challenges

Infrastructure and Capacity Regions in the developing world were shown to lack infrastructure and capacity for the management of animal genetic resources (Table 9).

Between 71% and 100% of African countries had limited or no capacity in the seven categories that were considered. The situation in Asia was more variable. The central and southeastern parts had limited capacity, while conditions in the east and south were somewhat better. The capacity of the Southwest Pacific, Central America, and the Near and Middle East was also limited with 78–100% of the countries having limited or no capacity in the categories that were considered. The capacity of the South American and the Caribbean regions were intermediate in this respect, with between 30% and 70% of the countries having no or limited capacity in one or more of the categories under consideration. In contrast, the developed countries in Europe and the Caucasus and North America consistently had moderate to good capacities in 50% or more of the categories under consideration. The situation in North America in particular is good, with 100% of the countries having medium to good capacity in all categories except for the existence of laws and political programs.

The scenario set out in Table 9 led to an assessment by Brockhaus [63] that the strategic action as pertaining to genetic resource management has a sound basis in North America, Europe and the Caucasus, and, to a lesser extent, in Latin America and the Caribbean. Legislation in the former regions is well underway to regulate animal genetic resources. Weaknesses in the developing world are not only present at a strategic level but also at the basic operational and organizational levels. It thus seems that efforts to improve the vast and diversified livestock genetic resource of the developing world are constrained by several factors [64]. On the positive side, a growing level of awareness is present in the other regions [63]. This provides an opportunity to identify countries and stakeholders that may act as catalysts of development in other countries of the regions lagging behind. Local, regional, and international stakeholders that could play a role in this respect were listed by the latter literature source.

Structured Programs From Table 9 it is evident that the capacities to ensure structured livestock improvement may be lacking in most countries of the developing world. The emphasis placed on structured breeding programs for the livestock species of interest in the different regions must thus be considered next. The species most likely to be involved in structured breeding programs worldwide is cattle, followed by sheep, goats, pigs, and chickens (Table 10). Breeding of both

Breeding in Developing Countries and Tropics. Table 9 Assessment of regional capacities for the management of animal genetic resources for food production

Region	Physical infrastructure		Intellectual infrastructure		Policy development		
	Capacity	Participation	Research	Knowledge	Awareness	Laws	Implementation
Africa							
North and West Africa	92	96	87	87	87	96	96
East Africa	71	100	71	86	71	86	100
Southern Africa	82	82	100	100	91	91	100
Asia							
Central Asia	100	100	100	100	100	100	100
East Asia	50	50	75	25	50	50	50
South Asia	43	71	43	85	43	71	86
Southeast Asia	75	100	75	75	75	50	75
Southwest Pacific	91	91	91	91	91	91	91
Europe and the Caucasus	31	31	36	33	31	36	46
Latin America and the Caribbean							
Caribbean	33	67	33	33	33	67	67
Central America	78	78	78	78	78	78	78
South America	30	70	30	50	50	60	50
North America	0	0	0	0	0	50	0
Near and Middle East	86	100	85	86	86	100	100

Source: Adapted from Brockhaus [63].

The percentage of countries having no or limited capacities are listed with reference to physical infrastructure, intellectual infrastructure, and policy development. The balance of countries would have medium to high scores in these respects.

dairy and beef cattle is also considered as a priority by the largest number of countries. Regions with below 50% countries involved in structured cattle breeding programs are Africa (31%), the Near and Middle East (14%), the Caribbean and Central America (17%), and the Southwest Pacific (13%). Structured sheep and goat breeding is practiced less widely, with the only regions where programs are in place in 50% or more of the countries being Europe and the Caucasus, as well as North America in the developed world, and only the Near and Middle East in the developing World. The situation for structured goat breeding is much the same except for fewer than 50% of the countries in the Near and Middle East having programs in place. It is of interest that sheep and goat breeding was only considered as a priority in four African countries [65]. This situation is perplexing, as Africa is the continent with the third highest sheep population and the second largest goat population worldwide (Table 4). Structured pig breeding is basically only being practiced in the developed world countries in the Europe and Caucasus and North American regions. The North American region is basically the only region where structured poultry breeding is practiced by 50% of the countries. The lack of activity in pig and poultry breeding in most regions can be related to breeding in these species being mostly entrusted to international breeding companies with worldwide marketing capabilities.

Breeding in Developing Countries and Tropics. Table 10 Percentages of countries reporting structured livestock breeding programs per species in the regions of interest

Region	Chickens	Cattle	Sheep	Goats	Pigs
Africa	2	31	10	10	6
Asia	16	58	30	32	19
Near and Middle East	14	14	57	43	0
Europe and the Caucasus	23	74	59	54	62
Caribbean and Central America	8	17	17	8	8
South America	20	60	10	10	10
North America	50	100	50	50	100
Southwest Pacific	9	13	40	0	18
World	14	47	33	27	27

Source: Adapted from Thieme [65].

Tools and Strategies in Livestock Breeding Breeding in pigs and poultry is mostly centralized and practiced at low levels in the developing world. These species will thus not be covered in this section.

There is clearly a lack of activities associated with animal genetic resource management of cattle in Africa, Asia, as well as in the Near and Middle East, when compared to the situation in the developed countries of Europe and the Caucasus (Table 11). Countries in Latin America and the Caribbean are well situated as far as identification, performance recording, and artificial insemination of cattle are concerned. However, most cattle breeds in this region lack a breeding goal and a breeding strategy.

Genetic resource management of sheep breeds in Africa, Asia, and the Near and Middle East is clearly hampered by a lack of identification of individuals and performance recording (Table 10). A third of Asian sheep breeds have a breeding goal and selection strategy in place. This is in contrast with Latin America and the Caribbean, where these aspects of animal resource management are lacking. However, the latter region performs better for the recording of individual animals, artificial insemination, and genetic evaluation. Artificial insemination and genetic evaluation of sheep are in fact better placed in Latin America and the Caribbean than in the developed regions of Europe and the Caucasus. About half the sheep breeds in these developed regions have breeding goals, selection strategies, as well as individual identification and recording in place.

The situation regarding goat breeds are quite similar to that in sheep, but with generally higher levels of uptake of genetic strategies and tools in African goat breeds (Table 11). In contrast, goat genetic resource management in the developed countries of Europe and the Caucasus are generally on lower levels compared to sheep. The lower uptake of artificial insemination in small ruminants compared to bovines is notable. This trend is probably related to the lack of a reliable trans-cervical insemination protocol in the small ruminant species.

The most important stakeholder in determining breeding goal definition was identified as research organizations in all regions [65]. The identification of individual animals also appears to be mostly driven by research institutions. The exception to this rule was in Europe and the Caucasus, where it was mostly driven by breeders and governments. Recording of animal performance is mostly driven by research organizations in Africa, by governments and breeders in Asia, by nongovernmental organizations in the Near and Middle East, and by breeders in Europe and the Caucasus. Genetic evaluation is seen as the responsibility of research organizations in all regions.

Breeding in Developing Countries and Tropics. Table 11 The percentage of cattle and sheep breeds applying tools integral to livestock breeding during the process of breed improvement

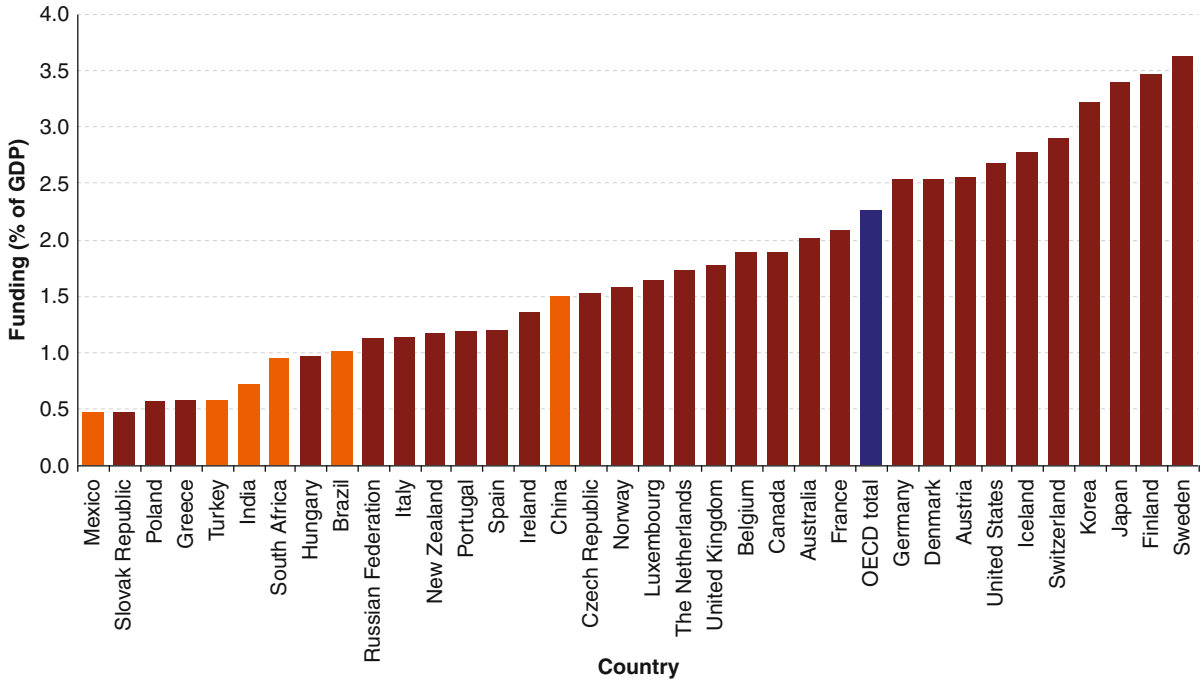
Region	Africa	Asia	Near and Middle East	Europe and the Caucasus	Latin America and the Caribbean
Cattle breeds with:					
Breeding goal	18	28	14	44	4
Strategy implemented	13	24	9	44	1
Individual identification	11	12	9	44	58
Performance recording	12	16	9	42	45
Artificial insemination	23	12	23	38	69
Genetic evaluation	9	12	5	38	24
Sheep breeds with:					
Breeding goal	14	33	16	52	5
Strategy implemented	9	33	8	50	5
Individual identification	9	2	8	45	31
Performance recording	8	2	8	45	14
Artificial insemination	2	17	0	12	35
Genetic evaluation	5	18	0	21	37
Goat breeds with:					
Breeding goal	21	12	13	28	12
Strategy implemented	15	12	13	25	12
Individual identification	18	3	6	33	27
Performance recording	21	3	13	30	22
Artificial insemination	5	3	0	5	31
Genetic evaluation	16	3	0	10	27

Source: Adapted from Thieme [65].

Crossbreeding programs to complement pure-breeding efforts were reported in all species and all regions [65]. Crossbreeding seems to be more prevalent in cattle and goats compared to sheep.

Research Expenditure The importance of research organizations in the evaluation of animal genetic resources has been highlighted in the previous section. It is therefore relevant to consider the state of research funding in countries of the developing world and in the developed world (Fig. 19). The developing countries (orange bars) with the highest research expenditure in terms of gross domestic product (GDP) is China at 1.49%. Other developing countries are Brazil (1.02%), South Africa (0.95%), India (0.71%), Turkey (0.58%), and Mexico (0.46%). Research expenditure of countries in the developed world (dark red bars) ranged from 0.47% of the GDP for the Slovak Republic to 3.63% of the GDP for Sweden. The average research expenditure for OECD countries (blue bar) amounted to 2.26% of GDP.

Figure 19 provides information on research in general. Agricultural research is often not the area of scientific development attracting the highest level of



Breeding in Developing Countries and Tropics. Figure 19
Research funding expressed as a percentage of the gross domestic product (GDP) for a number of countries. Developed countries are depicted in dark red, developing countries in orange, while the blue bar depicts the average of the OECD countries (Source: OECD [66]).

funding. Information on South Africa as an example of a fairly prosperous developing economy is provided to demonstrate this point. Of a total research budget of ~ZAR 16.5 billion in 2006/2007, funding of research and development in Agricultural Sciences amounted to 6.9% [67]. This figure was substantially lower than those for Engineering Sciences (21%), Medical and Health Sciences (15%), Information, Computer and Communication Sciences (14%), Applied Sciences and Technologies (11%) and Social Sciences (9.4%). Of the ~ZAR 1.14 billion invested in Agricultural Sciences, only ~ZAR 0.34 billion are dedicated to research on animal production and animal primary products. This amounts to approximately 2% of the overall research and development budget.

During 2006/2007, the contribution of different sectors of the South African economy to agricultural research and development (the gross value of research and development is provided in Table 12) amounted 3.0% for the business sector, 16.7% for the governmental sector, 4.6% for the higher education sector, 8.1% for nonprofit organizations and 19.0% for science councils. Compared to 2004/2005 figures, research and development expenditure increased by 37% in the business sector, by 98% in the government sector, by 30% in the higher education sector, and by 38% for science councils. No consistent increase was evident in the research and development expenditure of nonprofit organizations. The share of agricultural research remained relatively stable in the business and higher education sectors as well as in the science councils. In contrast, the percentage of governmental research and development funds devoted to agricultural research halved over the same period (Table 12). The gross value of governmental research on animal production and animal primary products increased slightly from ZAR 0.058 billion during 2004/2005 to ZAR 0.067 billion during 2006/2007. However, the percentage of the total budget devoted to these study fields declined from 11.2% to 7.0% over the same period [67]. A modest increase in the share of agricultural research was recorded for nonprofit organizations. The

Breeding in Developing Countries and Tropics.

Table 12 Changes in South African research and development expenditure for Agricultural Sciences in the recent past, expressed relative to the total expenditure on research and development in the business, governmental, and higher education sectors, as well as nonprofit organizations and science councils

Sector and expenditure	Year		
	2004/2005	2005/2006	2006/2007
Business			
Total R&D (ZAR billion)	6.766	8.244	9.243
Agriculture (%)	2.8	3.1	3.0
Governmental			
Total R&D (ZAR billion)	0.515	0.844	1.021
Agriculture (%)	33.9	18.5	16.7
Higher education			
Total R&D (ZAR billion)	2.534	2.732	3.299
Agriculture (%)	3.8	5.2	4.6
Nonprofit organizations			
Total R&D (ZAR billion)	0.198	0.227	0.212
Agriculture (%)	6.4	7.3	8.1
Science councils			
Total R&D (ZAR billion)	1.996	2.102	2.745
Agriculture (%)	19.7	18.4	19.0

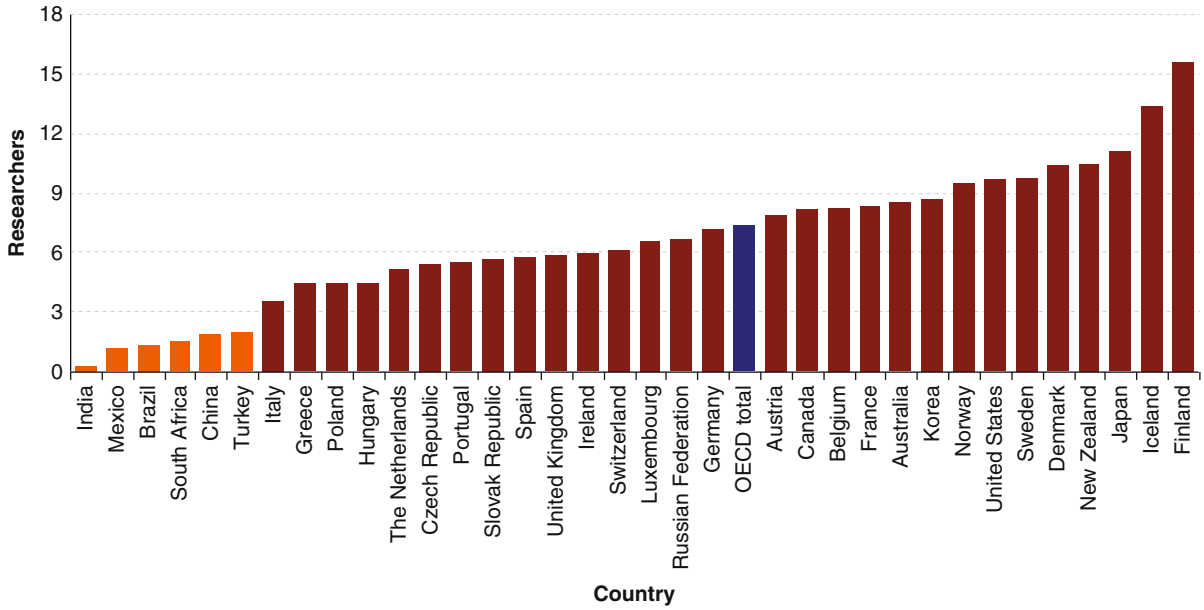
Source: Adapted from Anon [67].

contribution of this sector is probably not that important if it is considered that research by nonprofit organizations attract a relatively small fraction of the overall research funding (according to Table 12 only 1.2% of the overall research and development budget).

Given the importance of governments and other stakeholders in research in decision-making pertaining to livestock breeding [65], the reduction in the percentage of governmental funds allocated to agricultural research and development is disconcerting. If it is considered that South Africa has been identified as an emerging economy along with Brazil, Chile, China, India, Russia, and Ukraine [68], it is evident that the situation may be worse in other countries in the developing world. There thus seem to be a need for a concerted effort to consolidate funding for research pertaining to livestock genetic resources in the countries of the developing world.

Intellectual Capacity The previous heading was concerned with the funding of research in the developing world. Apart from the monetary needs, the functioning of all research organizations also depends on the number and quality of the researchers employed. The number of researchers per 1,000 members of the population in a number of developed and developing countries thus needs to be considered. It is clear that developing countries (orange bars) have substantially fewer researchers in employment per 1,000 population members when compared to developed countries (Fig. 20). The numbers of researchers per 1,000 population members in developing countries ranged from 0.3 in India to 1.9 in Turkey. In contrast, between 3.6 (Italy) and 15.7 (Finland) researchers were employed per 1,000 population members in the developed world, giving an average of 7.3 researchers per 1,000 population members for OECD member countries. The number of researchers per 1,000 population members in the developed world is thus substantially higher than in the developing world.

The discrepancy in the number of researchers per 1,000 population members is evident from Fig. 20. It is of interest to note the trend in the employment of researcher fulltime equivalents in a developing country like South Africa. Firstly, it is important to note that close to 50% of all the researcher fulltime equivalents in South Africa were employed by the business sector in 2006/2007 (Table 13). The higher education sector accounted for another 29%, science councils for 16%, the governmental sector for 6%, and nonprofit organizations for 1.6%. Overall, the number of researcher fulltime equivalents increased by 15% from 11,080 in 2004/2005 to 12,739 in 2006/2007 [67]. A similar increase was noted in the business sector, while the number of researcher fulltime equivalents in the governmental sector increased by a substantial 60%.



Breeding in Developing Countries and Tropics. Figure 20
The number of researchers expressed per 1,000 population members for a number of countries. Developed countries are depicted in dark red, developing countries in orange, while the blue bar depicts the average of the OECD countries (Source: OECD [66]).

The number of researcher fulltime equivalents in the higher education sector was fairly stable, while nonprofit organizations suffered a 13% decline in researcher fulltime equivalents. The number of researcher fulltime equivalents in science councils decreased by 15% from 2004/2005 to 2005/2006, but recovered to a level 28% higher than in 2004/2005 during 2006/2007. It is notable that researchers contributed about 50% to all research related personnel in the business sector (Table 13). Researchers formed a more commanding part of the personnel corps in the higher education sector (71–77%) and in nonprofit organizations (56–69%). In contrast, the support personnel appeared to have the lion's share of the personnel corps at the expense of researchers in the government sector (30–44%) and in science councils (31–40%). The latter similarity is not surprising, as the science councils are parastatal organizations receiving part of their budgets from the government coffers.

From the aforegoing, it can be deducted that scientists in the developed world are often faced with particular challenges relative to their counterparts in the developed world. One of these should be a heavier workload, to make up for the deficit in manpower as reflected in Fig. 20. It is therefore not surprising that South African professionals were found to spend longer times at the office compared to their colleagues in the United States, United Kingdom, and Australia in a survey conducted between June 2010 and July 2010 [69]. In 25% of the professionals interviewed, this situation was aggravated by inconsistent internet access. In view of South Africa's assessment as a developing economy [68], it is expected that the situation in many other developing countries could be worse.

It needs to be stated that the results provided in Fig. 19 and in Table 13 are for research in general and not for agricultural research in particular. Unfortunately, the information pertaining to researcher fulltime equivalents was not broken down to the level of research field as for the expenditure on research reported in Table 12. The increase in the number of researchers has to be viewed positively, noting that research and development in a country is needed for sustained growth and global competitiveness [67].

Breeding in Developing Countries and Tropics. Table 13 Changes in the number of South African researchers (expressed in full-time equivalents – FTE) in the recent past, expressed relative to the total number of FTE active within research and development (R&D) in the business, governmental, and higher education sectors, as well as nonprofit organizations and science councils

Sector and researcher fulltime equivalents	Year		
	2004/2005	2005/2006	2006/2007
Business			
Researcher fulltime equivalents (n)	5,300	5,896	6,111
Percent of personnel involved in R&D	46.9	48.2	48.5
Governmental			
Researcher fulltime equivalents (n)	491	651	784
% of personnel involved in R&D	29.5	43.9	37.9
Higher education			
Researcher fulltime equivalents (n)	3,506	3,555	3,658
Percent of personnel involved in R&D	77.1	72.1	70.8
Nonprofit organizations			
Researcher fulltime equivalents (n)	234	198	203
Percent of personnel involved in R&D	64.5	69.2	55.9
Science councils			
Researcher fulltime equivalents (n)	1,549	1,323	1,983
Percent of personnel involved in R&D	31.0	32.2	40.0

Source: Adapted from Anon [67].

On the other hand, it has already been shown that the emerging economy of South Africa invested only 6.9% of the total research and development budget in agricultural research (Table 12). As high-level researchers are often attracted to better funding opportunities, it is likely that the agricultural sector could find it difficult to recruit and retain the most promising young scientists. Moreover, the increase in the number of researcher full time equivalents is unlikely to be met with immediate success, as its impact still needs to filter through with time.

Against this background, it is not surprising that animal resource management is suboptimal in most countries in the developing world, as depicted in Tables 9–11. Therefore it is important to consider ways to ensure that services with respect to animal resource management are delivered to producers in these regions, given the important contribution of livestock to food security and rural sustainability. In this process it is fitting to have a look at how the developing world contributes to the global science output.

The Contribution of Developing Countries to Global Science The recent 9th World Congress on Genetics Applied to Livestock Production (WCGALP) was held from August 1 to August 6, 2010, in Leipzig, Germany. WCGALP provides an opportunity to take stock of global research efforts involving livestock production every 4 years. The relative contribution that stemmed from scientists in the developing world can be assessed simultaneously. The country of origin of the first author of each abstract of oral contributions during concurrent sessions and of posters displayed during poster sessions was recorded and summarized in

Breeding in Developing Countries and Tropics. Table 14 Contributions to the concurrent paper or poster sessions of the 2010 World Congress on Genetics Applied to Livestock Production per topic, with the contributions from developing countries as percentage

Topic	Papers[a]		Posters[a]		Total[a]	
	N	%	*N*	%	*N*	%
Species breeding	164	11.0	181	49.2	345	31.0
Genetics of trait complexes	101	4.0	79	27.8	180	14.4
Methods and tools	90	4.4	46	30.4	136	13.2
Genetic improvement programs	96	12.5	52	32.7	148	19.6
Special topics	24	12.5	2	0	26	11.5

[a]Number of contributions within categories (*N*) and percentage contributed by the developing world (%).
Source: Adapted from WCGALP [70].

Table 14. None of the eight plenary lectures that were presented during the congress was by scientists or groups of scientists from the developing world. The contribution of the developing world to papers presented during concurrent sessions will be considered next. The contribution of scientists from the developing world ranged from 4% in the case of the topic "genetics of trait complexes" to 12.5% for the topics "genetic improvement programs" and "special topics" (Table 14). Cases where contributions from the developing world exceeded 10% of all contributions within subtopics will be noted subsequently. Within the topic of "species breeding," the developing world contributed most toward "small stock breeding" (33.3% of 21 papers), "poultry breeding" (20.0% of 20 papers), and "aquaculture" (18.8% of 16 papers). The contribution of scientist groups in the developing world to small stock breeding should probably be seen in context with the projected and current trends in sheep meat in Fig. 12. Pertaining to the topic "Genetics of trait complexes" one of nine papers on "behavior" (11.1%) was presented by scientists from the developing world. The developing world does not seem to be active on the topic "methods and tools" as no subtopic attracted 10% of more of the papers in this case. When the topic "genetic improvement programs" was considered, groups from the developing world contributed most papers to the subtopic "breeding objectives and economics of selection schemes" (40% of 10 papers), "management of genetic resources" (26.1% of 23 papers) and "selection for harsh environments (e.g., the tropics)" (11.1% of 9 papers). Given the location of most developing countries, it is somewhat surprising that the major contribution of the latter subtopic still seemed to originate from the developed world. A notable lack of activity was observed for the subtopics "advances in selection theory, including experimental demonstrations" (0 of 7 contributions), "selection using molecular information" (1 out of 39 contributions) and "design of selection schemes exploiting additive and/or nonadditive effects" (0 out of 8 contributions). Within the "special topics" category, scientists from developing countries contributed to the subtopics of "animal breeding and the environmental challenges" (22.2% of 9 contributions) and "education and training" (14.3% of 7 contributions). Overall scientist groups from the developing world contributed 41 out of 475 papers during concurrent sessions, or 8.6% of the total number of papers. Developing countries contributing three or more of these papers were Brazil (8), South Africa (5), Iran (4), Israel (4), and Kenya (3).

The contributions of scientist groups from the developing world to the poster sessions are much more evident (Table 14). About half of the posters on the topic "species breeding" originated in the developing world, while between 27.8% (genetics of trait complexes) and 32.7% (breeding objectives and economics of selection schemes) of contributions were made by scientist groups in the developing world. Both posters

on special topics were presented by scientist groups of the developed world.

Subtopics with more than 30% contributions from the developing world are listed next. The contribution of the developing world to posters on the topic of "species breeding" amounted to 73.7% for "poultry breeding" (of 19 contributions), 71.4% for "beef cattle breeding" (of 42 contributions), 62.5% for "small stock breeding" (of 16 contributions), 58.7% of "dairy cattle breeding" (of 46 contributions), and 38.5% for "aquaculture" (of 13 contributions). When the topic "genetics of trait complexes" was considered, it was found that posters originating from the developing world exceeded 30% for the subtopics "growth, development, feed intake and efficiency" (55.6% of 18 contributions), "lactation" (42.9% of 7 contributions), and "behavior" (33.3% of 3 contributions). Under the topic "methods and tools," the contributions of scientists from the developing world exceeded 30% for "functional genomics and systems biology" (37.5% of 8 contributions), "statistical methods – linear and nonlinear models" (33.3% of 15 contributions) and "software and bioinformatics" (33.3% of 6 contributions). When the topic of "genetic improvement programs was considered, contributions from developing world exceeded 30% for "breeding objectives and economics of selection schemes" (50% of 10 contributions) and "management of genetic resources" (33.3% of 15 contributions). It is evident that scientists from the developing world were more likely to make their contributions by the way of posters. It could be by own choice in many instances, while the available slots for oral presentations could simply have been filled by contributions from developed countries in other cases. Of the 142 posters presented by scientists from the developing world, five or more posters were contributed by Brazil (57), China (17), Mexico (14), South Africa (12), Iran (11), and Kenya (5). The overall contribution of developing countries thus ranged from 11.5% for the topic "special topics" to 31.0% for the topic "species breeding" (Table 14).

Of the 837 contributions presented in concurrent sessions or as posters during WCGALP, 183 (or 21.9%) originated from the developing world. Most of these contributions were from Brazil (65), China (19), South Africa (17), Iran (15), Mexico (14), and Kenya (8). In total, 1,382 delegates from 60 countries attended the Congress. Of these, 229 delegates (or 16.6%) were from 32 developing countries. Developing countries represented by more than 10 delegates were Brazil (67), China (39), South Africa (19), and Mexico (19). It is notable that the average number of contributions per delegate were 183/229 = 0.799 for the developing world and 654/1,153 = 0.567 for the developed world. It thus seemed as if the scientists from the developing world were at least as willing to contribute to the scientific program as their peers from developed countries. Alternatively, a contribution at the congress could be a prerequisite for attendance for many of these scientists, given the limited funds that are available for these scientists (see Fig. 19).

From the aforegoing it is evident that the quantity and depth of research in the developing world make it difficult for scientists from these countries to compete with peers in the developed world. Kahi [64] also noted that studies from the developing world were underrepresented at the WCGALP. The fact that science groups in the developing world were much less likely to contribute papers on molecular genetics and genomic selection was also highlighted in the latter paper. More than a third of contributions at the WCGALP reported results of genomic selection [71]. The trend toward an increased investment in genomic selection is discussed later under the section "The technological age." However, the role of scientists in the developing world is anticipated to decline if they are not able to become involved in this study field. As noted before, the disparity in research funding and the availability of high-quality manpower (Fig. 20) doubtlessly contribute to this finding. However, it is also clear that a number of dedicated scientists from the developing world (based on the average number of contributions per delegate) still present their research findings at international forums. The capacity for change thus exists with such scientists acting as catalysts for the application of sound scientific applications in the developing world.

The Case for Conserving Animal Genetic Resources

It is estimated that 190 of approximately 7,600 livestock breeds became extinct over the past 15 years [72]. Roughly 60 breeds of cattle, goats, pigs, horses, and poultry were lost in the past 5 years. This section asks whether this should be a cause of concern or not.

Moreover, it should be debated whether the livestock breeds of the developed world should be treated as a strategic resource, and if so, why?

As an initial comment, it needs to be stated that the share of the developing world in global animal genetic resources trading is minimal [73]. Detailed records were available for trade flows of cattle semen, live cattle, and live pigs. Data for poultry, small ruminants, and aquaculture species are unfortunately scant. The bovine semen trade is dominated by OECD countries. Bovine semen is mostly exported by the United States (32.6% of the trade), Canada (31.5%), the Netherlands (7.4%), France (6.2%), Germany (5.6%), the United Kingdom (3.8%), Italy (2.5%), and other OECD countries (9.2%). In contrast, the share of developing countries in bovine semen exports is minimal at just over 1% of global trade. Developing countries exporting bovine semen are South Africa (0.5%), Argentina (0.3%), Brazil (0.3%), and others (0.2%). Much the same situation is in place for live cattle for breeding, where the contribution of the developing world amounts to only 7.5% of the global trade [73]. The contribution of the developing world to the trade of live pigs for breeding amounts to approximately 5%. The bulk of gene flow in farm animal genetic resources takes place between developed countries (60–70%). The rationale is that farming in these countries developed to depend on high-performance operations, operating at high levels of intensity and accuracy and at a reduced environmental variability [73]. It is argued that farm animal genetic resources from these systems should be adaptable to production systems worldwide. Based on this assumption, it is not surprising that gene flow from the developed countries to the developing countries constitutes the second most important trade pathways, accounting for between 20% and 33% in the trade of farm animal genetic resources. The exportation of genetic material from the developing world to the developed world constitutes between 1% and 2% of the global trade. There is also modest trade in farm animal genetic resources among developing countries, particularly with reference to low-input production systems [73].

A number of instances where genetic material from developing countries has been introduced to developed countries were reviewed by Blackburn and Gollin [74]. Neither Chinese Meishan pigs nor Zimbabwean Tuli cattle were able to penetrate the commercial livestock market in the United States. Although the Meishan pigs were prolific, unwanted growth and body composition traits led to a realization among pig geneticists in the United States that they could select for prolificacy within local pig breeds, without a need to cater for the unwanted characteristics of the Meishan. Tuli cattle similarly did not perform satisfactorily in terms of growth and feedlot performance, despite a good ranking for biological efficiency compared to Angus/Hereford cows. Interest in the breed thus dwindled, with only 150 registrations in 2000. In contrast, the South African Boer goat gained a foothold in the commercial goat market based on an improved body size, faster growth rate, and an improved conformation compared to local Spanish goats, which formed the basis of United States chevron production prior to the importation of Boer goats [74]. Adoption of the breed is supported by ~45,000 registrations in 2005, but Boer does were found to have a lower reproduction capacity than Spanish and Kiko does [75].

Against this background, it does not seem as if farm animal genetic resources of the developing world have a major role to play in the high-output production systems of the developed world. It even seems worthwhile to consider replacing the relatively low output farm animals of the developing world with more productive genotypes from the developed world. However, it has clearly been shown that these genotypes do not always perform according to expectations because of susceptibility to heat conditions [53–56], and typical diseases [24, 26, 37, 38] in the developing world. The often unpredictable environmental conditions inherent to farming systems in the developing world also contribute to a lack of adaptation of exotic genotypes [16, 19] under typical farming conditions in the developing regions. Samdup et al. [76] recently established that the crossing of indigenous dairy cattle in Bhutan with high-yielding Jersey cattle, as well as purebred Jersey cattle, resulted in a milk off-take of 2.4–4.6 times that achieved by local cows. However, the higher production levels of Jersey and Jersey crossbreds were offset by poor survival as well as low reproduction rates. Although crossbreeding contributed to higher livestock gross margins in the intensive areas, it has not yet resulted in meeting the demand for dairy products in Bhutan.

The downside of uncontrolled crossbreeding in farm animal genetic resources in the developing world is that the valuable adaptation traits like disease resistance may be lost. Based on the analysis of microsatellites, there is evidence that the genetic basis of the gastrointestinal nematode–tolerant Red Maasai breed is being eroded by crossing, predominantly with the exogenous Dorper breed [17, 77]. Based on the results of Baker et al. [37, 38], the ability of the Red Maasai to tolerate internal parasites may be lost in this way. Urgent intervention to halt the process has been advocated by Ole Kwallah et al. [77]. Fortunately, a nucleus flock under governmental control is still available to use in this process [17]. When Scholtz et al. [78] surveyed the South African beef cattle population in the communal sector, a high percentage (35%) of bulls that were used for breeding purposes were classified as nondescript or crossbred. This classification included the highest percentage of bulls, followed by the Nguni (22.5%), Brahman (18.2%), Afrikaner (9.9%), Bonsmara (5.1%), Drakensberger (2.8%), Simmentaler (2.1%), and various other breeds (4.4%). This observation also draws attention to a possible loss of genetic diversity because of random crossbreeding in the communal resource herds.

In this context, it is important to draw attention to fears that global livestock biodiversity will be compromised by the present animal breeding practices. These fears are supported by many breeds in different parts of the world with a lack of complete population data, as summarized in Table 15. It is clear that population data are not available for many breeds in the developing regions. Among the developing countries, information for Asia does not lag as far behind the developed world as for other developing regions, like Latin America and the Caribbean. The implication of this is that conservation status of many breeds is doubtful, simply because there is no information. According to Cardellino and Boyazoglu [79], the risk of extinction is not known for 30.0% of cattle breeds, 33.8% of goat breeds, 30.4% of pig breeds, and 29.6% of sheep breeds. Between 3.1% (goat) and 18.9% (pig) of breeds in all these species are already extinct, while between 12.7% (sheep) and 18.0% (pig) breeds are classified as at risk. The classification of no risk applies to 38.1% of cattle breeds, 49.5% of goat breeds, 32.6% of pig breeds, and 44.9% of sheep breeds. Against the background of these realities, it is important to consider ex situ conservation in the form of embryos and semen in some cases [80, 81]. The Brazilian animal germplasm bank has 60,000 doses of semen, more than 250 embryos, as well as over 700 DNA samples at present [81].

Breeding in Developing Countries and Tropics.

Table 15 The percentage of breeds with population data in the respective world regions according to class (*Mammalia* or *Aves*), and in total

Region	*Mammalia*	*Aves*	Total
Africa	32.5	21.4	30.5
Asia	55.9	41.4	52.5
Europe and the Caucasus	67.2	58.4	64.6
Latin America and the Caribbean	12.9	13.7	13.1
Near and Middle East	42.5	23.3	39.3
North America	61.3	97.8	69.3
Southwest Pacific	22.1	15.2	20.4
World	**47.2**	**44.2**	**46.5**

Source: Adapted from Cardellino and Boyazoglu [79].

It is hypothesized that biodiversity in farm animal genetic resources is needed to ensure that it is possible to react to major changes possibly brought about by major events, like global warming. Against this background, a plan was drawn up to conserve the existing animal genetic resources, and to ensure that adapted genetic resources remain available for the smallholder systems typical of the developing world [82]. In accordance with this quest, Villanueva et al. [83] developed an indicator of farm animal biodiversity. The system was applied to British sheep and cattle breeds, and allowed inferences that the biodiversity in cattle at least actually increased from 2001 to 2008. A similar trend in sheep was negated somewhat by an increased variability in the data. It has also been reported by Djemali et al. [84] that the Sicilo-Sarde Tunisian dairy sheep breed has actually been resurrected in the recent past, after having been on the verge of extinction.

These arguments lead to the important topic of conserving farm animal genetic resources in the

developing world. According to Cardellino and Boyazoglu [79], such efforts should incorporate the recording of phenotypic performance in local breeds. According to the latter authors, the use of molecular information has possibly been overemphasized in conservation efforts of genetic resources in the developing world in the past. The role that molecular characterization may play in the conservation of farm animal genetic resources is well understood, and described by Toro et al. [85]. The point made by Cardellino and Boyazoglu [79], however, is that efforts for animal breeding for the genetic improvement of populations have been compromised to some extent. The basic principles of recording phenotypic data and pedigree information in livestock production systems have been known for a long time [86], so there is no need to elaborate on this. However, this is not done on a routine basis in the developing world. While major gains have been achieved in livestock production systems in the developed world, the same cannot be said for the developing world. Aspects like routine recording of phenotypes and pedigrees become a major effort under communal and smallholder production systems [79]. This results in little genetic gain being achieved. This may be a blessing in disguise, as there are many burning questions in this respect that still need to be answered [79]. Some of these questions pertain to the following:

- Standard interventions aimed at improving production may be counterproductive.
- Genetic and environmental effects may not be adequately researched in harsh environments.
- The magnitude and direction of genotype by environment interactions in unfavorable environment may differ from those in good environments.
- All the crucial adaptive traits may not be recognized.
- Selection could move populations off their adaptation equilibrium and make matters worse.

These challenges should be addressed before major interventions are applied. There are thus a number of research opportunities to address these challenges in future [79]. Before these can be addressed, however, it is necessary to have a closer look at the successes and failures of programs that were implemented in developing countries in the past.

Animal Breeding in the Developing World: Successes and Failures This section summarizes existing breeding programs, and what could be learned from them. It was attempted to cite most examples from the developing regions, but comparisons with the developed world are unavoidable. Readers will notice that this section is biased toward experience with farm animal genetic resources in South Africa. The reason for this is that a well-developed commercial sector exists in South Africa alongside low-input systems with communal land use. With a producer support estimate of approximately 4% in comparison to the range of estimates from below 5% (in Australia and New Zealand) to more than 70% (in Iceland, Norway, and Switzerland) in OECD countries [8], the South African commercial livestock industry is relatively efficient and in many ways comparable to livestock industries in the developed world.

As a point of departure, it has been established that animal breeding in developed countries has resulted in major and verifiable contributions in improved livestock production, resulting in the demands of consumers being met in a cost effective and sustainable manner [87]. Genetic advances in the more intensive pig, poultry, and dairy industries are often cited as examples of what can be achieved by a structured breeding program for well-defined selection objectives. Recent results pertaining to the well-established Sheep Genetics Australia scheme indicated that participants make progress at an average rate that is comparable to what is attainable in theory in the dual-purpose and terminal sire schemes [88]. Wool sheep farmers are less successful and are progressing at a rate of ~30% of what is attainable in theory. Advances such as those reported by Swan et al. [88] for dual-purpose and terminal sire sheep breeds have led to selection objectives in developed countries having changed from production traits to traits associated with animal welfare, product safety, and preferences by consumers [65].

It is evident from the foregoing discussion that such advances have not been realized in the developing world. However, there are examples of animal improvement schemes in developing countries that are benchmarked with international schemes. An example is the South African dairy cattle scheme participating in Multiple Across-Country Evaluations (MACE). Test-day databases for the South African Ayrshire, Guernsey,

Holstein, and Jersey breeds were harmonized with those of other participating countries [89, 90], to allow participation of the local dairy industry in MACE. Bovine semen produced in South Africa has a small international market share of 0.5% [73].

In contrast, dairy contributed only about 10% to the reason why emerging and communal farmers keep cattle [91]. Communal farmers mostly milked nondairy breeds like the Nguni (34%), Brahman (22%), Afrikaner (20%), and Bonsmara (10%) under free-ranging conditions. Traditional dairy breeds like Friesians and Jerseys only contributed 3% to communal cattle used for milk production. Herd size averaged 6 for dairy cattle and 11 for dual-purpose breeds. At 16%, the percentage of traditional dairy cows was somewhat higher for emerging farmers, but most of the cows milked were still from nondairy breeds. Herd size averaged 39 head for dairy cattle and 42 head for dual-purpose cattle in this sector [91]. Details of levels of production were not provided but it is assumed to be fairly low. In this respect, McDermott et al. [2] identified the dairy and small stock enterprises in small-scale farming systems as amenable to intensification.

There is evidence that breeding decisions peculiar to regions and managerial capacity of farmers may hold advantages in the dairy production enterprises of developing countries. In this respect, it was demonstrated that the continuous importation of semen may not be economically viable in the Kenyan dairy production system, where smallholders living below the poverty line own 70% of the dairy cattle [92]. Systems where semen from local bulls was used were consistently more profitable than those relying predominantly on imported semen. Arguably this effects could result from better adaptation to environmental conditions, as more daughters of Kenyan, Israeli, and South African artificial insemination bulls survived involuntary culling under Kenyan conditions [16], as discussed previously. Based on the parameters of the mechanistic lactation functions, it was contended that lower-yielding Ayrshire cows were better adapted to low- and medium-input systems in Kenya than Holstein-Friesian cows [93]. In contrast, the latter breed would have distinct advantages in high-input production systems where the levels of management and husbandry were high. Finally, it should be noted than India became the world's largest producer of milk, mostly through smallholding systems [94]. It is thus clear that substantial quantities of milk could be produced by small-scale farmers. This appears to be the case particularly in countries like Kenya and India, where there is also substantial private sector involvement [94].

The bulk of agricultural land in South Africa is not arable. Large areas are thus suitable only for extensive grazing, mostly by beef cattle in the north-eastern part and by sheep in the southwestern part [8]. The commercial breeding sector of the South African beef industry is serviced by the National Beef Recording and Improvement Scheme managed by the Agricultural Research Council (ARC), as described by Bergh [95]. After a peak of ~450,000 weights in the mid-1980s, this Scheme annually recorded between ~170,000 and ~260,000 weights since 1992. Breeding values for a range of growth/production, size, and reproduction traits are available to breeders [96]. Additional reproduction traits have been developed for inclusion in the Scheme recently [97], indicative of continuous development according to consumer specifications. It is estimated that nine beef breeds (Afrikaner, Beefmaster, Bonsmara, Braford, Charolais, Drakensberger, Gelbvieh, Angus, and Sussex) have at least 80% of the available females participating in the recording scheme [78]. As for other schemes managed by the ARC, real-time information to producers is available on the Internet [95]. Other beef breeds in South Africa are being provided by similar services by Breedplan® International [98], providing a link to international genetics. At 0.8%, South Africa controls a small portion of the international trade in live cattle exports for breeding [73], placing the country in the third position after Panama (4.1%) and Lithuania (1.0%) among developing countries.

At 47%, the sale of live animals and beef was listed as the most important reason for keeping cattle in the communal cattle sector of South Africa [78]. Cattle were also listed as being important for investment (15.4%) and cultural and ceremonial practices (13.3%), while the production of milk (10.2%), draft power (4.1%), and other reasons (4.5%) were of secondary importance. The average herd size in the communal/emerging sector was 19 head, as compared to 413 head in the commercial sector [78]. The most important attributes looked for a bull by communal farmers were size (33.1%), followed by conformation

(22.0%) and performance (18.9%). When ranked by emerging farmers, the attributes were ranked somewhat differently, being performance (30.3%), size (23.5%), and conformation (19.3%). Corresponding attributes in the commercial sector were performance (33.2%), conformation (11.1%), and temperament (9.8%). Only 1.9% of communal and 36.8% of emerging farmers used a controlled mating season, compared to 88.6% in the commercial sector. Artificial insemination was used by 0.1% of communal farmers, 6.3% of emerging farmers, and 21.9% of commercial farmers [78]. Guidelines for the participation of communal farmers to the National Beef Recording and Improvement Scheme are available [99], but uptake is low.

In sheep, across flock genetic evaluation results have been reported for the South African Dorper breed [100], the South African Merino breed [101], and the South African Dohne Merino breed [102]. The South African Small Stock Scheme has recently been compared to the evaluation scheme provided by Sheep Genetics Australia to Australian livestock producers [8]. The schemes do not differ appreciably for the basic production traits (live weights, wool and fiber traits, as well as for reproduction), which are recorded on farm by livestock producers. Differences between the schemes involve traits associated with disease resistance (fecal worm egg counts and traits associated with blowfly challenge) and carcass quality traits (ultrasonic muscle and fat dimensions) which are recorded on a national basis in Australia, but not in South Africa. The personnel to record these traits are not available in the South African scheme [8], and it is therefore only recorded in experimental flocks. The shortage in qualified personnel is not unexpected if the trend in terms of researchers are compared (Fig. 20 and Table 13). Australia boasts 8.5 researchers per 1,000 community members, compared to 1.5 researchers in South Africa. With almost a sixfold advantage it is not surprising that the former country has the capacity to allow the recording of the additional traits on a national basis. Moreover, if it is considered that the investment in research on animal production and animal primary products constitutes only 2% of the national research budget in South Africa [67], it is clear that scientists in South Africa are under immense pressure to deliver services comparable to that in developed countries like Australia.

Still there is reason for positive thinking. Overall, the South African scheme recorded ~81,000 weights and ~33,000 wool records (including a fleece testing service) for 2006-born animals with four professional staff and 10 auxiliary staff [103]. It was also shown that the recording of weights increased by about 50% from 2003 to 2006 in the scheme. These records were derived from 13 breeds, but slightly more than half came from the two major wool breeds (Merino – 31.4% and Dohne Merino – 23.9%) and a further quarter from the Dorper meat breed (24.2%) [8].

Sustained improvements in the relative economic value of breeding animals have been reported in the South African Merino breed [101] and in other South African breeds [103]. Leading studs in almost all breeds showed additive genetic gains comparable to those achieved elsewhere in the world. It is important to note that similar sheep breeding schemes are operative elsewhere in the developing world. Across-flock breeding values are thus available on the Internet for several sheep-producing countries in South America (see review by Cardellino and Mueller [104]). Similar reports were forthcoming from developing countries in South America, Western Africa, and India [105]. Kosgey et al. [18] reviewed a number of small stock projects in the tropics. Objectives strived for mostly involved growth, live weight, disease resistance, and traits associated with reproduction. An Indian project that was highlighted, involved the introgression of the fecundity gene from the Garole breed into the Deccani breed. Failed programs mostly lacked farmer participation [18]. Predicted rates of genetic gain from simulations in Ethiopian sheep breeds were generally in good agreement with actual responses in on-station research [106]. These predictions are in the process of being validated in four communities from different Ethiopian agroecological systems, based on community participation. Traditional Brazilian sheep breeding systems generally used appearance as the main criterion for selection. Recently a recording and genetic evaluation scheme named Ovigol® was launched to provide progressive breeders with the option of selection for traits linked to the profitability of their operations [107]. Uptake during the first 18 months was good, with records of 5,195 performance recorded animals of 13 breeders having been entered into the system.

Breeding in Developing Countries and Tropics. Table 16 Relative performance of commercial and communal rams on properties in the commercial and communal areas of South Africa

Trait	Commercial properties		Communal properties	
	Commercial rams	Communal rams	Commercial rams	Communal rams
Clean fleece weight (kg)	2.8 (100)	2.4 (86)	2.0 (100)	1.7 (85)
Fiber diameter (μm)	18.4 (100)	20.0 (109)	19.4 (100)	20.5 (107)
Clean yield (%)	76.3 (100)	68.5 (90)	66.9 (100)	60.5 (90)
Wool income (ZAR/head)	92.67 (100)	60.09 (65)	54.87 (100)	37.53 (68)

Source: Adapted from Marais [108].
Figures in brackets are expressed as a percentage of performance of commercial rams.

The genetic merit of rams from the South African commercial sheep breeding sector was compared to that of the communal sector on commercial and communal properties [108]. Fleece weight of progeny from commercial rams exceeded that of communal rams by about 15%, while the fiber diameter of lambs sired by communal rams was between 7% and 9% broader than that of commercial rams (Table 16). This resulted in wool income from the progeny of commercial rams being about 35% higher than that of lambs sired by communal rams. This trend was fairly consistent at both localities, with performance at the commercial properties being improved relative to that at communal properties.

In another study, Marais [109] studied the reproduction of ~1,200 communal ewes on four locations either mated to commercial or communal rams under communal production conditions. Reproduction was low, with the lambing percentages (lambs born per 100 ewes mated) being 30% and 47% for ewes mated to commercial and communal rams respectively. The corresponding means for weaning percentage (lambs weaned per 100 ewes mated) were 27% and 37% respectively, while lamb survival (lambs weaned per 100 lambs born) averaged 88% and 79% respectively. It is clear that reproduction of ewes mated to communal rams were substantially better than that of ewes mated to commercial rams, while the progeny of commercial rams had a slight advantage in terms of survival. The poor mating performance of commercial rams possibly suggest that they were not adequately adapted to the low-input communal conditions. The progeny of commercial rams were 7.2% heavier than those of communal rams at an age of 7 months. Lambs marketed per 100 ewes mated averaged 34.2% in a Western Cape study on properties of emerging farmers, with ranges between 9.5% and 56.7% [110]. In contrast, lamb marketing percentage ranged from 90% to 116% in a study where five commercial Merino-type ewe lines were mated to Dormer or Suffolk rams in a terminal crossbreeding experiment [111]. Even if it is considered that no replacements were kept in the latter study (ewe replacements need to be bought in) the improvement is evident. Additionally, the percentage of lambs docked per 100 ewes mated (range in brackets) on commercial properties in the Western Cape amounted to 88% for Merinos (63–103%), 93% for Dohne Merinos (76–113%), and 113% for South African Mutton Merinos (105–124%) in the study of Fourie and Cloete [112]. Even if losses between docking and marketing are considered, it is evident that reproduction on commercial properties would be superior to that on communal properties.

Based on the above information, the South African wool industry embarked on a program to improve conditions in the communal small stock production areas in the Eastern Cape in the early 2000s. The dual focus was on the upgrading of existing communal shearing sheds as well as the genetic improvement of the local animal resources [113]. Approximately 20,000 breeding rams have been introduced from the commencement of the program in 2003. It was furthermore independently established that 28% of such communal sheds realized prices of better or equal to the national

average in 2008/2009, compared to 9% in 2005/2006. Socioeconomic studies also indicated that undernutrition of children in the region was reduced from 43% in 2004 to 28% in 2009. This change results from an improved product (meat and wool) income, which initially contributed 47% to total household income compared to 65% at present.

As pertaining to substitution of animals from a local population with animals from the same breed but from another region to ensure a higher output, it was shown that Turkish Awassi dairy sheep had an improved milk yield compared to local Awassi ewes maintained in Syria [114]. Crossbreeding with and substitution by Turkish breeding stock were thus advocated as avenues for the improvement of Syrian dairy sheep breeding stock. Selection based on performance in the current flock also resulted in immediate gains, demonstrating that these principles are robust for extension to the Syrian environment.

The performance testing of meat and dairy goats in South Africa and other regions were reviewed by Olivier et al. [115]. The bulk of meat goat weaning weight data was derived from the Boer goat breed, with 11,679 records. The vast majority of dairy goat lactations were derived from the Saanen breed, with 14,688 out of a total of 16,148 lactation records. Data from these two breeds (Boer and Saanen) combine pedigree information and performance and form part of the National Livestock Improvement Scheme in South Africa. A study of small-scale goat keepers revealed that uncontrolled mating practices were followed by 98.3% of communal farmers and 92.3% of emerging farmers, at respective doe to buck ratios of 11:1 and 30:1 [116]. The reasons for keeping goats in the communal sector were for cash or investment (43.0%), products (37.6%), and cultural purposes (18.7%). Corresponding figures for emerging goat farmers were, respectively, 34.4%, 40.7%, and 24.8%. Communal farmers mostly kept unimproved veld goats (53.3%), Angora goats (28.3%), and Boer goats (15.4%). Emerging farmers had a lower percentage of unimproved veld goats (1.0%), and more Angora and Boer goats (62.8% and 36.1% respectively). Flock sizes ranged from 9 to 29 in the communal sector and from 18 to 91 in the emerging sector. High mortality of kids and females was listed as a major constraint to goat farming in these sectors [116].

The South African commercial poultry industry is highly industrialized, with little information on performance in the public domain. It would suffice to say that approximately 16 billion ZAR out of a total livestock income of 37 billion ZAR was derived from this sector in 2005/2006 [8]. In contrast, the communal sector kept an average of 10.9 chickens per household, with a range from 9.7 to 17.0 [117]. The number of hens per household averaged 6.1, with ranges from 5.4 to 8.4. Chicks per household amounted to 1.3, with ranges from 1.0 to 2.3. Egg output was estimated at between 35 and 45 eggs per hen per year. Chickens were mostly kept for household meat (89.8% of cases) and eggs for home consumption (64.2% of cases). Minor reasons for keeping chickens were for selling to other parties, ceremonies, culture, and manure [117]. Indigenous chicken production is considered important throughout Africa, despite relatively low levels of output [118]. However, the latter reference still demonstrated significant genetic variation in egg weight and body weights to be exploited in well-designed breeding programs. Genetic progress can thus be achieved, even though production conditions are less than optimal.

Based upon arguments presented under the previous headings, it was expected that animal improvement in most of the developing countries will be constrained by a lack of capacity. However, success in the commercial livestock sector of South Africa and in other developing countries has been reported above. Relative information on small-scale farming operations in South Africa is also reported for comparison. The latter production systems are comparable with small-scale production elsewhere. However, it is clear that commercial successes are not common in such systems. Production is mostly focused on home consumption, while levels of production are relatively poor. Yet successes have been reported throughout the world, as reviewed by Kahi et al. [119]. Moreover, socioeconomic drivers leading to success in these case studies were listed by the latter study. These guidelines could be used as a blueprint for future schemes wanting to embark on successful breeding programs in the communal livestock resource.

From the foregoing it seems that sustainable animal genetic resource management is feasible in the developing world. Strategies to operate within the constraints imposed by capacity problems and other limitations in this region need to be considered, while

the improvement of production in small-scale, low-input systems should receive serious attention. It is suggested that this will be easier to achieve in the presence of a strong commercial sector.

The Technological Age

It is common knowledge that the available technology improved rapidly over the past couple of decades. Analyses on animal breeding and genetics data that required a mainframe computer 20 years ago for a series of single-trait analyses are now routinely run as a single multi-trait analysis on a laptop computer.

Data Capturing, Analysis, and Dissemination

Computing power and data storage capacities increased markedly over the past few years. Software for the on-farm capture of animal records is readily available. This software enables operators with only basic computer skills to effectively collect data for entrance in national or regional databases. The recording system can easily be managed to use open-source software for routine data entry, data editing, and genetic analyses [120, 121]. Custom-made software for a specific enterprise can also be used for routine recordings [122]. Moreover, excellent research tools for data manipulation, the assessment of environmental effects, genetic parameter estimation, as well as the computation of animal solutions are readily available [123–126]. It seems quite feasible to adapt existing software used for breeding value estimation to include a genomic relationship matrix for the estimation of genomic breeding values in a single step approach [127, 128]. The available software packages allow for the analysis of various traits, which could consist of normally distributed linear data or traits with more challenging distributions (for instance, binary traits or discrete scores with multiple thresholds) in single- or multiple-trait analyses. The partitioning of random effects in direct and maternal genetic components as well as maternal permanent environmental effects have also become commonplace, while standard genotype by environment interactions is easily modeled. Analyses of longitudinal data, for example, growth data, lactation test-day records or reaction-norm analyses to model genotype by environment interactions, by using random regression methods have also become routine [129–132]. Interactive systems, where participants can access information on their animals directly from the Internet, have also become commonplace [95, 104, 121]. As this process takes place in real time, delays are effectively dealt with. This infrastructure is at the disposal of all livestock producers, including those of the emerging/communal sectors, provided that all relevant information is recorded.

Genomic Selection

If progress can be considered as rapid in computer support systems and specialist software for genetic analysis, this probably applies in the superlative to progress in DNA-based marker systems and marker-assisted selection. The bovine genome has recently been completely mapped and allows the study of the genetic structure of cattle breeds worldwide [133]. High-density Single Nucleotide Polymorphism (SNP) chips of thousands of SNP markers evenly spaced across the genome are readily available for pigs [134, 135], sheep [136, 137], cattle [138–140], and poultry [141, 142]. It has indeed been suggested that full genome information may become as commonplace as SNP technology at the next WCGALP to be held in Vancouver in 2014 [143]. The utilization of the genomic information linked to phenotypes has recently been reviewed by Goddard et al. [144], to give an overview of methods and the future of the technology. Information on SNPs was used in a BLUP or BayesA framework to attain genomic breeding values for Australian progeny testing sires [145]. Compared to the tradition sire pathway EBV, the BLUP-based system improved the accuracy of selecting dairy sires for progeny testing from 0.38 to 0.44 for the Australian selection index, from 0.35 to 0.53 for the Australian profit ranking, from 0.28 to 0.45 for protein yield, from 0.20 to 0.29 for protein percentage, and from 0.16 to 0.18 for fertility. The BayesA method resulted in further improvements of 0.04, 0.02, 0.03, 0.05, and −0.04, respectively [145]. There is consensus among analysts that genomic information can greatly enhance genetic progress in dairy cattle breeding [144, 146–148]. However, it needs to be stated that price considerations at present will only allow males and elite females to be genotyped [147]. Naturally, this may change in future as the technology becomes more affordable.

The above advances will have a marked impact on animal breeding in the developed world. The question remains if it will become sufficiently affordable for routine use in the countries of the developing world, given the financial constraints in these countries (Fig. 19). The human capacity needed to make full use of these innovations is another topic that needs to be debated, given the dearth of people with adequate scientific training in these countries (Fig. 20). These and other issues will be debated in the next section.

Future Directions

Future of Livestock Breeding in the Developing Regions and Tropics

Breeding Objectives Given the environmental challenges faced by animals in the developing world and the tropics, there is little doubt that elements of adaptability, robustness, and fitness should form part of the selection objective in those areas. Fitness and/or adaptability can be defined in many ways as was reviewed by Barker [149], but a definition like "a measure of the ability of an organism to survive and reproduce in a particular environment" is probably a good compromise. Naturally, the concept includes stressors that are inherent to the particular environment maintaining the animals, for instance, climatic conditions and disease. As the prospects of breeding for resistance to disease as well as adaptation to stressful environments in terms of heat, water scarcity, and a variable food supply have already been discussed, they will not be revisited here. It is, however, important to mention that records pertaining to susceptibility to a disease are highly incidence dependent [150], which should be considered during analysis. The importance of assessing the genetic resources from the developing world and the tropics for disease resistance has been highlighted by Gauly et al. [151], while some guidelines as to how to proceed with the process were also presented. Bath and Van Wyk [152] recently proposed a practical check for helminth infestation of small ruminants, which was easily applied by both commercial and small-scale farmers. It was suggested that this check can be used to guide decisions pertaining to the selective treatment of animals for internal parasites.

The remaining elements of fitness are therefore survival and reproduction, as was defined by Goddard [153]. According to the latter author, these elements of fitness have largely been ignored during selection of farm animals, despite obvious economic value. Arguments against reproduction and survival are a low heritability, while it was also considered difficult to record. Reproduction is also sex-limited and usually assessed later in the life of animals than other production traits. The omission of including fitness traits in the breeding objective led to a general decline of fitness in those farm animals that were intensely selected for (say) milk production. Yet the genetic coefficient of variation of 6-week pregnancy rate in dairy cattle equals that of milk yield [153]. The latter author suggests that the correct way to handle this situation would thus be to include fitness traits together with the traits of interest for a particular livestock species in a selection index, with appropriate economic weights.

It is also appropriate to consider selection for reproduction and survivability in a number of livestock species farmed with in developing countries. A number of indicators of reproduction (days from calving to first service, days open, artificial inseminations per conception, and pregnancy rate) in South African Holstein cattle of 14 herds were considered by Muller et al. [154]. Heritability estimates ranged from 0.06 to 0.08, as was also reported by other authors from developed countries. Selection for number of lambs weaned per ewe mated of South African Merino ewes (a trait with a similar low heritability as that quoted above) resulted in a genetic response of ~2% per annum, which is of the same magnitude as is expected for other traits of economic importance [155]. This realized improvement was consistent with expectations based on the review of Snowder and Fogarty [156]. Stayability of South African Angus cattle was similarly shown to be heritable [157]. From these results from the developing world it is evident that selection for reproduction and herd life in farm animals should be attainable, should it be desired. Appreciable progress has in fact been demonstrated in the national analysis of the South African Merino breed [101]. Total weight of lamb weaned per ewe mated (as measure of reproduction) improved at a rate of 0.73% per annum in the best performing flock, while the average flock gained at 0.14% per annum. At the same time, fiber diameter was reduced by 0.48% per annum in the best flock and by 0.42% per annum in the national flock. During the same period, relative

economic values increased by 2.80 ZAR per annum in the best performing flock ($R^2 = 0.94$) and by 1.17 ZAR per annum in the national flock ($R^2 = 0.98$).

How to Achieve the Objective of Improving Fitness and Adaptability Having discussed the state of animal agriculture in the developing world, as well as successes and failures of projects in that region, the issue remains whether the capacity to allow sustained improvement in those regions exists. It needs to be stated that research priorities for this venture has been clearly outlined by Cardellino and Boyazoglu [79]. The proposed needs are summarized in Table 17 and include the development of suitable information systems, the characterization of resources, as well as a sound knowledge of genetic diversity, functional genetics, and animal breeding. The collection of phenotypic data is a prerequisite for the system to be successful. If these data could be linked to pedigrees it would be an added bonus. This objective may seem nearly unattainable at present. However, traceability based on individual identification is increasingly seen as a good agricultural practice for the assurance of safety and quality in global trade [158]. It is foreseen that the developing world will in future have to comply with such prerequisites for continued market access. It should thus not be seen as far-fetched to start to apply a principle like individual identification in the near future. Data containing phenotypes linked to pedigree information can initially be used for routine genetic improvement.

Breeding in Developing Countries and Tropics. Table 17 Research priorities to enable the breeding of livestock in developing regions to increasingly contribute to local food security and poverty relief

Area	Specific needs
Information systems	Upgrading of the existing farm animal genetic resources (FAnGR) information system
	Acquisition of data on population size and structure
	The geographical referencing of FAnGR
Characterization	Define traits and record adaptation and performance of indigenous populations
	Describe the environment to evaluate genotype x environment interactions
Genetic diversity	Define and determine the risk of extinction for FAnGR
	Adopt measures to halt the decline of genetic diversity
	Assess genetic diversity by using molecular markers
	Integrate data on phenotypes with molecular information
	Acquire global information on specific markers associated with production and fitness
Functional genetics	Ensure that the genetic basis of adaptation traits are properly understood, including disease resistance, fitness, and adaptability to challenging environments
	Apply the most recent tools for conventional genetic improvement and marker-assisted selection (MAS)
Animal breeding	Assess impact of selection under low-input conditions
	Study the consequences of introducing exotic breeds
	Devise breeding structures for a low impact environment where little or no organizational structure exists
	Research stable crossbreeding systems with a role for indigenous breeds
	Implement MAS selection where applicable, that is, in the case of disease resistance
	Gain insight in genetics of adaptation to systems with a low and variable nutrient supply

Source: Adapted from Cardellino and Boyazoglu [79].

If it is affordable it could also be linked to genomic information in future. Goddard et al. [144] stressed the importance of being able to link genomic information to phenotypes for the accurate prediction of genomic breeding values. It should be noted that recommendations on animal welfare have been left out from Table 17 as it will be dealt with separately at a later stage.

The challenges of such a venture will be enormous. The requirements and structure of performance recording and genetic evaluation for low-input agriculture have been well documented [159] but their application in practice is lagging behind. Open nucleus breeding systems have often been advocated as a solution to the lack of sustainable genetic improvement in the developing regions [18, 160]. This solution will only be feasible if the nucleus herds/flocks are under central control of accomplished scientists at a governmental, academic, or parastatal research institution. Having said this, it is equally important to have the support of the stockowners at grassroots level. Failure of livestock breeding projects in the developing world has commonly been attributed to a lack of community involvement [18], whereas successful efforts generally benefitted from community involvement [119]. The importance of close involvement with the community stems from the multifunctional role of livestock in communities in the developing world [94]. Broad stakeholder involvement has been suggested, with collaboration of local committees, researchers and development practitioners [161]. Close interaction of these diverse partners are considered to be critical for success. The importance of participatory development approaches was also stressed by Nesamvuni et al. [162]. According to the latter authors, the integration of coexisting research and development programs is highly preferable to research and development programs operating in isolation.

Using the Kenyan Boran breed as an example, Rewe et al. [163] assessed open and closed nucleus systems of registered breeders with or without gene influx from commercial breeders to ensure that adaptation traits (mainly disease tolerance) are not compromised. The introduction of germplasm from the commercial breeders was profitable in both schemes. The study demonstrated that the success of a breeding program depended on the production system, and that adaptation could be introduced from the indigenous herd, as was suggested in Table 17.

It will be a bonus if a strong commercial sector, operating on par with conditions in the developing world, can be included in the genetic evaluation system. The successful establishment of public–private partnerships to enable the system to work is a further prerequisite for success. This blueprint seems to be effective in the South African small stock sector, as was described previously. It also appears to be relevant to dairy production in Kenya [94]. The real success with it will, however, only become known with the passage of time.

It needs to be stressed that this whole process can be orchestrated with existing tools for quantitative genetics, using known theory for the sustained genetic improvement of livestock. The emphasis on fitness traits may depart slightly from what has been practiced traditionally. Yet the approach to select for adaptation and fitness would be fairly similar to what has been proposed by Goddard [153] for general application across the globe.

Table 17 also refers to the possible role that crossing with indigenous livestock may play in commercial production. The benefits of well-planned crossbreeding systems in terms of direct and maternal heterosis are well known [164]. Structured studies are needed to apply these to livestock breeds in the developing world, where it needs to be balanced with the need to conserve the indigenous genetic resources. The substantial differences between breeds that form part of the genetic resource, as well as the relative advantage of crossbred ewes under challenging conditions in France compared to purebreds was confirmed in the literature [165, 166]. More structured research on this topic is needed for the optimal utilization of local, adapted farm animal genetic resources in the developing world. The role of cortisol and the functioning of the hypothalamic-pituitary-adrenocortical axis in breeding for an increased robustness have been highlighted recently [167]. Future studies should thus focus on the underlying causes of genetic variation at this level.

It is also important to consider the role that molecular genetics and marker-assisted selection may play in this process [167]. Constraints in monetary resources, infrastructure, and suitably qualified scientists place immediate earth-shattering breakthroughs beyond the ability of developing countries at present. Further

issues like intellectual property rights also complicate immediate application on a wide scale in the developing world [168, 169]. However, scientists in the developing world should take cognizance of progress in the application of marker-assisted selection in the developed world. The rapid development of technology may allow for inputs from this part of the world in future, as the technology becomes more affordable. Fitness traits like disease resistance and reproduction, which need to be selected for in the emerging regions, is particularly well suited to marker-assisted selection [168, 170]. The provision here is that sufficient monetary investment in the genotyping of animals should be available at that stage. Therefore it is also recommended that DNA samples from phenotyped animals in the nucleus flocks/herds be collected and stored for possible future usage. The general concept has been applied in the Australian Information Nucleus flock [171–173], which has already produced a number of outcomes [174]. Scientists in the developing world are well aware of the importance of combining genetic and environmental information for selection decisions [64]. It should thus be feasible for these scientists to adapt to the requirements listed above, should the opportunity arise.

Readers will notice that most of the examples listed in this section involved grazing ruminants. It is conceded that pigs and poultry played an important role in the production of protein in both the developing and developed world, and will continue to do so in the future (see Fig. 13). However, these industrialized livestock systems are in many cases served by multinational breeding companies, as stated previously. Moreover, the prospects of selecting for "robustness" or "fitness" in poultry and pigs have been covered in some detail in other entries in this encyclopedia [175, 176]. The issues dealt with in the latter entries are complex, and it would have been impossible to deal with them comprehensively in this entry.

Finally, it is clear that the system that is proposed would not only need monetary inputs, but also the human capacity to successfully orchestrate the flocks/herds [119]. In case of severe threat to some genetic resources, the option of ex situ conservation should also be an option [80, 81]. The need for collaboration across institutional and national boundaries, to ensure that the various forms of information generated in this way are optimally utilized, cannot be overstressed [168, 169]. It is therefore appropriate to ensure that the intellectual capacity to drive all the processes is available, as discussed under the following heading.

Broadening the Knowledge Base It is evident that the objectives above are unlikely to be achieved without a sound knowledge base. Based on the disadvantage of developing countries in terms of human capacity (Fig. 20), it is obvious that the people active on research and development of farm animal genetic resources should be well-trained. The limitation in human resources results in several challenges for scientists in this region, such as an increased workload in terms of teaching, and a reduced capacity for conducting research [177]. Sustainable farming and the improved use of farm animal genetic resources were targeted in a joint venture between the International Livestock Research Institute (ILRI) and the Swedish University of Agricultural Sciences. Training by the project team reached 137 university lecturers and researchers in developing countries so far [177]. Agriculture is not a favored study direction, as it is associated with poverty and an inability to improve the livelihood of families. A typical curriculum for students in animal science in South Africa (as an example of a developing country) was provided by Casey [178]. The intention with the training of students is to prepare them for a professional career in animal production, with appropriate registration. It is also noted that Sub-Saharan Africa has many universities that teach animal production. However, there is a lack of communication and collaboration between these role players [178]. In contrast, six European universities joined hands to form the 2-year European Master of Science program in Animal Breeding and Genetics [179]. The intention of the course is to assist in increasing livestock and fish production, development of sustainable animal breeding programs, the improvement of health and welfare, and the preservation of natural resources. The program also draws students from developing countries, but with ~20 scholarships per year, the impact is limited. Of students starting the program between 2007 and 2009, 46% were from Asia, 31% from Africa, 9% from the Americas, and 13% from within Europe. In Latin America, two universities from Peru, Bolivia, Mexico, and Spain each collaborate with universities

in Argentina and Austria to present a master degree in Animal Science [180]. Original assessment indicates that there is heterogeneity in the curricula, grading systems, and duration. A program for harmonization has been proposed.

In view of the challenges listed above, steps need to be taken to rectify the situation in the developing world. The obvious prerequisite is to ensure a skilled and motivated core of senior mentors, to pass on knowledge to the next generation. This will be facilitated by closer collaboration between role-players, both among institutions in the developing world and with institutions in developed countries [64, 119, 168, 169, 177]. Joint appointments of senior researchers at science and technology institutions at higher education institutes could also benefit training in farm animal genetic resources, by making better use of expertise [181]. To assist in these quests, there is a wealth of Internet-based training modules that can easily be accessed [182–184]. Collaboration between research groups and those involved in training is also proposed [177]. This approach will ensure that the studies embarked upon will be relevant. It is also important to attempt to recruit and retain well-qualified and dynamic persons for careers in animal agriculture, both in training and research. The so-called brain-drain from developing countries needs to be stopped and preferably turned around. This will only become feasible if scientists see a future for themselves in animal agriculture in developing countries, both in monetary terms and in job satisfaction. Alternatively, well-trained international scientists could be used as trainers when they return to their home countries for holiday or familial responsibilities [177]. There ought to be opportunities for improving the local knowledge base in developing countries when all these avenues are fully exploited.

"New" Issues

Finally, it is necessary to give attention to issues that have not previously impacted on animal agriculture in the developing world. Both topics are specialized study fields, worthy of an exhaustive discussion in their own right. However, this chapter will only briefly deal with the new challenges, within the developing world context.

Climate Change There is consensus that the global temperatures are on the increase, and that it is probably related to higher atmospheric concentrations of the so-called greenhouse gases. It is expected that global warming will affect extensive pasture systems to a greater extent than intensive, industrialized systems [62]. This implies that animal production in the developing world will be more vulnerable than in the developed world [4]. The carbon dioxide emissions of the developing world, in particular, are modest compared to emissions in the developed world [2]. Hoffman [80] pointed out that livestock production is contributing to global warming, but also that it will be affected by the consequences of the phenomenon. It is estimated that agriculture produces between 10% and 12% of the total global anthropogenic greenhouse gas emissions [185]. Between 50% and 60% of methane and nitrous oxide could be derived from livestock activities. The review by Eckard et al. [185] focuses on the possibility to reduce greenhouse gas emissions by ruminants through the manipulation of the animals, their diet, and their rumen microbes. Manipulation of animals is centered about breeding (the focus of this entry) and on changing managerial systems. Eckard et al. [185] cited sources that quoted differences between animals in methane production, hinting at possible genetic differences. These allegations were substantiated by Robinson et al. [186], reporting that methane production in sheep were repeatable (0.47) and heritable (0.30). Adjustment for live weight resulted in these parameters being reduced to respectively 0.32 and 0.13. Adjustment for live weight also eliminated the effect of sire breed upon methane production, while correlations with rumen volatile fatty acids did not support a contention that the latter could be used as a proxy for methane production. Cassandro et al. [187] predicted methane production of dairy cows from dry matter intake, and derived a heritability of 0.12, with upper and lower confidence limits of respectively 0.03 and 0.28. Genetic correlations of methane production with milk yield and butterfat content were high (respectively 0.92 and 0.67), but protein content and somatic cell count were not genetically correlated to predicted methane production (0.14 in both instances).

Alternatively, selection for an improved efficiency would also indirectly benefit methane excretions per

unit product from ruminants, as reviewed by Eckhard et al. [185], Hegarty and McEwan [188], and Herrero et al. [189], as would improvements in feed conversion and residual feed intake. Lamb production systems based on crossbreeding would be more effective in terms of methane produced per unit product than a wool and hogget production system [188]. Alternatively, methane emissions per unit product could also be reduced by an improved reproduction rate, the early disposal of unproductive animals and the longer retention of productive animals in flocks or herds. As dairy products are produced at lower levels of greenhouse gas emissions than beef, there may be a shift to dairy in a situation where greenhouse gasses need to be reduced [80].

Possibly more relevant to conditions in the developing world is the adaptation of animals to higher ambient temperatures and to more frequent severe climatic events, which are predicted to be a consequence of climate change [80]. The possible genetic improvement of adaptability, robustness, and heat tolerance has already been discussed and will not be reiterated here. Unforeseen catastrophic climatic episodes like floods, droughts, and heat waves could have a major and unpredictable impact on local farm animal genetic resources. For instance a heat wave in 1995 in the Midwestern United States resulted in an economic loss of $31 million in the state of Iowa alone [62]. Such events should be managed when they occur, as it is impossible to plan for them. However, the need for a disaster strategy to be in place in areas likely to be affected cannot be overemphasized. Thornton and Gerber [4] suggested that up-to-date weather information will play a role in mitigating the effects of climate change. State-supported schemes reminiscent of a sort of insurance scheme may have a role to play in disastrous events, while species substitution toward more hardy animals may be contemplated (i.e., from cattle to camels in dry parts of Kenya).

Animal Welfare Pressure on output traits, particularly regarding the more intensive livestock industries, resulted in the welfare of farm animals being compromised [189]. This topic is also extensively covered by Knap [175] in this issue, and interested readers are also referred to this paper. Because of this, discerning consumers have ensured that animal welfare is among the important considerations in production systems in developed countries, like Europe [190, 191]. Welfare in farm animals are usually defined as freedom from hunger, thirst, discomfort, pain, injury, fear, and distress, as well as an ability to express normal behavior [189]. Several routine husbandry procedures are considered to infringe on these fundamental freedoms, for example, the Mules operation in Australia [28, 30]. In this case, popular opinion has prevailed that the pain, fear, and distress of the operation outweighs the possible later advantages in terms of better resistance of animals to breech strike. This has resulted in pressure on Australian livestock producers to phase out the procedure. The same reasoning applies to tail docking of lambs. An alternative genetic solution was thus sought to enable the abolishment of this practice in the Netherlands in the two sheep breeds where conditional approval to dock tails are still granted [192]. Genetic variation seems to exist for tail length and selection for shorter tails may thus be possible. Issues in the developed world mostly involve the quality of life of farm animals in more intensive systems [193]. Aspects like survival, adequate nutrition, and protection against intense cold or heat are more important under extensive conditions. Sørenson and Fraser [193] proposed a self-regulatory process of auditing livestock operations for animal welfare, agreed on by all role-players.

Aspects of importance to this discussion are the impact breeding for welfare traits could have on genetic gains in other traits. First of all, it is fair to say that animals, where welfare is compromised, will in many cases not be capable of high levels of production. Because of the emphasis of herd health in dairy cattle selection schemes in Norway along with production traits, cows are much more likely to reconceive than in European countries. In response to this, longevity as well as health and fertility became much more important in the United Kingdom dairy indexes as well [189, 190]. Impaired mobility of dairy cattle because of poor claw health also leads to direct and indirect costs. Claw health traits of Finnish Ayrshire cows were lowly heritable [194]. Some leg conformation traits had favorable genetic correlations with claw health, and could serve as indicator traits.

A simulation study on outdoor pig production indicated that genetic gains had to be compromised

when welfare traits (leg conformation, mothering ability, and longevity) were included in the selection index [195]. Apart from a slightly slower growth rate and a reduced fat depth, free-range pigs performed as well as pigs in a conventional housing system [196]. The former system produced pork of a similar quality as that observed in a conventional system. Observations made at lambing were evaluated as possible indicators of ovine lamb survival by Brien et al. [174] and Lemmon et al. [197]. Although some observations were favorably related to survival, none were obvious candidates for immediate industry application in the former study. The latter study suggested that alternative methods of improving lamb survival should be investigated. It is however, notable that divergent selection for the ability of sheep to rear multiple offspring led to divergence in age-specific lamb survival in South Africa [198]. Hatcher et al. [199] also reported scope for the improvement of lamb survival by selection, although low direct and maternal heritability estimates were derived.

Animal welfare of the mostly free-ranging animals in the developing world is probably not compromised to the same extent as those animals in more industrialized systems. The exceptions are probably freedom of hunger and thirst, which cannot be guaranteed. It is nonetheless important for scientists in developing countries to take cognizance of the emphasis on welfare, and ensure that information on the impact of the selection strategies for livestock (that were previously discussed) on animal welfare are also considered. Intuitively, selection for adaptability, robustness, and fitness is unlikely to compromise welfare. However, challenge of animals by environmental stressors (heat, parasites, and uncertain food supply) typical of the developing world may lead to some ethical concerns. It is reassuring that the need to ensure a value system throughout the entire production chain is also recognized in the developing world [200].

Conclusion and Recommendations

From the foregoing it is evident that livestock industries in the developing world are faced by numerous challenges involving the environment, infrastructure, funding, and the availability of a sufficient number of well-trained scientists specializing in breeding and farm animal genetic resources. Despite these drawbacks, scientists in the developing world seem to be willing to contribute to the advancement of science in the region in spite of the considerable odds against them.

Within the constraints in terms of funding and manpower, it is proposed that major efforts should be put into obtaining phenotypic records from the farm animal genetic resource managed in the developing world. Wherever possible these records should be linked to pedigree information, which could follow on from the identification of animals for traceability purposes. Should this vision prove to be impossible to implement on a wider scale, it should be applied to genetic resources maintained by governmental, academic, and parastatal institutions within the developing world. The decision as pertaining to broader community involvement will largely be determined by local conditions, and need to be considered with care on the grassroots level. A prerequisite is that supportive institutional research actions should be conducted in environments conforming to those used by those resource-poor stockkeepers standing to benefit from it. Secondly, it has to be ensured that all initiatives involving the latter groups must have their full support for the intended projects to be successful. This structure will form the basis for simple animal recording and genetic evaluation schemes under communal and emerging farm systems. These schemes are highly unlikely to be cost effective and need to be subsidized from funds supplied by local government, higher education institutions or external sources. However, such evaluation is integral for local food security, sustainable development, and rural stability, thereby increasing leverage for the acquisition of funds dedicated to this cause. Should the envisaged scheme become successful and gather momentum, it would be easy to integrate into existing livestock improvement schemes for commercial agriculture, should the infrastructure exist.

Being in the developing world and the tropics, breeding should focus on traits associated with fitness and adaptability. However, traditional production and product quality traits should also be recorded, to monitor the impact of selection decisions on these traits.

It is not foreseen that genomic selection will change the way animal genetic resources in the developing world are selected in a drastic way in the immediate

future. However, it is advised that samples for the extraction of DNA are acquired for as many animals with phenotypes as possible in the system. After the extraction of DNA, these samples should be kept under safe conditions for possible future use. Given the appreciable advances in the technology, it may be possible to utilize DNA-based methods for the evaluation and selection of farm animal genetic resources in the developing world in the foreseeable future. Should this vision become a reality, the scene should be set for the immediate application of this technology on a broader basis. The need for adequate phenotypic data to link to genomic information cannot be overemphasized.

Finally, it is conceded that new issues such as global warming and the insistence of customers on assurance of traceable and welfare-based production systems are likely to affect animal breeding and genetics in the developing world in future. The animal breeding sector in developing countries should preempt these possible challenges, and react to it in a constructive way. Modern societies embrace change. Animal breeders in the developing world thus need to ensure that the challenges imposed by these issues are turned into opportunities.

Bibliography

1. Millennium Development Goals Report, 2005. United Nations, New York
2. McDermott JJ, Staal SJ, Freeman HA, Herrero M, Van der Steeg JA (2010) Sustaining intensification of smallholder livestock systems in the tropics. Livest Sci 130:95–109
3. Pilling D, Rischkowsky B, Cuesta ML (2007) Uses and values of animal genetic resources. In: Rischkowsky B, Pilling D (eds) Part 1: The state of agricultural biodiversity in the livestock sector – The state of the World's Animal Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome, pp 77–100
4. Thornton PK, Gerber PJ (2010) Climate change and growth of the livestock sector in developing countries. Mitig Adapt Strateg Glob Change 15:169–184
5. Thornton PK, Kruska RL, Henninger N, Kristjanson PM, Reid RS, Atieno F, Odero AN (2002) Mapping poverty and livestock in the developing world. ILRI (International Livestock Research Institute), Nairobi, 124 p
6. Faber M (2010) Nutrition in vulnerable communities in economically marginalized societies. Livest Sci 130:110–114
7. McAfee AJ, McSorley EM, Cuskelly GJ, Moss BW, Wallace JMW, Bonham MP, Fearon AM (2010) Red meat consumption: an overview of the risks and benefits. Meat Sci 84:1–13
8. Cloete SWP, Olivier JJ (2010) South African sheep and wool industries. In: Cottle DJ (ed) The international sheep and wool handbook. Nottingham University Press, Nottingham, pp 95–112
9. FAO (2010) Food security statistics. http://www.fao.org/economic/ess/food-security-statistics/en/. Accessed 6 July 2010
10. FAO (2009) FAO Statistical yearbook for 2009. http://www.fao.org/economic/ess/publications-studies/statistical-yearbook/en/. Accessed 6 July 2010
11. OECD-FAO (2008) Agricultural outlook 2008–2017. The Organization for Economic Co-operation and Development and the Food and Agricultural Organization of the United Nations. OECD Publications, Paris
12. Hanotte O (2007) Origin and history of livestock diversity. In: Rischkowsky B, Pilling D (eds) Part 1: The state of agricultural biodiversity in the livestock sector – The state of the world's animal genetic resources for food and agriculture. Food and Agriculture Organization of the United Nations, Rome, pp 5–22
13. Rischkowsky B, Pilling D, Scherf B (2007) Status of animal genetic resources. In: Rischkowsky B, Pilling D (eds) Part 1: The state of agricultural biodiversity in the livestock sector – The state of the world's animal genetic resources for food and agriculture. Food and Agriculture Organization of the United Nations, Rome, pp 23–49
14. Ndlovu L (2010) The role of foods of animal origin in human nutrition and health. In: Swanepoel F, Stroebel A, Moyo S (eds) The role of livestock in developing communities: enhancing multifunctionality. Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen, pp 77–92
15. Dubeuf J-P, Boyazoglu J (2009) An international panorama of goat selection and breeds. Livest Sci 120:225–231
16. Menjo DK, Bebe BO, Okeyo AM, Ojanjo JMK (2009) Survival of Holstein-Friesian heifers on commercial dairy farms in Kenya. Appl Anim Husb Rural Dev 2:14–17. www.sasas.co.za/aahrd/
17. Muigai AWT, Okeyo AM, Kwallah AK, Mburu D, Hanotte O (2009) Characterization of sheep populations of Kenya using microsatellite markers: implications for conservation and management of indigenous sheep populations. S Afr J Anim Sci 39(Suppl 1):93–96
18. Kosgey IS, Baker RL, Udo HMJ, Van Arendonk JAM (2006) Successes and failures of small ruminant breeding programmes in the tropics: a review. Small Rumin Res 61:13–28
19. Mirkena T, Duguma G, Haile A, Tibbo M, Okeyo AM, Wurzinger M, Sölkner J (2010) Genetics of adaptation in domestic farm animals: a review. Livest Sci 132:1–12
20. Silanikova N (2000) The physiological basis of adaptation in goats to harsh environments: a review. Small Rumin Res 35:191–193
21. Nigussie E, Rottman OJ, Pirchner E, Rege JEO (2000) Allometric growth coefficients and partitioning of fat depots in indigenous Ethiopian Menz and Horro sheep breeds. In: Merkel RC, Abebe G, Goetsch AI (eds) Proceedings of the Conference on the opportunities and challenges of enhancing goat

production in East Africa, Debub University, Awassa, 10–12 November 2000, pp 151–163
22. Otte J, Roland-Holst D, Pfeiffer D, Soares-Magalhaes R, Ruston J, Graham J, Silbergeld E (2007) Industrial livestock production and global health risks. Research Report, Pro-Poor livestock policy initiative, a living from livestock. RR Nr 07–09, June 2007. http://www.fao.org/AG/AGAINFO/programmes/en/pplpi/docarc/rep-hpai_industrialisationrisks.pdf. Accessed 18 May 2010
23. Gummow B (2010) Challenges posed by new and re-emerging infectious diseases in livestock production, wildlife and humans. Livest Sci 130:41–46
24. Van der Waaij EH (2001) Breeding for trypanotolerance in African cattle. PhD dissertation, Wageningen Institute of Animal Sciences, Wageningen
25. Bigalke RD (1980) The control of ticks and tick-borne diseases of cattle in South Africa. Zimb Vet J 11:20–21
26. Schwalbach LMJ, Greyling JPC, David M (2003) The efficacy of a 10% aqueous Neem (*Azadirachta indica*) seed extract for tick control in Small East African and Toggenburg female goat kids in Tanzania. S Afr J Anim Sci 33:83–88
27. Budeli MA, Nephawe KA, Norris D, Selapa NW, Bergh L, Maiwashe A (2009) Genetic parameter estimates for tick resistance in Bonsmara cattle. S Afr J Anim Sci 39:321–327
28. Greeff JC, Karlsson LJE (2009) Opportunities to breed for resistance to breech strike in Merino sheep in a Mediterranean environment. Proc Assoc Adv Anim Breed Genet 18:272–278
29. Greeff JC, Karlsson LJE, Schlink AC (2010) Genetic parameters of breech strike and its indicator traits in Merino sheep in a Mediterranean environment. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0954.pdf. Accessed 20 Jan 2011
30. Scholtz AJ, Cloete SWP, Van Wyk JB, Misztal I, Du Toit E, Van der Linde TCdeK (2010) Genetic (co)variances between wrinkle score and absence of breech strike in mulesed and unmulesed Merino sheep, using a threshold model. Anim Prod Sci 50:210–218
31. Scholtz AJ, Cloete SWP, Van Wyk JB, Kruger ACM, Van der Linde TCdeK (2010) Influence of divergent selection for reproduction on the occurrence of breech strike in mature Merino ewes. Anim Prod Sci 50:203–209
32. Brown DJ, Swan AA, Graser H-U (2010) Genetic relationships between indicator traits for fly strike resistance and production traits in Merino sheep. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0924.pdf. Accessed 20 Jan 2011
33. Pickering NK, Dodds KG, Blair HT, Hickson RE, Johnson PL, McEwan JC (2010) Heritability of dags and its genetic relationships with fleece traits and live weights. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0294.pdf. Accessed 20 Jan 2011
34. Morris CA (2009) Review of genetic parameters for disease resistance in sheep in New Zealand and Australia. Proc Assoc Adv Anim Breed Genet 18:263–271
35. Mortimer SI, Atkins KD, Raadsma HW (1998) Responses to selection for resistance and susceptibility to fleece rot and body strike in Merino sheep. In: Proceedings of the 6th world congress on genetics applied to livestock production, Armidale, vol 27, pp 283–386
36. Pfeffer A, Morris CA, Green RS, Wheeler M, Shu D, Bisset SA, Vlassoff A (2007) Heritability of resistance to infestation with the body louse, *Bovicola ovis*, in Romney sheep bred for differences in resistance or resilience to gastro-intestinal nematode parasites. Int J Parasit 37:1589–1597
37. Baker RL, Mwamachi DM, Audho JO, Aduda EO, Thorpe W (1999) Genetic resistance to gastro-intestinal parasites in Red Maasai, Dorper and Maasai x Dorper ewes in the sub-humid tropics. Anim Sci 69:335–344
38. Baker RL, Mugambi JM, Audho JO, Charles AB, Thorpe W (2002) Comparison of Red Maasai and Dorper sheep for resistance to gastro-intestinal nematode parasites, productivity and efficiency in a humid and semi-arid environment in Kenya. In: Proceedings of the 7th world congress on genetics applied to livestock production, Montpellier, 19–23 August 2002, vol 31, pp 639–642
39. Nimbkar C, Ghaisasi PM, Swan AA, Walkden-Brown SW, Kahn LP (2003) Evaluation of growth rates and resistance to nematodes of Deccani and Banmur lambs and their crosses with Garole. Anim Sci 76:503–515
40. Cloete SWP, Olivier JJ, du Toit E, Dreyer FH (2007) Genetic analysis of faecal worm egg count in South African Merinos under natural challenge. S Afr J Anim Sci 37:237–247
41. Karlsson LJE, Greeff JC (2006) Selection response in worm egg counts in the Rylington Merino parasite resistant flock. Aust J Exp Agric 46:809–811
42. Woolaston RR, Piper LR (1996) Selection of Merino sheep for resistance to *Haemonchus contortus*: genetic variation. Anim Sci 62:451–460
43. Williams AR, Greeff JC, Vercoe PE, Dobson RJ, Karlsson LJE (2010) Merino ewes bred for parasite resistance reduce larval contamination onto pasture during the peri-parturient period. Animal 4:122–127
44. Greeff JC, Karlsson L, Underwood N (2006) Breeding Merino sheep for worm resistance increases profit in a Mediterranean environment. In: Proceedings of the 8th world congress on genetics applied to livestock production, Bello Horizonte, Brazil. Communication 15–08
45. Bishop SC (2009) Interpretation and prediction in disease genetics. Proc Assoc Adv Anim Breed Genet 18:255–262
46. Eding H, De Jong G (2010) Predicting Mastitis resistance breeding values from somatic cell count indicator traits. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN

978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0437.pdf. Accessed 20 Jan 2011
47. Urioste JI, Franzen J, Strandberg E (2010) Genetic characterization of new candidate traits derived from test-day somatic cell counts. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0121.pdf. Accessed 20 Jan 2011
48. Van Hulzen K, Nielen M, Koets AP, De Jong G, Van Arendonk J, Heuven HCM (2010) Within herd test prevalence affects genetic variation in antibody response to *Mycobacterium avium* subspecies paratuberculosis in milk of Dutch Holstein Friesians. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0301.pdf. Accessed 20 Jan 2011
49. Bermingham M, More S, Good M, Cromie A, Higgins I, Berry D (2010) Genetic correlations between susceptibility to *Mycobacterium bovis* infection and performance in Irish Holstein Friesian dairy cows. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0766.pdf. Accessed 20 Jan 2011
50. Anon (2010) Free state: rift valley fever spreads. http://www.health24.com/news/Infectious_diseases/1-922,55166.asp. Accessed 11 Aug 2010
51. Anon (2010) 17 dead from rift valley fever. http://www.news24.com/SouthAfrica/News/17-dead-from-Rift-Valley-Fever-20100507. Accessed 11 Aug 2010
52. Anon (2010) SA wool exports to China stopped. http://www.fin24.com/Business/SA-wool-exports-to-China-stopped-20100817?pageNo=1. Accessed 18 Aug 2010
53. Marai IFM, El-Darawany AA, Fadiel A, Abdel-Hafez MAM (2007) Physiological traits as affected by heat stress in sheep – A review. Small Rumin Res 71:1–12
54. McManus C, Prescott E, Paluda GR, Bianchini E, Louvandini H, Mariante AS (2009) Heat tolerance in naturalized Brazilian cattle breeds. Livest Sci 120:256–264
55. Singh M, Rai AK, More T, Dhaliwal JS (1980) Note on comparative physiological response of sheep and goats to high ambient temperature. Indian J Anim Sci 50:202–205
56. El-Sheikh AS, Salem MH, Ibrahim II, Mohamed AA, El-Sherbini AA, Yousef MK (1981) Relative adaptability of some local and foreign breeds of sheep to Sahara desert. Egypt J Anim Prod 21:109–120
57. Boonkum W, Misztal I, Tsuruta S, Duangjinda M, Pattarajinda V, Tumwasorn S, Sanpote J, Buaban S (2010) Genetics of days open in Thai crossbred Holsteins. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0255.pdf. Accessed 20 Jan 2011
58. Misztal I, Aguilar I, Tsuruta S, Sanchez JP, Zumbach B (2010) Studies on heat stress in dairy cattle and pigs. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0625.pdf. Accessed 20 Jan 2011
59. Schmidt-Nielsen B, Schmidt-Nielsen K, Houpt TR, Jarnum SA (1956) Water balance of the camel. Am J Phys 185:185–194
60. Schmidt-Nielsen K (1955) Investigations on the Physiology of the Camel: Preliminary report. http://unesdoc.unesco.org/images/0014/001486/148616eb.pdf. Accessed 27 July 2010
61. Bayer W, Feldman A (2003) Diversity of animals adapted to smallholder systems: conservation and sustainable use of agricultural biodiversity. http://www.eseap.cipotato.org/UPWARD/Agrobio-sourcebook.html. Accessed 21 Aug 2010
62. Nardone A, Ronchi B, Lacetera N, Ranieri MS, Bernabucci U (2010) Effects of climate changes on animal production and the sustainability of livestock systems. Livest Sci 130:57–69
63. Brockhaus M (2007) Institutions and stakeholders. In: Rischkowsky B, Pilling D (eds) Part 3: The state and capacities in animal resource management – The state of the world's animal genetic resources for food and agriculture. Food and Agriculture Organization of the United Nations, Rome, pp 187–214
64. Kahi AK (2010) Developing countries and the 9th world congress on genetics applied to livestock production. J Anim Breed Genet 127:333
65. Thieme O (2007) Structured breeding programmes. In: Rischkowsky B, Pilling D (eds) Part 3: The state and capacities in animal resource management – The state of the world's animal genetic resources for food and agriculture. Food and Agriculture Organization of the United Nations, Rome, pp 215–241
66. OECD (2009) OECD Factbook 2009: Economic, environmental and social statistics, Paris. ISBN 92-64-05604-1
67. Anon (2009) National survey of Research and Experimental development (R & D): 2006/2007. Compiled by the Centre for Science, Technology and innovation indicators and the Human Sciences Research council for the Department of Science and Technology. The Department of Science and Technology, Pretoria, South Africa
68. OECD (2009) Agricultural policies in emerging economies: monitoring and evaluation. http://www.oecd.org/tad/support. Accessed 14 Oct 2009
69. Anon (2010) South Africans at work longer – survey. http://www.fin24.com/printArticle.aspx?iframe&aid=b17e6777-d714. Accessed 20 Dec 2010
70. WCGALP (2010) Book of abstracts. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010. http://www.wcgalp2010.org/download/wcgalp_hauptprogramm-web.pdf. Accessed 20 Jan 2011
71. Habier D (2010) More than a third of the WCGALP presentations on genomic selection. J Anim Breed Genet 127:336–337
72. Cardellino RA (2009) Editorial: introduction and overview to the special issue on animal genetic resources. Livest Sci 120:163–165

73. Gollin D, Van Dusen E, Blackburn H (2009) Animal genetic resource trade flows: economic assessment. Livest Sci 120:248–255
74. Blackburn H, Gollin D (2009) Animal genetic trade flows: the utilization of newly imported breeds and gene flow of imported animals in the United States of America. Livest Sci 120:240–247
75. Browning R, Leite-Browning ML, Byars M (2010) Reproductive and health traits among Boer, Kiko, and Spanish meat goat does under humid subtropical conditions of the southeastern United States. J Anim Sci 89:648–660
76. Sandup T, Udo HMJ, Eilers CHAM, Ibrahim MNM, Van der Zijpp E (2010) Crossbreeding and intensification of smallholder crop-cattle farming systems in Bhutan. Livest Sci 132:126–134
77. Ole Kwallah ABO, Okeyo AM, Mburu D, Hanotte O, Muigai AWT (2010) Genetic diversity and relationships of sheep breeds of Kenya: preliminary results and evidence of dilution of the indigenous Red Maasai. In: Proceedings of the 9th World Congress on Genetics Applied to Livestock Production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0828.pdf. Accessed 20 Jan 2011
78. Scholtz MM, Bester J, Mamabolo JM, Ramsay KA (2008) Results of the national cattle survey undertaken in South Africa, with emphasis on beef. Appl Anim Husb Rural Dev 1:1–9. www.sasas.co.za/aahrd/
79. Cardellino RA, Boyazoglu J (2009) Research opportunities in the field of animal genetic resources. Livest Sci 120:166–173
80. Hoffmann I (2010) Climate change and the characterization, breeding and conservation of animal genetic resources. Anim Genet 41(Suppl 1):32–46
81. AdaS M, MdoSM A, Egito AA, McManus C, Lopes MA, Paiva SR (2009) Present status of the conservation of genetic resources in Brazil. Livest Sci 120:204–212
82. FAO (2007) Report of the technical conference on animal genetic resources for food and agriculture. Interlaken, Switzerland, 3–7 September 2007, ITC-AnGR/07/REP. http://www.fao.org/AG/againfo/programmes/en/genetics/ITC_docs.htm. Accessed 18 May 2010
83. Villanueva B, Sawalha RM, Roughsedge T, Rius-Vilarrasa E, Wooliams JA (2009) Development of a genetic indicator of biodiversity for farm animals. Livest Sci 129:200–207
84. Djemali M, Bedhiaf-Romdhani S, Iniguez L, Inounou I (2009) Saving threatened native breeds by autonomous production, involvement of farmer organization, research and policy makers: the case of the Sicilo-Sarde breed in Tunisia, North Africa. Livest Sci 120:213–217
85. Toro MA, Fernandez J, Caballero A (2009) Molecular characterization of breeds and its use in conservation. Livest Sci 120:174–195
86. Holst PJ (1999) Recording and on-farm evaluations and monitoring: breeding and selection. Small Rumin Res 34:197–202
87. Djemani M, Wrigley J (2002) Tayloring genetic improvement to meet the overall livestock development objective. In: Proceedings of the world congress on genetics applied to livestock production, Montpellier, 19–23 August 2002, vol, 33, pp 299–306
88. Swan AA, Brown DJ, Banks RG (2009) Genetic progress in the Australian sheep industry. Proc Assoc Adv Anim Breed Genet 18:326–329
89. Mostert BE, Theron HE, Kanfer FHJ, Van Marle-Köster E (2006) Comparison of breeding values and genetic trends for production traits estimated by a lactation model and a fixed regression Test-day Model. S Afr J Anim Sci 36:71–78
90. Mostert BE, Theron HE, Kanfer FHJ, Van Marle-Köster E (2006) Test-day models for South African dairy cattle for participation in international evaluations. S Afr J Anim Sci 36:58–70
91. Grobler SM, Scholtz MM, Bester J, Mamabola JM, Ramsay KA (2008) Dairy production systems in the emerging and communal sectors of South Africa: Results of a structured survey. Appl Anim Husb Rural Dev 1:25–30. www.sasas.co.za/aahrd
92. Okeno TO, Kosgey IS, Kahi AK (2010) Economic evaluation for improvement of dairy cattle in Kenya. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0610.pdf. Accessed 20 Jan 2011
93. Wasike CB, Magothe TM, Peters KJ, Kahi AK (2010) Describing the lactation process in Ayrshire and Holstein Friesian cattle in Kenya using a mechanistic lactation function. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0402.pdf. Accessed 20 Jan 2011
94. Moyo S, Swanepoel FJC (2010) Multifunctionality of livestock in developing communities. In: Swanepoel F, Stroebel A, Moyo S (eds) The role of livestock in developing communities: enhancing multifunctionality. Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen, pp 1–11
95. Bergh L (2010) The national beef recording and improvement scheme. In: Scholtz MM (ed) Beef breeding in South Africa, 2nd edn. Agricultural Research Council, Pretoria, pp 55–70
96. Rust T, Theron HE, Scholtz MM, Bergh L (2010) The practical use of breeding values in beef cattle breeding. In: Scholtz MM (ed) Beef breeding in South Africa, 2nd edn. Agricultural Research Council, Pretoria, pp 115–128
97. Rust T, Schoeman SJ, Van der Westhuizen J, Van Wyk JB (2009) Non-linear model analysis of categorical traits related to female reproduction efficiency in beef cattle. S Afr J Anim Sci 39:260–266
98. Anon (2010) Performance information for breeds that do not participate in the national beef recording and improvement scheme. In: Scholtz MM (ed) Beef breeding in South Africa, 2nd edn. Agricultural Research Council, Pretoria, pp 267–291
99. Nenghovela NB, Maiwashe A, Matjuda LE (2010) Breeding principles and guidelines for the emerging beef farmer. In: Scholtz MM (ed) Beef breeding in South Africa, 2nd edn. Agricultural Research Council, Pretoria, pp 139–148
100. Olivier JJ, Cloete SWP (2006) Genetic analysis of the South African Dorper sheep. In: Proceedings of the 7th world congress on genetics applied to livestock production, Bello Horizonte. Communication 04–10

101. Olivier JJ, Cloete SWP (2007) Genetic change in some South African Merino studs participating in the Merino plan. Proc Assoc Adv Anim Breed Genet 17:324–327
102. Van Wyk JB, Swanepoel JW, Cloete SWP, Olivier JJ, Delport GJ (2008) Across flock genetic parameter estimation for yearling body weight and fleece traits in the South African Dohne Merino population. S Afr J Anim Sci 38:31–37
103. Schoeman SJ, Cloete SWP, Olivier JJ (2010) Returns on investment in sheep and goat breeding in South Africa. Livest Sci 130:70–82
104. Cardellino RC, Mueller JP (2009) Fibre production and sheep breeding in South America. Proc Assoc Adv Anim Breed Genet 18:366–373
105. Madalena FE, Agyemang K, Cardellino RC, Jain GL (2002) Genetic improvement in medium to low-input systems of animal production. Experiences to date. In: Proceedings of the world congress on genetics applied to livestock production, Montpellier, 19–23 August 2002, vol 33, pp 299–306
106. Tadele M, Duguma G, William A, Haille A, Iñiguez L, Wurzinger M, Sölkner J (2010) Indigenous sheep genetic improvement schemes for Ethiopian smallholder farmers and pastoralists. Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0937.pdf. Accessed 20 Jan 2011
107. Amer PR, Santos BFS, Byrne TJ, Campbell AW (2010) Breeding objectives for a developing sheep industry in Brazil. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0523.pdf. Accessed 20 Jan 2011
108. Marais PG (2007) Evaluation of the genetic potential for growth and wool production of typical Transkei ewes and rams. Research report of the Grootfontein Agricultural Development Institute, 2007, Middelburg, pp 33–34
109. Marais PG (2006) Evaluation of the genetic potential for growth and wool production of typical Transkei ewes and rams. Research report of the Grootfontein Agricultural Development Institute, Middelburg, pp 33–34
110. Grobler HJF (2008) How are our LRAD farmers doing? AgriProbe 5(2):4–5. Research and news magazine of the Western Cape Department of Agriculture, Elsenburg
111. Cloete SWP, Cloete JJE, Herselman MJ, Hoffman LC (2004) Relative performance and efficiency of five Merino and Merino-type dam lines in a terminal crossbreeding system with Dormer or Suffolk sires. S Afr J Anim Sci 34:135–143
112. Fourie AJ, Cloete SWP (1993) Reproductive performance of commercial Merino, Dohne Merino and SA Mutton Merino flocks in the Southern Cape. S Afr J Anim Sci 23:104–110
113. Cape Wools SA (2010) Annual report 2008/2009. http://www.capewools.co.za/index.php?option=com_docman&task=cat_view&gid=36&Itemid=92. Accessed 2 Sept 2010
114. Iñiguez L, Hilali M (2009) Evaluation of Awassi genotypes for improved milk production in Syria. Livest Sci 120:232–239
115. Olivier JJ, Cloete SWP, Schoeman SJ, Muller CJC (2005) Performance testing and recording in meat and dairy goats. Small Rumin Res 60:83–93
116. Bester J, Ramsay KA, Scholtz MM (2009) Goat farming in South Africa: findings of a national survey. Appl Anim Husb Rural Dev 2:9–13. www.sasas.co.za/aahrd/
117. Mtleni BJ, Muchadeyi FC, Maiwashe A, Phitsane PM, Halimani TE, Chimonyo M, Dzama K (2009) Characterisation of production systems for indigenous chicken genetic resources in South Africa. Appl Anim Husb Rural Dev 2:18–22. www.sasas.co.za/aahrd/
118. Magothe TM, Muhuyi WB, Kahi AK (2010) Genetic parameter estimates for egg and body weights of indigenous chicken in Kenya. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0195.pdf. Accessed 20 Jan 2011
119. Kahi AK, Bett RC, Rewe TO (2010) Socio-economic drivers of successful breeding programs in developing countries. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0011.pdf. Accessed 20 Jan 2011
120. Newman SAN, Amyes N, Cruickshank GJ, Young MJ (2002) Sheep Improvement Limited – the national sheep performance scheme in New Zealand. In: Proceedings of the 9th world congress on genetics applied to livestock production, Montpellier, 19–23 August 2002, vol 33, pp 733–736
121. Szkotnicki WJ, Kennedy D, Miller SP, Schaeffer LR (2010) A web 2.0 system for managing sheep records and genetic evaluations. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0447.pdf. Accessed 20 Jan 2011
122. Olivier JJ (2003) Shepherd. Sheep breeding support software. http://gadi.agric.za/software/shepherd/shepherd.htm. Accessed 24 Aug 2010
123. Gilmour AR (2009) Mixed models in animal breeding: where to now? Proc Assoc Adv Anim Breed Genet 18:394–397
124. Gilmour AR, Gogel BJ, Cullis BR, Thompson R (2006) ASReml User Guide. Release 2.0. VSN International, Hemel Hempstead
125. Gianola D, de los Compos G, Gonzalez-Recio O, Long N, Okut H, Rosa GJM, Weigel KA, Wu X-L (2010) Statistical learning methods for genome based analysis of quantitative traits. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0014.pdf. Accessed 20 Jan 2011
126. Misztal I (2008) Reliable computing in estimation of variance components. J Anim Breed Genet 125:363–370

127. Aguilar I, Misztal I, Legarra A, Tsuruta S (2010) Efficient computations of genomic relationship matrix and other matrices used in the single step evaluation. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0768.pdf. Accessed 20 Jan 2011
128. Misztal I, Aguilar I, Legarra A, Tsuruta S, Johnson DL, Lawlor TJ (2010) A unified approach to utilize phenotypic, full pedigree, and genomic information for genetic evaluation. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 16 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0050.pdf. Accessed 20 Jan 2011
129. Nephawe KA (2004) Application of random regression models to the genetic evaluation of cow weight in Bonsmara cattle in South Africa. S Afr J Anim Sci 34:166–173
130. Cardoso FF, Alencar MM, Tempelman RJ (2010) Genotype by environment interaction and prediction of genetic merit. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0015.pdf. Accessed 20 Jan 2011
131. Lewis CRG, Hermesch S, Bunter KL (2010) A random regression analysis of sow lactation feed intake and the effect of temperature on intake. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0670.pdf. Accessed 20 Jan 2011
132. Wolc A, Barczak E, Wòjtowski J, Ślòsarz P, Szwaczkowski T (2010) Variance components of growth in Polish sheep described by random regression models. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0408.pdf. Accessed 20 Jan 2011
133. The Bovine Hapmap Consortium (2009) Genome-wide survey of SNP variation uncovers the genetic structure of cattle breeds. Science 324:528–532
134. Onteru S, Fan B, Garrick D, Stalder K, Rothschild M (2010) Whole- genome association analysis for sow lifetime production, reproduction and structural soundness traits using the porcine SNP60 beadchip. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0273.pdf. Accessed 20 Jan 2011
135. Souza CA, Ramayo Y, Megens HJ, Rodriguez MC, Loarca A, Caal E, Soto H, Melo M, Revidatti MA, De la Rosa SA, Shemereteva IN, Okumura N, Cho, IC, Delgado JV, Paiva SR, Crooijmans RPMA, Schook LB, Groenen MAM, Ramos-Onsins SE, Pèrez-Enciso M (2010) Porcine colonization of the Americas: a 60K SNP story. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0510.pdf. Accessed 20 Jan 2011
136. Dodds KG, Auvray B, Pickering N, McEwan JC (2009) Quality control for ovine SNP50 beadchip genotypes. Proc Assoc Adv Anim Breed Genet 18:296–299
137. Magee DA, Park SDE, Scraggs S, Murphy AM, Doherty ML, Kijas JW, International Sheep Genomics Consortium, MacHugh DE (2010) High fidelity of whole-genome amplified sheep (*Ovis aries*) deoxyribonucleic acid using a high-density single nucleotide polymorphism array-based genotyping platform. J Anim Sci 88:3183–3186
138. Khatkar MS, Tier B, Hobbs M, Khatkar D, Cavanagh JAL, Crump R, Moser G, Raadsma HW (2009) Genome structure in Australian Holstein Friesian cattle revealed by combined analysis of three high density SNP panels. Proc Assoc Adv Anim Breed Genet 18:247–250
139. Raadsma HW, Zenger KR, Khatkar MS, Crump R, Moser G, Solkner J, Cavanagh JAL, Hawken RJ, Hobbs M, Barris W, Nicholas FW, Tier B (2007) Genome wide selection in dairy cattle based on high density genome-wide SNP analysis: from discovery to application. Proc Assoc Adv Anim Breed Genet 17:231–234
140. Vinet A, Touze JL, Bichot R, Hestault O, Bellayer C, Boussaha M, Fritz S, Guillaume F, Sapa J, Bodin L, Phocas F (2010) Genome-wide scan for bovine ovulation rate using a dense SNP map. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0459.pdf. Accessed 20 Jan 2011
141. Avendaňo S, Watson KA, Kranis A (2010) Genomics in poultry breeding – from Utopias to deliverables. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0049.pdf. Accessed 20 Jan 2011
142. Gärke C, Ytournel F, Bed'hom B, Gut I, Lathrop M, Weigend S, Simianer H (2010) Differentiation power of SNPs for assessing the phylogenetic structure of populations. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0722.pdf. Accessed 20 Jan 2011
143. Perez-Enciso M (2010) Leipzig: the dawn of the genomes era. J Anim Breed Genet 127:338
144. Goddard ME, Hayes BJ, Meuwissen THE (2010) Genomic selection in farm animal species – Lessons learn and future perspectives. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0701.pdf. Accessed 20 Jan 2011
145. Hayes BJ, Bowman PJ, Chamberlain AJ, Goddard ME (2009) Invited review: genomic selection in dairy cattle: progress and challenges. J Dairy Sci 92:433–443

146. Liu Z, Seefried F, Reinhardt F, Thaller G, Reents R (2010) Dairy cattle genetic evaluation enhanced with genomic information. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0009.pdf. Accessed 20 Jan 2011
147. Weigel KA, De los Campos G, Vazquez AI, Van Tassell CP, Rosa GMJ, Gianola D, O'Connell JR, VanRaden PM, Wiggans GR (2010) Genomic selection and its effects on dairy cattle breeding programs. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0119.pdf. Accessed 20 Jan 2011
148. Wiggans GR, Cooper TA, VanRaden PM, Silva MV (2010) Increased reliability of genetic evaluation for dairy cattle in the United States from use of genomic information. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0476.pdf. Accessed 20 Jan 2011
149. Barker JSF (2009) Defining fitness in natural and domesticated populations. In: van der Werf J, Graser H-U, Frankham R, Gondro C (eds) Adaptation and fitness in farm animal populations: evolutionary and breeding perspectives on genetic resource management. Springer, Dordrecht, pp 3–14. www.springer.com
150. Bishop SC, Woolliams JA (2010) Understanding field disease data. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0401.pdf. Accessed 20 Jan 2011
151. Gauly M, Besbes B, Pinard-van der Laan MH, Hoffman I, Greeff J, Thevenon S, Baker L, Tibbo M, Bishop S, Mugambi J, Dempfle L, Sidibe I, Mandonnet N, Amarande AFT, Miller JE (2010) Ruminant genetic resources and their resistance/tolerance to parasitic diseases. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0445.pdf. Accessed 20 Jan 2011
152. Bath GF, Van Wyk JA (2010) The Five Point Check© for targeted selective treatment of internal parasites in small ruminants. Small Rumin Res 86:6–13
153. Goddard M (2009) Fitness traits in animal breeding programs. In: van der Werf J, Graser H-U, Frankham R, Gondro C (eds) Adaptation and fitness in farm animal populations: evolutionary and breeding perspectives on genetic resource management. Springer, Dordrecht, pp 41–52. www.springer.com
154. Muller CJC, Cloete SWP, Potgieter JP and Zishiri, OT (2010) Genetic parameters for fertility traits in South African Holstein cows. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0734.pdf. Accessed 20 Jan 2011
155. Cloete SWP, Gilmour AR, Olivier JJ, Van Wyk JB (2004) Genetic and phenotypic trends and parameters in reproduction, greasy fleece weight and live weight in Merino lines divergently selected for multiple rearing ability. Aust J Exp Agric 44:745–754
156. Snowder GD, Fogarty NM (2009) Composite trait selection to improve reproduction and ewe productivity. Anim Prod Sci 49:9–16
157. Maiwashe A, Nephawe KA, Theron HE (2009) Analysis of stayability in South African Angus cattle, using a threshold model. S Afr J Anim Sci 39:55–60
158. GLOBALGAP (2010) Control points and compliance criteria – integrated farm assurance: All livestock base. English version V.3.0-4_Mar10. GLOBALGAP, Köln (Cologne), Germany. http://www.globalgap.org/cms/upload/The_Standard/IFA/English/CPCC/GG_EG_IFA_CPCC_LB_ENG_V3_0_4_Mar10.pdf. Accessed 2 Sept 2010
159. Olivier JJ, Moyo S, Montaldo HH, Thorpe W, Valle Zarate A, Trivedi KR (2002) Integrating genetic improvement into livestock development in medium- to low-input productions systems. In: Proceedings of the world congress on genetics applied to livestock production, Montpellier, 19–23 August 2002, vol 33, pp 365–372
160. Philipsson J, Rege JEO, Okeyo AM (2006) Sustainable breeding programmes for tropical farming systems. In: Ojango JM, Malmfors B, Okeyo AM (eds) Animal Genetics Training Resource, version 2. International Livestock Research Institute, Nairobi, Kenya/Swedish University of Agricultural Sciences, Uppsala, Sweden
161. Pell AN, Stroebel A, Kristjanson P (2010) Livestock development projects that make a difference: what works, what doesn't and why. In: Swanepoel F, Stroebel A, Moyo S (eds) The role of livestock in developing communities: enhancing multifunctionality. Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen, pp 13–29
162. Nesamvuni EA, Swanepoel FJC, Stroebel A (2010) Interactions between gender, environment, livelihoods, food, nutrition and health. In: Swanepoel F, Stroebel A, Moyo S (eds) The role of livestock in developing communities: enhancing multifunctionality. Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen, pp 93–105
163. Rewe TO, Herold P, Kahi AK, Valle Zarate A (2010) Genetic improvement and monetary returns in alternative closed and open nucleus breeding programs for Boran cattle reared in the semi-arid tropics. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0850.pdf. Accessed 20 Jan 2011
164. Fogarty NM (2006) Utilization of breed resources for sheep production. In: Proceedings of the 8th world congress on genetics on applied livestock production, Bello Horizonte, Brazil. Communication 04–10
165. Bouix J, Jacquin M, Foulquiè D, Autran P, Guillouet P, Hubert D, Bibè B (2002) Genetic effects on sheep production in harsh environment and extensive management system. In: Proceedings of the world congress on genetics on applied

livestock production, Montpellier, 19–23 August 2002, vol 29, pp 441–444
166. Francois D, Boissy A, Jacquiet P, Allain D, Bibė B, Rupp R, Moreno CR, Bodin L, Hazard D, Bouix J (2010) Genetics of adaptation traits for harsh environment in sheep. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0453.pdf. Accessed 20 Jan 2011
167. Mormède P, Foury A, Terenina E, Knap PW (2010) Breeding for robustness: the role of cortisol. Animal 5:651–657
168. Naqvi AN (2007) Application of molecular genetic technologies in livestock production: potentials for developing countries. Adv Biol Res 1:72–84
169. Robinson J, Ruane J (2007) Marker-assisted selection as a potential tool for genetic improvement in developing countries: debating the issues. FAO invited book. In: Guimaraes EP, Ruane J, Scherf BD, Sonnino A, Dargie JD (eds) Marker-assisted selection: current status and future perspectives in crops, livestock, forestry and fish. FAO, Rome, pp 427–440. ftp://ftp.fao.org/docrep/fao/010/a1120e/a1120e04.pdf. Accessed 28 July 2010
170. Van der Werf JHJ (2007) Marker-assisted selection in sheep and goats. FAO invited book. In: Guimaraes EP, Ruane J, Scherf BD, Sonnino A, Dargie JD (eds) Marker-assisted selection: current status and future perspectives in crops, livestock, forestry and fish. FAO, Rome, pp 229–247. ftp://ftp.fao.org/docrep/fao/010/a1120e/a1120e04.pdf. Accessed 28 July 2010
171. Fogarty NM, Banks RG, Van der Werf JHJ, Ball AJ, Gibson JP (2007) Te information nucleus – a new concept to enhance sheep industry genetic improvement. Proc Assoc Adv Anim Breed Genet 17:29–32
172. Mortimer SI, Pearce KL, Jacobs RH, Hopkins DL, Warner RD, Geesink GH, Hocking Edwards JE, Pethick DW, Van der Werf JHJ (2009) The information nucleus – genetically improving Australian lamb production. Proc Assoc Adv Anim Breed Genet 18:426–429
173. Van der Werf JHJ, Banks RG (2010) A genomic information nucleus flock to accelerate rates of genetic improvement in sheep. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0046.pdf. Accessed 20 Jan 2011
174. Brien FD, Hebart ML, Hocking-Edwards JE, Greeff JC, Hart KW, Refshauge G, Gaunt G, Behrendt R, Thomson K, Hinch GN, Geenty KG, Van der Werf JHJ (2009) Genetics of lamb survival: preliminary studies on the information nucleus flock. Proc Assoc Adv Anim Breed Genet 18:108–111
175. Knap PW (2011) Pig breeding for sustainability. Encyclopedia for Sustainability, Science and Technology, Springer, Germany (in press)
176. Eitan Y, Soller M (2011) Poultry breeding: the broiler chicken as harbinger of the future. Encyclopedia for Sustainability, Science and Technology, Springer, Germany (in press)
177. Ojango JM, Panandam JM, Bhuiyan AKFH, Khan MS, Kahi AK, Imbayarwo-Chikosi VE, Halimani TE, Kosgey IS, Okeyo AM (2010) Higher education in animal breeding in developing countries – challenges and opportunities. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 6 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0073.pdf. Accessed 20 Jan 2011
178. Casey NH (2009) Integrated higher learning – an investment in intellectual capital for livestock production. Livest Sci 130:83–94
179. Van Arendonk JAM, Malmfors B, Verrier E, Thaller G, Solkner J, Klemetsdal G, Lont DE (2010) European Master in Animal Breeding and Genetics: international collaboration to face future needs. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0561.pdf. Accessed 20 Jan 2011
180. Wurzinger M, Toro M, Villaroel M, Serradilla JM (2010) Reform and desighn of Masters programs "Animal Science" at seven Universities in four Latin American countries. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0868.pdf. Accessed 20 Jan 2011
181. Ojango JMK, Malmfors B, Okeyo AM, Philipsson J (2009) Capacity building for sustainable use of animal genetic resources in developing countries. Appl Anim Husb Rural Dev 2:23–26. www.sasas.co.za/aahrd
182. Bullock D, Lewis R, Weaber R, Lockee B, Strohbehn D, Garrick D, Pollak J (2010) Distance education: A short time solution to training graduates and producers with a shrinking population of quantitative geneticists. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0916.pdf. Accessed 20 Jan 2011
183. Lewis R, Lockee B, Krishnamurthy L, Meese J, Boynton D (2010) Experiential learning online: Cyber sheep. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0930.pdf. Accessed 20 Jan 2011
184. Van der Waaij L, Oldenbroek JK (2010) Teaching Animal Breeding digitally in professional Agricultural education. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0980.pdf. Accessed 20 Jan 2011
185. Eckard RJ, Grainger C, De Klein CAM (2010) Options for the abatement of methane and nitrous oxide from ruminant production. Livest Sci 130:47–56
186. Robinson DL, Goopy J, Hegarty R (2010) Repeatability, animal and sire variation in 1-hour methane emissions and relationships with rumen volatile fatty acid concentrations. In:

Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0712.pdf. Accessed 20 Jan 2011

187. Cassandro M, Cecchinato A, Battagin M, Penasa M (2010) Genetic parameters of methane production in Holstein Friesian cows. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0837.pdf. Accessed 20 Jan 2011
188. Hegarty R, McEwan JC (2010) Genetic opportunities to reduce methane emissions from ruminant livestock. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 8 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0515.pdf. Accessed 20 Jan 2011
189. Herrero M, Thornton PK, Gerber P, Van der Zijpp A, Van de Steeg J, Notenbaert AM, Lecomte P, Tarawali S, Grace D (2010) The way forward for livestock and the environment. In: Swanepoel F, Stroebel A, Moyo S (eds) The role of livestock in developing communities: enhancing multifunctionality. Centre for Agricultural and Rural Cooperation (CTA), Wageningen, pp 51–76
190. Conington J, Gibbons J, Haskell MJ, Bünger L (2010) The use of breeding to improve animal welfare. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 12 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0057.pdf. Accessed 20 Jan 2011
191. Veissier L, Butterworth A, Bock B, Roe E (2008) European approach to ensure good animal welfare. Appl Anim Behav Sci 113:279–297
192. Bohte-Wilhelmus DI, De Haas Y, Veerkamp RF, Windig JJ (2009) Genetic selection as alternative to tail docking in Hampshire Down and Clun Forest. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0205.pdf. Accessed 20 Jan 2011
193. Sørenson JT, Fraser D (2010) On-farm welfare assessment for regulatory purposes: issues and possible solutions. Livest Sci 131:1–7
194. Häggman J, Juga J, Sillanpää MJ, Thompson R (2010) Genetic parameters for claw health an leg conformation traits in Finnish Ayrshire cows. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, 1–6 August 2010, 4 p. ISBN 978-3-00-031608-1. http://www.kongressband.de/wcgalp2010/assets/pdf/0308.pdf. Accessed 20 Jan 2011
195. Gourdine JL, De Greeff KH, Rydmer L (2010) Breeding for welfare in outdoor pig production: a simulation study. Livest Sci 132:26–34. doi:10.1016/j.livsci.2010.04.017
196. Hoffman LC, Styger E, Muller M, Brand TS (2003) The growth and carcass and meat characteristics of pigs raised in a free-range or conventional housing system. S Afr J Anim Sci 33:166–175
197. Lemmon KL, Hebart ML, Brien FD, Hynd PI (2009) The genetics of temperament traits in Merino sheep. Proc Assoc Adv Anim Breed Genet 18:96–99
198. Cloete SWP, Misztal I, Olivier JJ (2009) Genetic parameters and trends for lamb survival and birth weight in a Merino flock divergently selected for multiple rearing ability. J Anim Sci 87:2196–2208
199. Hatcher S, Atkins KD, Safari E (2010) Lamb survival in Australian Merino sheep: a genetic analysis. J Anim Sci 88: 3198–3205
200. Mushi DE, Mtenga LA, Eik LO (2007) Some factors affecting the quality of meat from ruminants and their relevance to the Tanzanian meat industry. Tanz J Agric Sci 8:173–192

Breeding in Horses

Thorvaldur Árnason
Agricultural University of Iceland, Borgarnes, Iceland
International Horse Breeding Consultant,
Morgongava, Sweden

Article Outline

Glossary

Animal model (AM) Statistical models that are used for evaluation of breeding values of all individuals in the population of interest.

Best linear unbiased prediction (BLUP) Standard statistical method for estimating breeding values in populations. BLUP adjusts for systematic fixed environmental effects and accounts for genetic relationships among animals.

Breeding value (BV) Mean additive genetic value of an individual relative to all members of the (base) population.

Effective population size (N_e) Number of individuals that would give rise to calculated sampling variance, or rate of inbreeding, if they bred in the manner of the idealized population with complete random mating, no selection, no migration, and no mutations.

Estimated breeding value (EBV) Estimate of the mean additive genetic value of an individual for a quantitative trait.

Generation interval (L) Average age of the parents when their progenies that will replace them in breeding are born.

Heritability (h^2) Measures the extent to which the phenotypes are determined by genetic factors. It is expressed as the ratio between additive genetic variance and the total phenotypic variance.

Inbreeding coefficient (F) Measure of the probability that two genes at any locus in an individual are identical by descent. It refers to an individual and expresses the degree of genetic relationship between the individual's parents.

Riding horse The narrow definition of riding horse or sport horse refers to horses of "Warmblood" breeds or Thoroughbred crosses, which compete in the classical equestrian sporting events of dressage, show jumping, and eventing. However, the broad definition of a riding horse refers to a horse used for any type of riding and will be used here.

Selection Any natural or artificial process favoring the survival and propagation of certain individuals in a population.

Selection intensity (i) Function of the proportion of animals selected for breeding relative to the total number of animals available for selection. The smaller the proportion selected, the higher the selection intensity.

Timeform handicap ratings An estimate of racing capacity of Thoroughbred horses (in Great Britain). Express racing merit as weights in pounds that the compiler believes the horse should carry in an average free handicap race.

Trotter A horse trained for harness racing. The horse races in trot, which is a two-beat springing gait with a suspension phase (no ground contact) between two diagonal supporting pair of legs.

Definition of the Subject

Animal breeding can be defined as a human activity with the deliberate purpose to change existing populations of animals in some desired directions, so that future generations of these animals become more valuable in some sense. A prerequisite for successful animal breeding is that there is a genetic variation in the population in the traits one want to improve in future generations. Other requirements are: clear definitions of the breeding goals, good pedigree files, valid measures of the desired traits, methods of genetic evaluations that can combine information from the pedigree and the measured trait, and finally effective selection of breeding animals with high estimated genetic values (BV = breeding values) and therefore

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

presumably with relatively large proportion of valuable genes affecting the desired traits.

The selected parents will transmit their genes to the next generation, and depending on the amount of genetic variation in the traits, accuracy of estimated genetic evaluations, and the selection intensity, the average level of the offspring generation will be raised as compared with the parent generation.

All general principles and methods of animal breeding obviously apply equally to the breeding of horses. There are however few structural and demographic characteristics specific to horse breeding that should be highlighted:

- Thorough registration of pedigree files
- Low rate of reproduction
- Long generation intervals
- Important traits are recorded on both males and females
- Wide overlapping of age groups among breeding candidates
- Considerable nonrandom mating practiced
- Extremely large differences in economic value depending on assumed genetic merit

All these factors support the use of methods for genetic evaluations that combine all pedigree information in an optimal way. In particular, the use of animal models (AMs) to obtain best linear unbiased predictions (BLUP) for genetic evaluation of breeding animals has become an important tool in modern horse breeding.

Introduction

Humans began to breed horses when the horse was domesticated some 6,000 years ago. Tamable animals were then selected from wild flocks and broken in. Stallions and mares with suitable temperament and desired characteristics were spared and got offspring in their new environment. The fitness measure of natural selection in the wild life was replaced by a selective value in the new role of serving humans. This artificial, and to lesser degree natural selection, of horses over 6,000 years has resulted in gradual genetic changes and subdivision into breeds adapted to different purposes [1, 2].

Throughout the history horses have been used for variety of purposes, such as riding, driving, carrying packs, and pulling agricultural equipments. Horses were also for a long time the most important means of transport in wars and have played a great role in the political and social development of many civilizations and even for the conquest of whole continents. Horses have also played a major role in the mythology of many religions but have as well been kept for their grace and were commonly used as a symbol of material status. The Vikings even used stallions for fighting contests. In many cultures, horses have been used for milk and meat production. The life of nomads in central Asia, of cowboys grazing and driving big herds of cattle, and of Indians hunting wild buffalos on the savannas in North America would have been impossible without horses. In brief, the human history would have been very much different without the company of the horse [3].

Today there are about 60 million horses in the world [4]. While horses are still used for agricultural work and transportation in many parts of the world, the main role of horses in modern societies is to serve humans as pleasure or sport companions. The economic and social impact of the worldwide horse sector is tremendous and often underrated. In Europe, three to six horses (varies between countries) create full employment for one person. So within the European Union alone, with almost six million horses, about one million people are directly or indirectly employed in horse-related activities. About 4% of European agricultural land is dedicated to horse breeding [5].

Horses are used and will undoubtedly be used in the future for various competitive performance events, sport events, and pleasure riding or driving. Modern knowledge of animal breeding, genetics, statistics, reproduction technology, and computer science will certainly be used for adapting the different horse populations of the world to their intended purpose.

Horse Breeding

Breeds

Horses are members of *Equus ferus caballus*. In fact, there is no strict scientific definition of the term breed, but the elusive concept of breed as a definition of a population of "purebred" animals is commonly used among horse breeders. The classic definition of a autochthonous breed refer to a distinctive set of

animals associated with a restricted geographical area in which it was developed to meet human needs under particular local conditions [6]. Often horse breeds are defined by breeding societies or registries that record pedigrees and maintain a studbook for a selected subset of horses based on geographical origin, phenotype, or function [3]. New synthetic horse breeds have been created by genetic selection according to a common breeding goal across several older horse breeds. As an example, many European Warmblood breeds have evolved this way. At the Web site "Breeds of Livestock: Horse Breeds" (http://www.ansi.okstate.edu/breeds/horses) detailed description on over 300 different horse breeds all over the world can be accessed.

Breeding Goals

Horse breeding, as all animal breeding, starts with a clear definition of breeding goals that usually includes many traits. The breeding objectives are a statement of the relative values of genetic change in all the desired traits that are included in a breeding plan. The overall objective (aggregate genotype) can be expressed as $H = \alpha_1 A_1 + \alpha_2 A_2 + \ldots + \alpha_n A_n$, where α_i is the relative (economic) weight and A_i is the animal's breeding value for the ith trait. The α_i values are often linear or nonlinear functions of the profit (return – cost) [7, 8]. Horse breeders often have a rather clear idea as to which traits they want to improve by genetic selection. However, the exact definitions of the relative weights on the different traits included in the breeding goal are often vague and determined by preferences of the individual breeder. Meanwhile, it is important that breeders should be provided with genetic evaluations for all the major traits that might be included in the breeding goal [9].

Registration of Pedigree Files

Unique identification of all members of a population has become a prerequisite for all rational breeding schemes using modern animal breeding methodology. The same identification number must refer to the same horse anywhere in the comprehensive pedigree files and in all files containing the registered traits. As horse breeding becomes increasingly more internationalized, the communication between horse registers in different countries is vital for successful breeding schemes. It is important that the identification number given to a newborn foal follow the individual animal throughout its entire life, even if the animal is exported to, or competes in, another country. It should contain codes for the country of birth, year of birth, and possibly the sex of the animal. The Web-based "universal equine life number" (UELN) system (www.ueln.net) offers a solution to this problem.

Measured Traits and Estimation of Genetic Parameters

An important part of a horse breeding scheme is to develop and use measures with good correlations with the traits in the breeding goal. The measurements need to be collected on a large or a random part of the population to be used in the selection and breeding process. Most traits that are important in breeding of horses are governed by a large number of genes and many environmental factors. They may therefore be analyzed with genetic and statistical methods that apply to quantitative traits [10]. Measurements of performance traits in horses are often not normally distributed and need to be transformed to approach the normal distribution. Sometimes conversion to ranks is more favorable [11]. Measures of performance in racing and other equine competition sport events are frequently based on functions of earnings, ranks, or racing times. Occasionally, the measures are summarized in handicap ratings or cumulated competition points.

In many populations, the recorded traits are scored on a linear scale, often by subjective judgment, in special field or station tests. These traits may involve measurements of conformational details, temperament, performance in special gaits, or performance at various ranch tasks.

Before the registered traits can be used as a basis for genetic evaluations, the genetic parameters (variation, heritability, genetic correlations, and environmental correlations) among the traits must be estimated. The methods used for estimating genetic parameters in horse populations are usually based on maximum likelihood techniques for mixed animal models [12, 13]. In some recent cases, Bayesian Markov Chain Monte Carlo (MCMC) methods have been applied [14, 15].

Heritability of racing performance traits based on earnings and rank functions have frequently been estimated in the range of 0.3–0.4. Time measures in trotter races and shorter gallop races generally show heritability estimates of nearly the same magnitude (0.2–0.4), while heritability estimates of racing time in longer Thoroughbred races are usually considerably lower or in the region 0.1–0.2 [12, 13].

Heritability estimates of competition variables for jumping and dressage performance in riding horses have been found to be lower than in racing or about 0.1, on average. Data on competition results in riding horses is often subjected to strong preselection, which can be the main reason for the low heritability estimates [13].

The average heritability estimates for field test traits measuring gaiting ability and free jumping in riding horses are in the range of 0.2–0.3. Corresponding average heritability estimates of traits registered at station tests for stallions are generally much higher or in the range of 0.4–0.6 [13].

Heritability estimates of sport competition scores in Icelandic horses were found to be around 0.2–0.3, while heritability of field test traits within the same breed was estimated in the range 0.2–0.4 with the average value of 0.34 [16, 17].

Heritability of cutting performance in Western riding competitions has been estimated in the range of 0.04–0.19 [18].

This brief summary confirms that genetic variation and thus scope for genetic improvement has been found in the most important traits in many horse breeds.

Genetic Evaluations and Selection

Traditionally, selection of horses was for a long time based on phenotypic observations on performance and body conformation in combination with subjective evaluation of the pedigree. Since mid-1980s, the BLUP method with animal model (AM) has gained popularity as a superior and standard method for combining information from phenotypic observations and pedigree [19]. Estimated breeding values (EBVs) obtained by AM-BLUP are now used as the main selection criteria in many horse populations.

Estimation of breeding values in a population begins with definition of a linear statistical model. The model should fit the data as well as possible but it should also include as few parameters as possible. The model should account for all major factors affecting variation in the data in a systematic way. In the simplest form, the model may be written for a single trait as

$$y_{ij} = \mu + b_i + a_j + e_{ij}$$

where y_{ij} is the phenotypic observation on the jth horse belonging to the ith class of fixed (systematic) effects affecting the trait; μ represents the overall mean of the (base) population; b_i represents the deviation from the population mean caused by the ith class of fixed effects; and a_j represents the breeding value of the jth horse. The breeding values are random normally distributed deviations from the genetic mean of the base population and have the variance $\mathbf{A}\sigma_A{}^2$, where $\mathbf{A}$ is the additive genetic relationship matrix. Finally e_{ij} denotes the remainder of the model, which is assumed to include independent and randomly distributed environmental effects pertaining to the jth horse.

Practical application of AM-BLUP in horse populations are usually based on far more complicated models than that shown above. They often include many fixed environmental factors, which affect systematically the variation in phenotypic records. As the breeding goal usually includes many correlated traits, multiple trait (MT) AM-BLUP procedures are commonly used for genetic evaluation of horses. Sometimes additional random factors are included in the model. This may be for genetic evaluation of repeated records on the same trait, or in other cases for better estimation and adjustment of genetic or environmental effects. As an example, the model may include both permanent environmental and genetic effects of the dam (maternal effects), or permanent environmental effects of riders, drivers, or trainers. Random regression models as an alternative to repeatability models for genetic evaluation of traits of longitudinal nature have good prospect in horse breeding [20, 21]. The trajectories may for example represent age development, racing distance, or competition classes.

Important traits in horse breeding are commonly scored on an ordered categorical scale. If there are at least several categorical classes and histograms of the

distribution show good approximation to the normal distribution then the data can be analyzed by linear models without any significant loss in precision. If, however, there are as few as 2–4 classes, then nonlinear threshold models may be more appropriate. This particularly applies to binary traits with low or variable frequency across the fixed effect classes of the model. But sometimes linear models (AM-BLUP) are used in such cases in large data, since the computations in the linear models are much simpler.

With the AM-BLUP method, the effects of fixed factors and the breeding values are estimated simultaneously by solving a large set of mixed model equations (MME) with equally as many unknown solutions as there are equations. The estimated breeding values become adjusted for fixed effects that are correspondingly adjusted for differences in breeding values of the horses with records in the various fixed effect subclasses. The use of the additive genetic relationship matrix inverse ($\mathbf{A}^{-1}$), computed from the pedigree list, ensures that sharing of genes between related animals is correctly accounted for in the model. In addition, the EBVs of animals that are parents of recorded animals are adjusted for in the EBVs of the mate. That is a very important property in horse breeding where the allocation of mares to breeding stallions is generally far from random in terms of genetic merit.

The pedigree file used as data for computing $\mathbf{A}^{-1}$ is a list of all registered animals in the population. Each row consists of unique identification number of the individual animal and both parents. First, the inbreeding coefficients are calculated for all animals with one of the several efficient algorithms (e.g., [22, 23]) that have been developed from the original algorithm of Quuas [24]. Then the elements of $\mathbf{A}^{-1}$ are build up by simple rules depending on the number of identified parents of each animal [25]. Animals without identified parents build the base population. In most horse populations, pedigrees are well filled and the pedigree of the youngest animals can be traced back through many generations. In such situations, one common base population can reasonably be assumed. Sometimes horse populations are either: mixtures from many base populations (e.g., synthetic breeds), or there is importation of breeding stock from other countries or populations, or there are many animals with incomplete pedigrees. In such cases, an appropriate genetic grouping by creating several base populations becomes essential [26].

The AM-BLUP method has been applied for genetic evaluations in many horse breeds throughout the world. Most applications have been within Europe, where AM-BLUP methods are routinely used for genetic evaluations in riding horses, trotters, Icelandic horses, and Thoroughbreds in many countries. In some breeds, the method has been in practical application for up to more than 2 decades. The published index values for the most important traits of the breeding goals have been widely used by breeding organizations, and by individual breeders, for facilitating their selection decisions. In many cases, the breeders have access to Web applications where BLUP indices can be searched and listed in various ways. These have become valuable tools for the breeders and a large help in their mating plans. Analysis of genetic trends in these populations have invariably shown that the rate of genetic progress have been boosted by the introduction of the AM-BLUP method.

As an alternative to the BLUP method, Ricard [13] has developed a nonlinear approximate Bayesian animal model method for genetic evaluation of horses based on ranks in competitions. The method provides estimated breeding values for an underlying normally distributed liability variable from the likelihood of ranking according to some phenotypic observations in any single race or competition event. The estimated breeding values are obtained as the mean (or mode) of the marginal joint posterior density of the combined prior information and the likelihood function according to Bayesian principles. A statistical inference drawn from the resulting EBVs can be given by probability statements in Bayesian fashion. The method adjusts the likelihood for systematic environmental effects and utilizes additive genetic relationship between animals in the same way as the AM-BLUP method does. The method has only been applied for genetic evaluation of trotters and riding horses in France [27, 28]. The method is computationally demanding and general software for the method is not yet readily available. Developments of the concept of ranks (Thurstonian model) into full Bayesian analyses using MCMC Gibbs sampler methods have

recently been proposed [29, 30]. The development of this methodology and modeling will continue and seems to have great potential in horse breeding.

Genetic evaluations of horses are often biased due to censoring of records [31, 32]. Horses are frequently preselected before they enter races, riding competitions, or field testing events. Information on the preselection criterion is usually lacking. However, in horse populations pedigree information is generally available on all horses, even on those without records. Therefore, the trait racing-status or test-status can be defined as an all-or-non trait, with the value one for tested animals and zero for culled or non-tested horses. Test-status has been confirmed a highly heritable trait in several horse populations (e.g., [32–34]). Environmental correlations between test-status and the recorded traits are not estimable because all animals with test-status equal to zero lack phenotypic records on the tested traits. However, genetic correlations between test-status and the recorded traits can be estimated if environmental covariances are constrained to some predefined values (e.g., zero). Indications of moderate to high genetic correlations between test-status and performance traits in horses have been confirmed in some populations [32, 33]. Inclusion of test-status in a multiple trait AM-BLUP framework was shown to increase the accuracy of selection and increase the rate of genetic progress in Swedish trotters, where the procedure has been routinely applied for a long time [33]. Ignorance of the problem with censoring of records results undoubtedly in bias in genetic evaluations and substantial loss of genetic progress in many horse populations.

International Genetic Evaluations

Breeding of sport horses has become a big global industry. Many breeding organizations of Warmblood riding horses in Europe have implemented modern methods for genetic evaluation of breeding horses on a national scale. The increased international trade with valuable breeding horses has led to important exchange of genetic material across populations having similar breeding goals.

The Interstallion project (http://www.interstallion.org) was established in 1998 to harmonize and improve exchange of information between Warmblood breeding organizations within Europe. The main aims have been to: (a) describe breeding objectives, test procedures, and genetic evaluation methods, (b) recommend improvements of national genetic evaluation systems, and (c) study methods of comparing genetic evaluations across countries. The project has resulted in several publications on these subjects [35–40].

Genetic evaluations for the global population of the Icelandic horse are computed regularly and published on the Internet (http://www.worldfengur.com) [41].

Selection and Genetic Progress

Factors Affecting Genetic Progress

The rate of genetic improvement by genetic selection in horse populations depends on:

1. Additive genetic variability in the selected traits (σ_A)
2. Intensity of selection (i)
3. Accuracy of selection (correlation between the true and the estimated breeding value, R_{TI})
4. Generation interval (L)
5. Inbreeding depression (d)

The genetic improvement per year can be expressed as

$$\Delta G/\text{year} = \sigma_A i R_{TI}/L - \Delta d$$

Increased intensity and accuracy of selection will increase the rate of genetic improvement if it is not leading to longer generation intervals nor increased homozygosity in loci affecting the traits and the general vigor (inbreeding depression). Generation intervals are generally very long in horse breeding (8–12 years). Shortening of the generation interval often means reduction in the accuracy in genetic evaluations (R_{TI}) but the selection intensity is usually affected in positive direction. The selection intensity, generation interval, and accuracy of selection are often different for stallions and mares. Therefore, the formula above is often modified to include two or four paths [42].

Multistage Selection in Horses

In horse breeding, large increase in the rate of response to selection can often be gained by an effective scheme for genetic evaluation of young horses that is based on pedigree information and early performance testing [43, 44]. The selection of stallions and usage for

breeding is usually of a multistage nature as they are selected repeatedly within the same generation [45]. The first step in stallion selection is based on pedigree information (ancestry and collateral relatives) and determines which colts get performance tested and thus undergo the second and the most important selection step for traits having medium to high heritability. Selection intensity should be high at the second selection stage, but unfortunately testing capacity is often limited, especially for riding horses. The cost and labor of keeping stallions instead of geldings is usually high and many colts are castrated at a young age before they can express their abilities. The third and final selection step is based on EBVs when the offspring results provide the main source of information. The value of the progeny information increases for traits with low heritability.

Effect of Selection on Effective Population Size and Inbreeding

Several generations of constant directional selection, in large populations with moderate selection intensity, will lead to a measurable reduction (10–30%) in the genetic variability (σ_A), which thereafter is assumed to stay reasonably constant due to an equilibrium between the reduction in genetic variance and the variation rebuilt by recombination [46]. Therefore, in large populations with negligible inbreeding, selection is expected to be effective for changing the population mean in the desired direction over many generations.

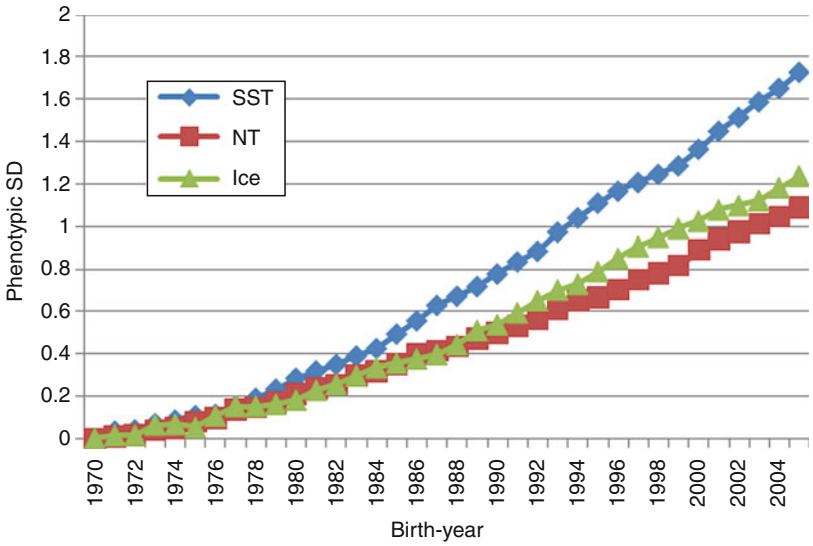
Intense selection in closed populations (no migration) will build up increased relationship among the future members of the population. That corresponds to reduction in the effective population size (N_e) and leads to mating of related individuals, which is defined as inbreeding. Small N_e and consequently heavy inbreeding results in random change in gene frequencies across generations and a general reduction in the Mendelian sampling variance term and thus decreased genetic variability (σ_A). Accumulation of inbreeding (Δd) in small closed populations will reduce the scope for genetic improvement in the selected traits. In addition, increased homozygosity in many loci in the population will presumably affect fitness traits negatively (fertility, health, vigor, etc.) and increase frequency of genetic diseases in the population [10].

Many horse populations are at risk of getting small effective population size, due to small actual population size, intensive selection on EBVs alone, and large variation in family size. In such populations, the risk of reduced genetic variability and inbreeding depression should not be ignored and the breeding schemes should aim at sustainable long-term progress, where breeding animals are to be selected in an optimum way such that genetic improvement in the breeding goal traits and effects on future inbreeding are simultaneously considered [47–49]. Sustainable breeding plans aiming at maintenance of genetic variation should direct the selection such that future inbreeding is avoided at the cost of some loss in short-term genetic progress.

Obtainable and Observed Genetic Progress in Horse Populations

The rate of genetic response in large horse populations depends mainly on how accurately the traits reflecting the breeding goal can be measured (heritability), the amount of variation in the aggregate genotypes (genetic variability), the quality of the method used for genetic evaluation (accuracy of selection), the testing capacity (selection intensity), and the age when the trait comes to an expression and can be measured (generation interval) [10].

In horse populations with onset of testing or racing performance at 3–6 years of age, and a conventional breeding scheme, the generation intervals are normally about 8–10 years for males and 10–12 years for the females. The selection intensity could correspond to ca 5% (1–10%) for males and ca 60% (40–80%) for females. When the selection criteria are EBVs that are obtained from the AM-BLUP method, then the source of information is a combination of pedigree, own performance, and offspring results. The average accuracy of selection on the male side (R_{TI}) could be about 0.8 for trait with $h^2 = 0.2$ and about 0.9 for trait with $h^2 = 0.4$. Higher accuracy would normally require more emphasis on progeny performance, which would prolong the generation interval. The accuracy of selection on the female side depends heavily on how large proportion of mares is tested for their own performance. A reasonable assumption could be $R_{TI} = 0.45$ for trait with $h^2 = 0.2$ and $R_{TI} = 0.63$ for $h^2 = 0.4$.



Breeding in Horses. Figure 1

Realized progress in the aggregate genotype, expressed in phenotypic standard deviation (σ_P) units, in three horse populations (*SST* Swedish Standardbred Trotter, *NT* Nordic Trotter, Ice Icelandic horse)

Given the assumptions above, the annual genetic progress in horse populations could correspond to 4–4.5% of the phenotypic standard deviation for traits (or combination of traits) with $h^2 = 0.2$ and to 6.5–7.5% for traits with the higher heritability of $h^2 = 0.4$. For readers interested in evaluation of different combinations of factors affecting the genetic progress in large populations, where inbreeding is ignored, a Java applet is available free at the following link: www.ihbc.se/web/contents/ihbcWebApplets/GenResponseApplet.html.

Estimates of genetic trend have been reported in several horse populations. The estimated annual genetic gain has been ranging from zero up to 6% of the phenotypic standard deviation of selected traits. Estimates of genetic trend in three different horse populations may serve as examples (Fig. 1). The Swedish Standardbred Trotter (SST) is a synthetic breed originating from imported American Standardbred Trotters and French Trotters. About 5,000 foals are born annually in the SST breed. The Nordic Trotter (NT) is a heavier type (cold-blood) originating from Norway and northern part of Sweden. About 1,300 Nordic Trotter foals are born annually (850 in Norway, 450 in Sweden). The Icelandic Horse is a closed population of small, compact, and strong horses that has been purebred in Iceland for more than thousand years. It is the only horse breed in Iceland where about 7,000 foals are born annually. Both the trotter breeds are used for harness racing and the breeding goal consists of several traits expressing racing performance ability and sustainability. The Icelandic Horse is used for riding and is known for its gaiting abilities, willingness, good character, and hardiness. The breeding goal for the Icelandic Horse consists of a linear function of 16 traits (8 conformation traits and 8 traits measuring riding ability [gaits and temperament]). The traits are registered at special field tests.

Genetic evaluations based on BLUP methods have been available as a guide for selection in these three breeds since mid-1980s. Between 1970 and 2005, the mean of the aggregate genotype (weighted mean of EBVs for several racing variables) in the SST population has been raised by 1.73 σ_P units, corresponding to the average annual ΔG/year = 0.049 over the entire period. The rate of genetic gain has increased gradually over the period as seen by the increased steepness in the slope showing the trend. For the last 10 years, the rate of genetic gain has been ΔG/year = 0.062, or 6.2% of the phenotypic standard deviation. In the smaller population of Nordic Trotter, the population average level

has lifted 1.09 σ_P units since 1970. That corresponds to ΔG/year = 0.031 over the entire period and to ΔG/year = 0.042 for the last 10 years. The population mean of Icelandic horses has been raised to 1.73 σ_P units over the 35-year period or by 3.5% σ_P per year. The average rate of response over the last decade has increased by 1% of the phenotypic standard deviation, or to 4.5% σ_P per year.

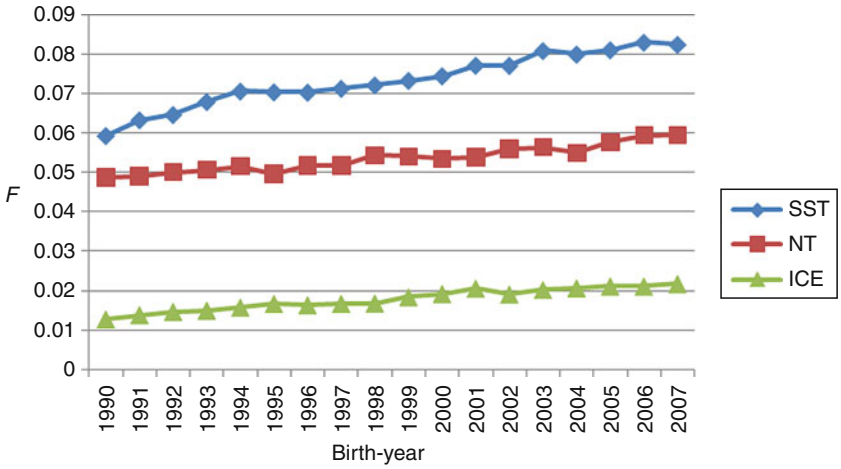
In the comparison of the three breeds, the efficiency of the selection has apparently been largest in the Standardbred Trotter. This larger genetic progress can mainly been attributed to higher selection intensity, particularly on the male side, where relatively few stallions are selected on the basis of results from races where many colts compete. The high selection intensity in SST is obtained by high testing capacity (races), widespread use of artificial insemination so that each selected stallion is able to cover more mares, and excessive use of information on results and pedigree. This is clearly reflected in the larger short-term response. However, the long-term sustainability of the breeding plan in the SST breed may be questioned as explained below when considering inbreeding and effective population size.

Long-Term Genetic Progress

An important aspect of sustainable animal breeding plans aiming at long-term genetic progress is to maintain genetic diversity (i.e., variation) within the population. In closed populations, gene frequencies fluctuate randomly from one generation to another as a result of the finite sampling of gametes. This phenomenon, called genetic drift, is quantified by the term effective population size, N_e [10, 50]. In a closed population, with no migration, the N_e is dependent on the number of parents in each generation, the variance of parental family sizes and selection. One way to estimate N_e is to measure the rate of increase of inbreeding over the different generations: $1/2N_e = \Delta F = (F_t - F_{t-1})/(1 - F_{t-1})$, where F_t is the mean coefficient of inbreeding for generation t (e.g., [10]).

The development of inbreeding in the three horse populations shows different levels that have evolved since the respective base populations (Fig. 2). The figure demonstrates difference in the rate of increase of inbreeding over time across the populations and thus gives a measure of their effective population sizes. According to the rate of inbreeding and the corresponding generation intervals, the effective population size (N_e) is approximately 40 for the Swedish Standardbred Trotter (SST), 50 for the Nordic Trotter (NT), and 100 for the Icelandic horse (Ice). The rate of inbreeding per generation, ΔF, is 1.3% in SST, 1.0% in NT, and 0.5% in Ice. In guidelines from FAO [51], the minimum recommended N_e is 50 corresponding to ΔF = 1.0% in livestock populations. Otherwise there is a great risk of loss in genetic variation, which will suppress long-term genetic progress and increase the risk for homozygosity of deleterious alleles, thus affecting health and fitness traits negatively. The SST breed is not only showing alarmingly high level of inbreeding (F ≈8%), but clearly surpassing the recommended rate of inbreeding per generation. The genetic constitution of the current generation of the SST breed is 94% of American Standardbred Trotter origin, while the remaining 6% can be traced to French Trotter base animals [33]. The American Standardbred Trotter is highly inbred with average inbreeding coefficient above 10%. The inbreeding in American Standardbred trotters and pacers is mainly due to remote inbreeding, where the horses are connected to one another by numerous paths through a small number of remote common ancestors. At the same time, breeders have deliberately avoided close consanguineous mating [52, 53]. Swedish breeders of Standardbred trotters have followed the same strategy. The average relationship among SST born in 2000 was R = 16.4% with a standard deviation of 5.2% [54]. Random mating within the population will result in average inbreeding coefficient above 8%. In order to reduce the rate of increase in inbreeding in the future, the variation in relationship among breeding candidates must be utilized in the mating strategy. Criteria for selection of breeding animals that involve EBVs modified such that the average relationship between selected animals is restricted have been proposed [47–49]. Increased migration of genetic material of French origin is also a recommended option for the breeders of Standardbred trotters in Sweden.

Breeders of the Nordic trotters have become increasingly aware of the risks associated with inbreeding in small closed populations. Negative effects of inbreeding (inbreeding depression) have been confirmed for reproductive traits, health traits, and racing



Breeding in Horses. Figure 2
Trend and level of inbreeding coefficients (*F*) in three horse populations (*SST* Swedish Standardbred Trotter, *NT* Nordic Trotter, Ice Icelandic horse)

performance in the Nordic trotter [55, 56]. Quotas for maximum number of mares that can be mated to each stallion have been put into effect and the rate of increase in inbreeding has reduced considerably compared with the period of 1970–1990, when the rate of inbreeding corresponded to $N_e = 32$ [57].

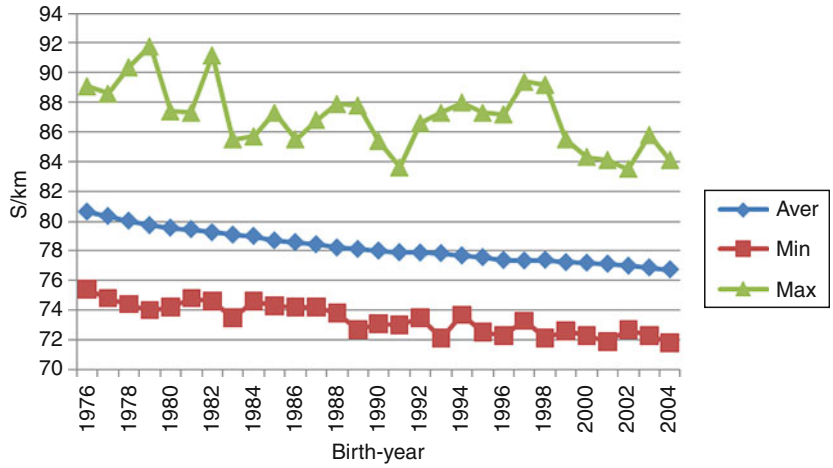
The inbreeding in the Icelandic horse is still at a low level and the rate of increase in the rate of inbreeding (ΔF) is yet not alarming. The breeding goal is broad as it includes very many traits, which helps keeping broad genetic variation among selected breeding individuals. However, awareness of the importance of maintaining low average genetic relationship among the selected parents is vital for the future of this breed, since import of genetic material to the closed horse population in Iceland is not a feasible option.

Genetic Progress in Racing Speed

One of the most interesting and debated questions concerning horse breeding is the "paradoxical" lack of improvement in winning times in the classical races for Thoroughbred horses over the last 50 years, in spite of significant genetic variation in racing performance variables and intense selection on racing performance [58–65].

The wild ancestors of modern horses were grazing flight animals and natural selection for speed and ability to traverse long distances were certainly important factors for the survival of the fittest. The Thoroughbred horses were founded by English and African horses screened for galloping speed and have been subject to an intense artificial selection on racing performance for over three centuries (30 horse generations). As a consequence, Thoroughbred horses are the fastest racehorses in the world galloping over distances of 1 to 2 miles. The breeding animals have not been selected directly on racing speed or racing time. Rather they have been selected on some function of ranks in races and special weight has been put on the ability to win races. Thoroughbred horses racing in the classical races all belong to the fastest segment of horses in the population.

Gaffney and Cunningham [61] estimated genetic change in Timeform handicap ratings of Thoroughbred horses in Great Britain. They confirmed effective selection on Timeform ratings and estimated annual genetic gain corresponding to almost 5% of the phenotypic standard deviation of 3-year-old Timeform ratings. This genetic progress is not reflected in winning times of the classic races, but the authors concluded that correlated genetic improvement in speed had been achieved in the Thoroughbred population as a whole but the fastest individuals in the population had reached the physiological limits for racing speed over the distance of the classic races. This conclusion assumes asymmetry in the distribution of racing speed.



Breeding in Horses. Figure 3

Observed trend in best average racing time records as 3–5-year-olds over 1 km for the average (aver), fastest (min), and slowest (max) Standardbred male trotters born in Sweden

The genetic evaluations of the Swedish Standardbred Trotter involve several variables that are functions of ranks in races (earnings, order at finish in the race) and racing time. As ranks in races are measures of racing performance that are relative to contemporaries racing at the same time, a genetic progress in these variables cannot be projected to any observable scale with clear physical meaning. On the other hand, the genetic progress in racing performance should be reflected in faster racing times. The best average racing time over 1 km has indeed improved continuously for the Swedish Standardbred Totter since recording started for the horses born in 1976 (Fig. 3). The limits for racing speed in trotters have apparently not been reached yet. However on the observed untransformed scale (s/km), the rate of improvement has slightly reduced over time and it seems increasingly harder to break the records. The dispersion of the slowest and fastest racing time records in the population around the average is also apparently asymmetrical. Arnason [66, 67] has previously argued that genetic evaluation of racing time records should be based on scaled logarithmic transformation of racing time records as $y^*_i = \ln(y_i - x)$, where y_i is the observed racing time record on the ith horse in s/km units, x is the asymptotic physiological limits for trotting racing speed in s/km in races over the traditional distance of 1 mile. When x is equal to 68.2 the distribution of y^*_i is almost perfectly normal in the population of Swedish Standardbred male trotters and the trend in average log transformed racing time records is linear.

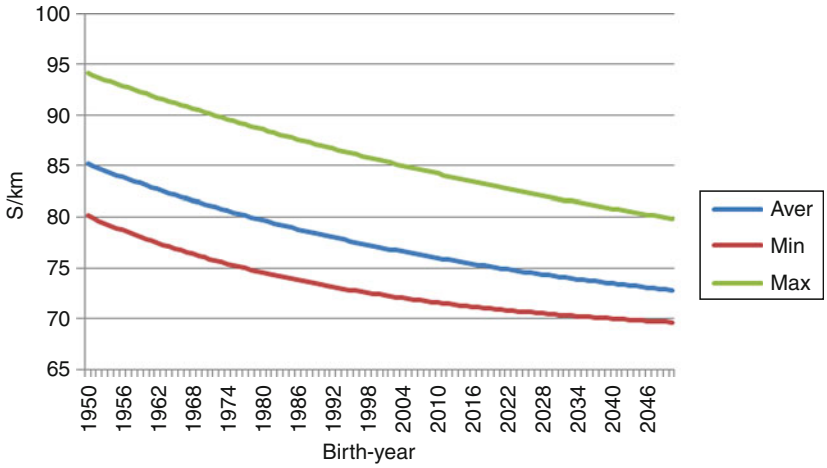
The trend in statistics (mean, minimum (fastest), maximum (slowest)) of fastest racing time records of 3–5-year-old males in the population of Swedish Standardbred Trotter can be described with the expression: $\text{s/km} = x\,(1 + e^{-pt})$, where x is the ultimate asymptotic limit for the fastest racing time records in the population as defined above, p is a positive constant, and t = birth-year – z, where z is a time scaling constant [67]. The constants p and z were estimated from recent data on racing results in the population of SST leading to the following equations for prediction of the development of the trend in the average, fastest, and slowest racing times in the population:

$$\text{s/km}_{(\text{aver})} = 68.2\left(1 + e^{(-0.013(\text{birth}-\text{year}-1843)}\right)$$

$$\text{s/km}_{(\text{min})} = 68.2\left(1 + e^{(-0.021(\text{birth}-\text{year}-1867)}\right)$$

$$\text{s/km}_{(\text{max})} = 68.2\left(1 + e^{(-0.008(\text{birth}-\text{year}-1829)}\right)$$

The predicted trend is revealed in Fig. 4. The racing time records in the population are expected to improve (become faster) at a linear rate on the logarithmic transformed scale, but at a diminishing rate on the untransformed linear scale of racing time (s/km). The presented racing times refer to "volt start," which is



Breeding in Horses. Figure 4

Predicted trend in best average racing time records as 3–5-year-olds over 1 km for the average (aver), fastest (min), and slowest (max) Standardbred male trotters born in Sweden

the most common starting procedure for trotting races in Sweden. Records from the flying "auto start" are adjusted by adding 2 s to the racing time.

The history of selection for trotting racing performance in Standardbred trotters is much shorter than that of selection for gallop racing performance in Thoroughbreds, and probably reflects a difference of at least 20 generations. If genetic variation can be maintained in the trotter population and the selection for trotting racing performance will be continued in the same manner over the next 200 years, the prediction equations above give the following estimates: Average racing time record for males born 2210 = 68.78 s/km; fastest racing time = 68.25 s/km; and slowest racing time = 71.44 s/km. This can be compared with the corresponding observed average, minimum, and maximum racing times of males born 2004 in the SST population: 76.74, 71.8, and 84.1 s/km. A parallel between this expected scenery for the development of racing speed in trotters and that already observed in Thoroughbreds is highly tempting. The average records (and the slowest) will continue to improve, however at ever decreasing rate, far after an apparent stagnation in the improvement of the fastest records. A slight improvement of the best records will be increasingly harder to identify due to the inappropriate scale. The small difference in racing speed between the fastest horses becomes less important as relative to fighting spirit and the mental and physical ability of the horse to react to the signals of the driver (or jockey) and to variations in speed at different phases of the race.

If there are some asymptotic limits to racing speed, they must relate to a nonlinear accumulation of biological and physical constraints on racing performance. Such factors might be: energy supply, energy transport rate, neural functions, oxygen supply, oxidative enzyme activity, accumulation of lactic acid in the blood, exponential increase in energy requirement for overcoming air resistance, and generally a nonlinear risk of stress on the locomotion (tendons, bones and ligaments), respiratory and cardiovascular systems, with increasing racing speed [13, 68].

Hypothetically, an insertion of a gene with positive effect on racing performance on an inferior racing horse would be likely to have more marked effect on its racing performance than if the same gene was inserted into an outstanding racer, which would already carry many genes with positive effects on racing performance. Such scale effects can often be removed by appropriate transformation and traditional quantitative genetic analysis methods may apply. Alternatively, a nonlinear polynomial genetic model for finite number of independent loci (geometric progression) might be attempted [66, 67]. The geometric progression model assumes constantly diminishing marginal substitution effects of the alleles affecting the trait.

In conclusion, the fact that racing times in classic Thoroughbred races have not improved markedly in more than a half century in spite of available genetic variation and selection is not necessarily a "paradox." It could merely be a result of the use of improper scale for measuring racing times in horses approaching their biological limits.

Future Directions

Methods of organized animal breeding using BLUP animal models and selection on EBV for important traits have proved useful in many horse populations worldwide. Genetic progress at the annual rate of 3–6% of the phenotypic standard deviation has been reported in several populations. Sustainable breeding schemes yielding this rate of constant response over a long period and at the same time maintaining sufficient genetic variability in the population can be accomplished with established knowledge and relatively cheap technical equipments.

In many parts of the world are rational and organized breeding plans still lacking in large and locally important horse breeds. In some societies, this can be explained by poverty and lack of higher level of education. In others is the social culture, including reluctant conservatism, a hindrance to the introduction of new technique and scientific influence on the traditional horsemanship and "horse culture." This will certainly change and introduction of new and modern organized breeding plans for horses will undoubtedly be seen in many countries in near future.

The increased international trade with valuable breeding horses and exchange of genetic material across horse populations has created need for international genetic evaluations, where EBVs can be fairly compared across countries. The need for international genetic evaluations will certainly grow in the near future.

Methodological developments in genetic evaluation procedures and statistical models will undoubtedly enhance future breeding in horses. Of particular foreseen interest is genetic evaluation of ranks in competitions based on Bayesian Thurstonian models [29, 30] and use of random regression models for evaluation of traits with repeated measurements [20, 21].

Recently, the horse genome has been fully sequenced and the results are available to the scientific community [69, 70]. This has created optimism that genomic selection, which is selection on genomic breeding values (GEBVs), might revolutionize future horse breeding. The GEBV are calculated as the sum of the effects of dense genetic markers across the entire genome on the trait of interest. The genetic (DNA) markers are in the form of a large collection of chromosome fragments, so-called SNP (single nucleotide polymorphism) chips. Since GEBV can be obtained already in young foals, the possibility to shorten the long generation intervals in traditional horse breeding and to hinder castration of genetically valuable individuals is obvious. Genomic selection has the most potential for genetic improvement of traits that are manifested late in life and of traits with low heritability. Breeding for performance traits in sport horses, longevity, and various health traits in horses could benefit enormously if genomic selection can be practiced in a cost-effective way.

More methodological as well as technical development is required before genomic selection can gain widespread practical application in horse breeding. The first step is the development of a sufficient number of high-density SNPs across the entire horse genome and the second step is to genotype large reference populations in different horse breeds with phenotypic records so that the effects of the markers on the phenotypes can be analyzed. In subsequent generations, a large number of horses will need to be genotyped for the markers to determine which chromosome segments they carry. Then the estimated effects of the segments can be summed across the whole genome to predict the GEBV [71]. This new technology is already revolutionizing dairy cattle breeding in several countries [72, 73] and an efficient algorithm for computing the relationship matrix by use of genomic information has been invented [74]. A combination of a traditional selection on EBVs and a selection on GEBVs is likely to be adapted soon in the most valuable sport and racing populations, where the cost of genotyping is little in relation to the value of the breeding animals and organized breeding plans with registration of data on phenotypes and pedigrees are already in function. As a dramatic reduction in the cost of genotyping can be anticipated in the future, a practical and widespread use of genomic selection may become reality in

breeding of horses throughout the world sooner than expected in the light of current status and prizes. An important challenge will be to meet unforeseeable snags that may rise from the use of the new technique and difficulties in managing future genetic variation and long-term genetic gain.

Bibliography

Primary Literature

1. Anthony D, Telegin DY, Brown D (1991) Origin of horseback riding. Scientific Am 225:44–48
2. Bökönyi S (1984) Horse. In: Mason IL (ed) Evolution of domesticated animals. Longman, London, pp 162–173
3. Bowling AT, Ruvinsky A (2000) Genetic aspects of domestication, breeds and their origins. In: Bowling AT, Ruvinsky A (eds) The genetics of the horse. CABI Publishing, Wallingford, pp 25–51
4. FAOSTAT Database (2008) faostat.fao.org
5. EU Equus (2009) www.equus2009.eu
6. Mason IL (1996) A world dictionary of livestock breeds, types and varieties, 4th edn. CAB International, Wallingford
7. Hazel LN (1943) The genetic basis for constructing selection indexes. Genetics 28:476–490
8. Amer PR, Fox GC, Smith C (1994) Economic weights from profit equations: appraising their accuracy in the long run. Anim Prod 58:11–18
9. Árnason T, Van Vleck LD (2000) Genetic improvement of the horse. In: Bowling AT, Ruvinsky A (eds) The genetics of the horse. CABI Publishing, Wallingford, pp 473–497
10. Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longmans Green, Harlow
11. Ricard A (1998) Developments in the genetic evaluation of performance traits in horses. In: Proceedings of the 6th world congress on genetics applied to livestock production, Armidale, NSW. University of New England, Armidale, vol 24, pp 388–395
12. Tolley ES, Notter DR, Marlowe TJ (1985) A review of the inheritance of racing performance in horses. Anim Breed Abstr 53:163–185
13. Ricard A, Bruns E, Cunningham EP (2000) Genetics of performance traits. In: Bowling AT, Ruvinsky A (eds) The genetics of the horse. CABI Publishing, Wallingford, pp 411–438
14. Correa MJM, Mota MDS (2007) Genetic evaluation of performance traits in Brazilian Quarter Horse. J Appl Genet 48: 145–151
15. Stock KF, Distl O, Hoeschele I (2007) Bayesian estimation of genetic parameters for multivariate threshold and continuous phenotypes and molecular genetic data in simulated horse populations using Gibbs sampling. BMC Genet 8:19
16. Albertsdóttir E, Eriksson S, Nasholm A, Strandberg E, Árnason T (2007) Genetic analysis of competition data on Icelandic horses. Livest Sci 110:242–250
17. Albertsdóttir E, Eriksson S, Nasholm A, Strandberg E, Árnason Th (2008) Genetic correlations between competition traits and traits scored at breeding field-tests in Icelandic horses. Livest Sci 114:181–187
18. Burns EM, Enns RM, Garrick DJ (2004) The status of equine genetic evaluation. In: Proceedings, Western Section, American Society of Animal Science, pp 82–86
19. Kennedy BW, Schaeffer LR, Sorensen DA (1988) Genetic properties of animal models. J Dairy Sci 71(suppl 2):17–26
20. Bugislaus AE, Roehe R, Willms F, Kalm E (2006) The use of a random regression model to account for change in racing speed of German trotters with increasing age. J Anim Breed Genet 123:239–246
21. Menendez-Buxadera AM, Mota MDS (2008) Variance component estimations for race performance of thoroughbred horses in Brazil by random regression model. Livest Sci 117:298–307
22. Meuwissen THE, Luo Z (1992) Computing inbreeding coefficients in large populations. Genet Sel Evol 24: 305–313
23. Sigurdsson Á, Árnason T (1995) Predicting genetic trend by uni- and multitrait models. Acta Agric Scand Sect A 45:1–10
24. Quaas RL (1976) Computing the diagonal elements and inverse of a large numerator relationship matrix. Biometrics 32:949–953
25. Henderson CR (1976) A simple method for computing the inverse of a numerator relationship matrix used in prediction of breeding values. Biometrics 32:69–83
26. Westell RA, Quaas RL, Van Vleck LD (1988) Genetic groups in an animal model. J Anim Sci 66:2817–2825
27. Tavernier A (1990) Estimation of breeding value of jumping horses from their ranks. Livest Prod Sci 26:277–290
28. Tavernier A (1991) Genetic evaluation of horses based on ranks in competition. Genet Sel Evol 23:159–173
29. Gianola D, Simianer H (2006) A thurstonian model for quantitative genetic analysis of ranks: a Bayesian approach. Genetics 174:1613–1624
30. Ricard A, Legarra A (2010) Validation of models for analysis of ranks in horse breeding evaluation. Genet Sel Evol 42:3
31. Klemetsdal G (1992) Estimation of genetic trend in racehorse breeding. Acta Agric Scand 42:226–231
32. Albertsdóttir E, Eriksson S, Sigurdsson Á, Árnason T (2009) An all-or-none trait to account for pre-selection in Icelandic horse breeding. In: Book of Abstracts of the 45th annual meeting of the European association of animal production, 24–27 August 2009, Barcelona. Universityof Wisconsin, Madison, p 217
33. Árnason T (1999) Genetic evaluation of Swedish standardbred trotters for racing performance traits and racing status. J Anim Breed Genet 116:387–398
34. Bugislaus AE, Roehe R, Willms F, Kalm E (2005) Multivariate genetic analysis to account for preselection and disqualified races in the genetic evaluation of racing performances in German trotters. Acta Agric Scand Sect A 55:49–56

35. Koenen EPC, Aldrige LI, Philipsson J (2004) An overview of breeding objectives for warmblood sport horses. Livest Prod Sci 88:77–84
36. Thorén-Hellsten E, Viklund A, Koenen EPC, Ricard A, Bruns E, Philipsson J (2006) Review of genetic parameters estimated at stallion and young horse performance tests and their correlations with later results in dressage and show-jumping competition. Livest Sci 103:1–12
37. Ruhlman C, Bruns E, Fraehr E, Koenen EPC, Philipsson J, Janssens S, Quinn K, Thorén-Hellsten E, Ricard A (2009) Genetic connectedness between seven European countries for performance in horse jumping competitions of warmblood riding horses. Livest Sci 120:75–86
38. Thorén-Hellsten E, Jorjani H, Philipsson J (2008) Connectedness among five European sport horse populations. Livest Sci 118:147–156
39. Thorén-Hellsten E, Jorjani H, Philipsson J (2009) Genetic correlations between similar traits in the Danish and Swedish Warmblood sport horse populations. Livest Sci 124:15–20
40. Ruhlman C, Janssens S, Philipsson J, Thorén-Hellsten E, Crolly H, Quinn K, Manfredi E, Ricard A (2009) Genetic correlations between horse show jumping competition traits in five European countries. Livest Sci 122:234–240
41. Árnason T, Sigurdsson Á, Lorange JB (2006) Global genetic evaluations of the Icelandic horse and genetic connectedness between countries. In: Proceedings of the 8th world congress on genetic applied to livestock production, Belo Horizonte, 13–18 August 2006, CD-ROM communication 24-16
42. Rendel JM, Robertson A (1950) Estimation of genetic gain in milk by selection in a closed herd of dairy cattle. J Genet 50:1–8
43. Strom H, Philipsson J (1978) Relative importance of performance tests and progeny tests in horse breeding. Livest Prod Sci 5:303–332
44. Hugason K, Árnason T, Norell L (1987) Efficiency of three-stage selection of stallions. J Anim Breed Genet 104:350–363
45. Cochran WG (1951) Improvement by means of selection. In: Proceedings of Second Berkely Symposium on Mathematical, Statistics and Probability. University of California Press, Berkeley, pp 440–470
46. Bulmer MG (1971) The effect of selection on genetic variability. Am Nat 105:201–211
47. Wray NR, Goddard ME (1994) Increased long-term response to selection. Genet Sel Evol 26:431–451
48. Meuwissen THE (1997) Maximizing the response of selection with a predefined rate of inbreeding. J Anim Sci 75: 934–940
49. Grundy B, Villanueva B, Wooliams JA (2000) Dynamic selection for maximizing response with constrained inbreeding in schemes with overlapping generations. Anim Sci 70: 373–382
50. Wright S (1931) Evolution in Mendelian populations. Genetics 16:97–159
51. Woolliams JA, Gwaze GP, Meuwissen THE, Planchenault D, Renard JP, Thibier M, Wagner H (1998) Secondary guidelines for the development of national farm animal genetic resources management plans. Management of small populations at risk. Food and Agricultural Organization of the United Nations (FAO), Rome
52. MacCluer JW, Boyce AJ, Dyke B, Weitkamp LR, Pfennig DW, Parsons CJ (1983) Inbreeding and pedigree structure in Standardbred horses. J Hered 74:394–399
53. Cothran EG, MacCluer JW, Weitkamp LR, Pfennig DW, Boyce AJ (1984) Inbreeding and reproductive performance in Standardbred horses. J Hered 75:220–224
54. Árnason T (2001) Inbreeding – threat to trotting horses. In: Papers, 2001 World Trotting Conference, 6th to 15th July 2001, Suomen Hippos, Helsinki & Mikkeli
55. Klemetsdal G (1993) Demographic parameters and inbreeding in the Norwegian trotter. Acta Agric Scand Sect A 43:1–8
56. Anderson E, Nasholm A, Gerber E, Árnason Th (1998) Inbreeding and its impact on racing performance in Swedish and Norwegian coldblooded trotters. In: Book of Abstracts of the 49th annual meeting of the European association of animal production, 24–27 August 1998, Warsaw. Wageningen Publishers, Wageningen, pp 314
57. Klemetsdal G, Johnson M (1989) Effect of inbreeding on fertility in Norwegian trotter. Livest Prod Sci 21:263–272
58. Cunningham EP (1976) Genetic studies in horse populations. In: Proceedings of the international symposium on genetics and horse breeding. Royal Dublin Society, Dublin, pp 2–8
59. Robertson A (1976) What has genetic to contribute to horse breeding. In: Proceedings of the international symposium on genetics and horse breeding. Royal Dublin Society, Dublin, pp 64–70
60. Langlois B (1980) Heritability of racing ability in thoroughbreds – a review. Livest Prod Sci 7:591–605
61. Gaffney B, Cunningham EP (1988) Estimation of genetic trend in racing performance of thoroughbred horses. Nature 332: 722–724
62. Hill WG (1988) Why aren't horses faster. Nature 332:678
63. James JW (1990) Selection limits in thoroughbred horses. In: Proceedings of the 4th world congress on genetics applied to livestock production, Edinburgh, 23–27 July 1990, vol 16, pp 221–224
64. Langlois B (1994) An introduction to genetic analysis in the Thoroughbred. In: Book of abstracts of the 45th annual meeting of the European association of animal production, Rome 5–8 September 1994, Edinburgh, Rome, p 358
65. Langlois B (1996) A consideration of the genetic aspects of some current practices in thoroughbred horse breeding. Ann Zootechnie 45:41–51
66. Árnason T (1994) Simple (plausible) non-linear model for inheritance of racing speed and corresponding genetic changes from selection. In: Book of abstracts of the 45th annual meeting of the European association of animal production, Rome 5–8 September 1994, Edinburgh, Rome, p 360
67. Árnason T (2001) Trends and asymptotic limits for racing speed in standard bred trotters. Livest Prod Sci 72:135–145

68. Cunningham EP (1998) The genetics of track performance in thoroughbreds. In: Book of abstracts of the 49th annual meeting of the European association of animal production, 24–27 August 1998, Warsaw, Wageningen Pers, Wageningen, p 309
69. Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, Lear TL, Adelson DL, Baily E, Bellone RR, Blocker H, Distl O, Edgar RC, Garber M, Leeb T, Mauceli E, MacLeod JN, Penedo MCT, Raison JM, Sharpe T, Vogel J, Andersson L, Antczak DF, Biagi T, Binns MM, Chowdhary BP, Coleman SJ, Della Valle G, Fryc S, Guérin G, Hasegawa T, Hill EW, Jurka J, Kiialainen A, Lindgren G, Liu J, Magnani E, Mickelson JR, Murray J, Nergadze SG, Onofrio R, Pedroni S, Piras MF, Raudsepp T, Rocchi M, Roed KH, Ryder OA, Searle S, Skow L, Swinburne JE, Syvanen AC, Tozaki T, Valberg SJ, Vaudin M, White JR, Zody MC, Broad Institute Genome Sequencing Platform, Broad Institute Whole Genome Assembly Team, Lander ES, Kindblad-Toh K (2009) Genome sequence, comparative analysis and population genetics of the domestic horse. Science 326:865–867
70. Gu J, Orr N, Park SD, Katz LM, Sulimova G, MacHugh DE, Hill EW (2009) A genome scan for positive selection in Thoroughbred horses. PLoS ONE 4(6):e5767. doi:10.1371/journal.phone.0005767
71. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
72. Hayes BJ, Bowman PJ, Chamberlain AJ, Goddard ME (2009) Invited review: genomic selection in dairy cattle: progress and challenges. J Dairy Sci 92:433–443
73. VanRaden PM, Sullivan PG (2010) International genomic evaluation methods for dairy cattle. Genet Sel Evol 42:7
74. Legarra A, Aguilar I, Misztal I (2009) A relationship matrix including full pedigree and genomic information. J Dairy Sci 92:4656–4663

Books and Reviews

Bowling AT (1996) Horse genetics. CAB International, Wallingford
Bowling AT, Ruvinsky A (eds) (2000) The genetic of the horse. CABI Publishing, Wallingford
Mrode RA (2005) Linear models for the prediction of animal breeding values, 2nd edn. CABI Publishing, Wallingford
Van Vleck LD, Pollak EJ, Oltenacu EAB (1989) Genetics for the animal sciences. Freeman, New York

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of

Joao G. Ferreira[1], Jon Grant[2], David W. Verner-Jeffreys[3], Nick G. H. Taylor[3]
[1]Departamento de Ciências e Engenharia do Ambiente, Faculdade de Ciências e Tecnologia Universidade Nova de Lisboa, Monte Caparica, Portugal
[2]Department of Oceanography, Dalhousie University, Halifax, NS, Canada
[3]Cefas Weymouth Laboratory, Weymouth, Dorset, UK

Article Outline

Glossary

Dynamic models Models that incorporate a time-varying element. Such models may have no spatial dimension or extend to modeling spatial variation in all three dimensions.

Integrated multi-trophic aquaculture (IMTA) Cocultivation of aquatic species at different trophic levels, such as salmon, mussels, and kelp or scallop and sea cucumber, in order to maximize yield and minimize the environmental footprint.

Modeling framework Models are a representation of reality, which they aim to reproduce in terms of generality, realism, accuracy, and simplicity. As a rule, generality and simplicity are maximized. A model framework usually consists of two or more models, which are combined to address different levels of complexity.

Research models Models that are more detailed, usually complex to develop, implement, and apply. Aimed mainly at the scientist, rather than the manager.

Screening models Models that use a limited set of inputs, and provide highly aggregated outputs, such as an index of suitability or an environmental score card.

Virtual technology for aquaculture Any artificial representation of ecosystems that support aquaculture, whether directly or indirectly. Such representations, exemplified by mathematical models, are designed to help measure, understand, and predict the underlying variables and processes, in order to inform an Ecosystem Approach to Aquaculture.

Definition of the Subject and Its Importance

Aquaculture, defined simply as the cultivation of aquatic organisms, has many similarities to agriculture, most notably that it is based on the interaction between humans and other elements of the natural system, converting the latter (at least in part) into a *managed* system.

In parts of the world, such as Southeast Asia, the distinction between the two types of cultivation becomes increasingly fuzzy, especially if they take place on land. Cocultivation of rice and tilapia in paddy fields [1] or the combination of penaeid shrimp (e.g., the whiteleg shrimp *Penaeus vannamei*) and water spinach (*Ipomoea aquatica*) in earthen ponds is common, as are many other combinations (Fig. 1). Carrying capacity in such intensive systems, whether in monoculture or in integrated multi-trophic aquaculture (IMTA), might at first glance seem equivalent to assimilative capacity.

Aquaculture in open water, whether in reservoirs, lakes, or coastal systems, must take into account the complexities of water circulation, together with the harmonization of different uses. In the context of organically extractive open-water culture, Bacher et al. [2] and Smaal et al. [3] defined carrying capacity as:

The standing stock at which the annual production of the marketable cohort is maximized.

Although this definition was proposed for bivalve shellfish culture, it is sufficiently broad to be relevant for production in open freshwater, coastal, and offshore environments, as well as in land-based systems using ponds or raceways. However, production carrying capacity needs to be further qualified, because in

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 1
Pigs might fly? Cocultivation of pigs and fish such as carp or rohu in India http://harfish.gov.in/technology.htm

economic terms the maximization of annual production is not the objective function, and brings with it increased environmental costs. Commercial production must seek to achieve optimal *profit*, well before the inflection point in the production function, where total physical product (TPP) maximizes *income* (e.g., [4, 5]).

This production-oriented view of carrying capacity for aquaculture, whether in terms of assimilative capacity for fed aquaculture such as finfish or shrimp or with respect to food depletion in the case of shellfish such as oysters, clams, or abalone, has been expanded over the last decade into a four pillar approach based on physical, production, ecological, and social carrying capacity [6, 7]. These pillars encompass the three elements of sustainability, viz., planet, people, and profit.

The terms carrying capacity and assimilative capacity are frequently associated with models of aquaculture impacts. Because these terms attempt to define the limits of sustainable aquaculture, predictive capability is highly sought (Fig. 2).

This chapter reviews the state of the art in modeling frameworks that assist with that prediction and support proactive management of aquaculture.

Introduction

The establishment of aquaculture activities in different geographical areas has historically been a bottom-up process, without any systemic planning or definition of a zoning framework. This is seen throughout the world, from the development of salmon cage culture in Scottish lochs to the incremental destruction of mangroves for construction of shrimp farms (Fig. 3).

This approach to licensing (or in many cases just to development) has been based on space availability and limits to production rather than on any environmental criteria, and has led to undesirable ecosystem effects, including habitat destruction both on land and in open waters, coastal eutrophication through increased nutrient loading from land, organic enrichment of sediments, loss of benthic biodiversity, and major outbreaks of disease.

In the last decade, better regulatory frameworks have led to a more stringent approach to licensing, most notably in the European Union, the United States, and Canada. The European Union's Marine Strategy Framework Directive (EU MSFD – EC [80]), together with guidelines for an Ecosystem Approach to Aquaculture (EAA – [8]), highlights the ecological component and aims to optimize production without compromising ecosystem services. Part of the challenge of determining carrying capacity is the quantification of negative externalities as a first step toward improved management.

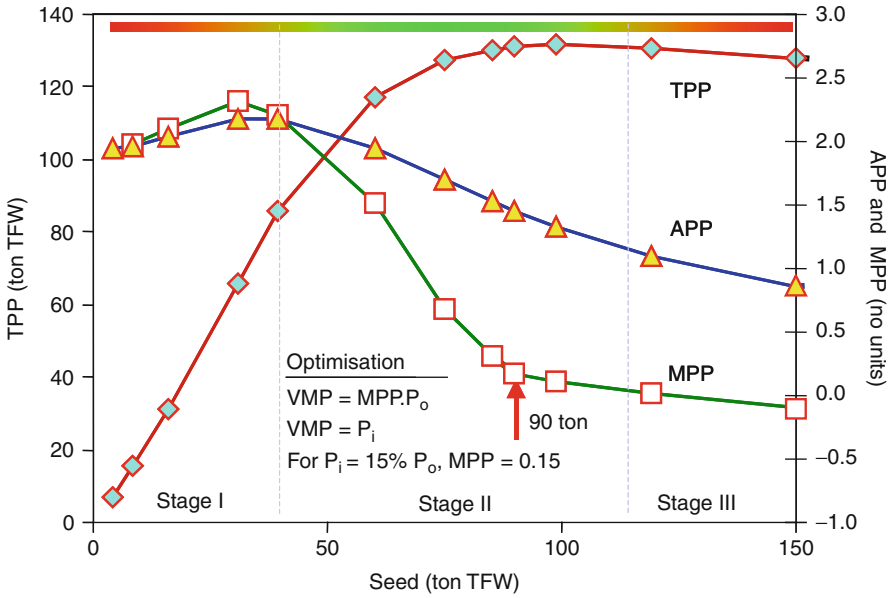
In Brazil, for instance, where aquaculture has grown very rapidly over the past decade (Fig. 4), environmental permitting of new tilapia farms in reservoirs is determined through the application of the Dillon and Rigler [9] phosphorus loading model, a rather simplistic view of carrying capacity.

A maximum limit of 30 μg L^{-1} of P has been established for reservoir waters, 5 μg L^{-1} of which is reserved for fish farming, to allow for multiple uses including cattle ranching, sugar cane production, urban discharges, and the natural background.

Fish farms are licensed sequentially, based on the contribution to P loading of their declared production.

Although this approach does address carrying capacity at the system scale (i.e., $\Delta P = 5$ μg L^{-1}), it does not consider any spatial or temporal variation, nor does it account for factors such as organic enrichment, disease, or impacts on biodiversity – all of which may be linked.

Whereas production and social (e.g., tourism) impacts are often local, ecological impacts *of* aquaculture can have far broader scales, as can large-scale effects *on* aquaculture, such as advection of harmful algae from offshore [10]. Sustainability is "easier to plan than to retrofit," [10] which makes a case for the



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 2
Marginal analysis indicates that the seed that provides maximum profit (*red arrow*) falls well to the left of the maximum production, shown by the *dotted line*. Production beyond the optimal profit point adds no value and potentially increases environmental pressure (Results from the FARM model, Adapted from [4])

analysis of carrying capacity at the system scale (e.g., Nobre et al. [11]), i.e., defining and quantifying the overall potential for different types of aquaculture prior to local-scale assessment (e.g., [12]) of new operations. Simulation models of varying degrees of complexity must play a role in the determination of carrying capacity for aquaculture, often combined as model frameworks, given the range of time and space scales and the number of processes involved.

Over the next decades, the growth of aquaculture will take place in developing nations [13–15], which makes it paramount that the digital divide, which is already considerable (as is the legal divide; see [16]), does not become wider.

Simulation technology that can support planning should be close to the production centers and be able to deal with data-poor environments and limited computational access and skills.

Modeling Frameworks for Carrying Capacity

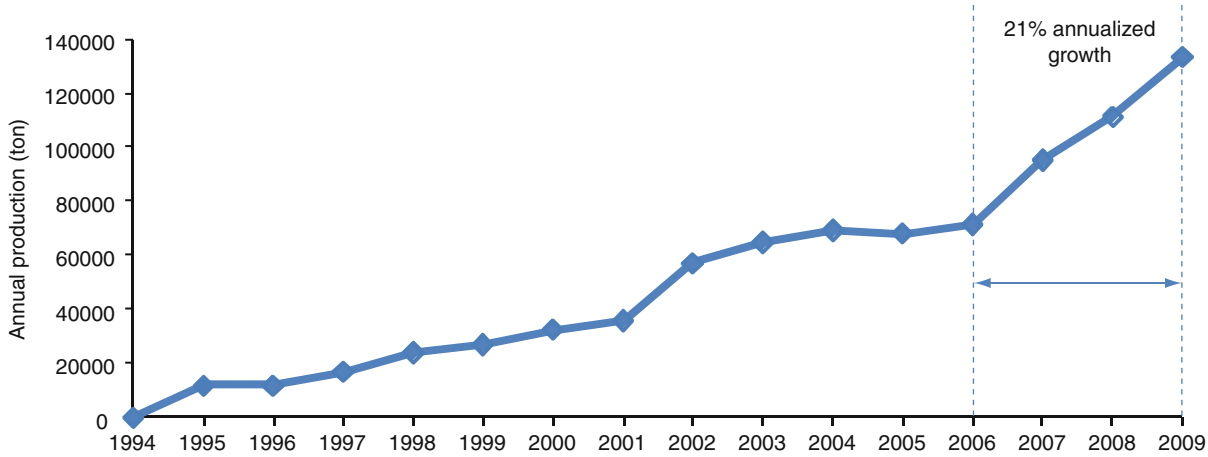
The concept of carrying capacity in aquaculture, based on four pillars of sustainability, has been adapted in Fig. 5 to include governance [16, 17]. This is considered more relevant than the physical element, which in many respects is encapsulated in the production pillar. Governance, on the other hand, is clearly missing from the original model of Inglis et al. [6], and the quality of balanced regulation, stakeholder involvement, and community-based management [18] often constitutes the difference between sustainable aquaculture and an environmental time bomb.

The social (here used in the context of social opposition to visual or other impacts of aquaculture development, such as conflict with leisure areas) pillar is at the forefront of decision-making for aquaculture in the EU, the US, and Canada and can frequently be identified as the single most important criterion for carrying capacity assessment and site selection (Fig. 5). By contrast, in Asia and other parts of the world where food production is the paramount concern, licensing criteria are more frequently based on the production pillar, with ecological considerations assuming less relevance. In China and Southeast Asia, the social component acts as a driver for increased aquaculture, for reasons of economy and food security. Governance is not usually

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 3
Expansion of shrimp ponds over a 13-year period in Estero Real, Nicaragua [83]

limited by a lack of legal instruments [16] but often by their adequacy and acceptance by stakeholders.

Two of the pillars illustrated in Fig. 5, production and ecology, are amenable to mathematical modeling, and two are not. This does not mean that those mathematical models will be entirely successful in describing growth, environmental effects, and particularly ecosystem responses, but they do make a significant contribution to the improved evaluation of carrying capacity. Part of the difficulty lies with our understanding of the relevant processes, parameters, and rates, part with other factors, such as the lack of a paradigm in ecology to support prediction. For instance, in the EU MSFD, as in other legal instruments, there are complex descriptors of ecosystem health such as biodiversity, and the scientific community struggles to establish meaningful classification systems and their relationship to anthropogenic pressures.



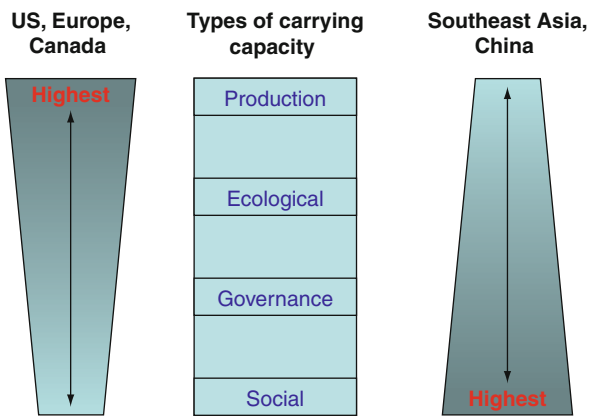
Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 4
Production of farmed tilapia in Brazil (MPA, personal communication, 2011)

There are also interactions with the human component of cultivation that constitute simulation challenges. Culture practice, for instance, is widely variable and can generally only be modeled in average terms [19].

Issues related to disease, which fall squarely between production and ecology and are extremely difficult to model, are a huge challenge in aquaculture and often include a significant element of poor governance, such as relaying of infected animals to hitherto uncontaminated areas.

Rearing large numbers of animals of the same species in close proximity to each other favors the establishment and spread of infectious diseases within those farmed populations [84]. Disease can affect both survival and growth of animals. Outbreaks of highly virulent bacterial and viral diseases can result in high mortality of an affected farm stock. For example, outbreaks of white spot syndrome virus (WSSV) in farmed whiteleg shrimp can result in greater than 60% mortality, with an attendant dramatic impact on the farms and regions dependent on farming this species.

Additionally, with ever increasing demands from consumers for high-quality products, the effects that disease can have on product quality are of increasing importance. For example, tilapia that have survived outbreaks of *Francisella* often have unsightly lesions in the fillets. There are also examples of diseases, such as Red Mark Syndrome in rainbow trout [85], that do not result in any significant mortality or morbidity, but still result in the product being downgraded or rejected by processors after harvest, imposing significant economic costs on the farmer.



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 5
The relative importance of the four pillars of carrying capacity (Adapted from [17])

The ecological effects of disease in aquaculture can also be profound. This includes spread of pathogens from farmed fish to wild fish and vice versa [86]. Disease agents that affect aquaculture species generally have their origins in wild aquatic animals [86]. Many systems (e.g., salmonid netcage and bivalve culture)

involve rearing the farmed animals in relatively open systems where the reared animals are in direct contact or close proximity with wild animals. Water is an ideal medium for the dissemination of many pathogens, leading to a high risk of transfer. For instance, in Norway and Scotland, exchange of sea lice *Lepeophtheirus salmonis* between farmed Atlantic salmon and wild salmonids has been implicated in causing significant declines in populations of wild Atlantic salmon and sea trout.

In order to examine modeling frameworks, the following sections review the definitions of, and distinctions between, the pillars of carrying capacity that are more amenable to mathematical modeling.

Assimilative Capacity and Carrying Capacity

Assimilative capacity is sometimes considered a subclass of the more general term carrying capacity, but other more specific definitions have been applied. We make a distinction as detailed below. Assimilative capacity refers to the ability of biological systems in the water column or sediments to process organic matter, nutrients, therapeutants, or contaminants without alteration of ecosystem state or function.

Carrying capacity refers to the biomass of cultured organisms that can be supported without altering system state or function measured by water or sediment quality standards and processes [20, 21]. The latter is thus determined by aquacultured biomass [22], where the former is independent of aquaculture and determined by biological properties of the habitat.

The application of standards to models of impact takes two forms:

1. Absolute criteria determined by regulatory bodies such as the Water Framework Directive of the EU (EU WFD – EC [79])
2. Relative standards compared to reference conditions or the range of variation observed in the environment

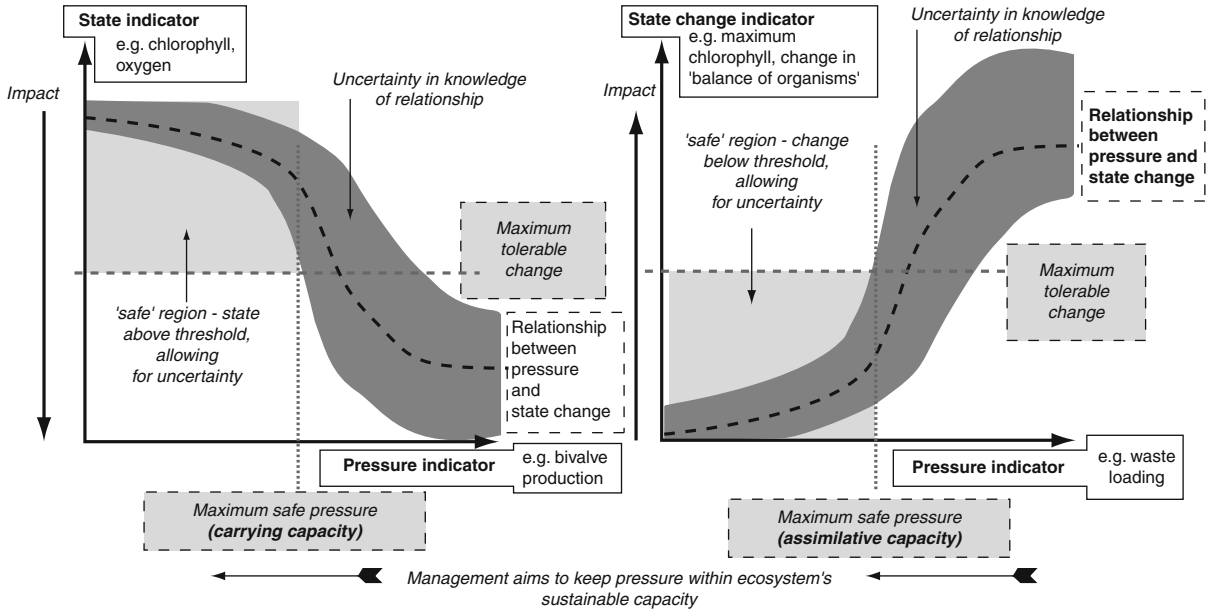
In both cases, there is an attempt to use these values as sustainability criteria. In the case of absolute standards, the background of the environmental conditions is not considered. For example, naturally eutrophic waters may show higher nutrient levels, with no relationship to aquaculture. This is particularly true of chlorophyll impacted by shellfish depletion. It may be more realistic to consider depletion in the context of the range of values observed in the environment as a means of establishing whether aquaculture signals can be detected against background noise. This type of standard has been applied to shellfish depletion of chlorophyll by Filgueira and Grant [20] and to shellfish biodeposits by Grant et al. [23].

Tett et al. [22] formalize both carrying capacity and assimilative capacity as the result of dose–response curves, couched in the terminology of DPSIR (Drivers-Pressure-State-Impact-Response; see also [24]; Fig. 6), where pressure is the farmed biomass and state is the system response modeled as a water quality standard. By comparing physical and geochemical rate processes, a net balance is determined, whereby the quantity of interest displays an increase (e.g., ammonia) or decrease (e.g., dissolved oxygen) relative to a water quality standard. When two of these processes are balanced, an index is created, constituting some of the earliest models in this research area, sometimes referred to as screening models.

A further distinction is that assimilative capacity be a function only of biological processing. Tett et al. [22] use the net results of water quality models which include advection–diffusion as well as biological processing to define assimilative capacity. Following bioenergetic lexicon, assimilation is a metabolic process involving digestion and/or decomposition, but excluding physical exchange.

There has been some decoupling of the measurements used to characterize aquaculture impacts in sediments (redox, sulfides; [25]) with models that are based on oxygen fluxes. Brigolin et al. [26] used a more classical early diagenesis model to examine coupled redox reactions at fish farm sites. This is one of the few cases where the field measurements used in regulation could be compared to model output including total sulfides and seabed oxygen consumption.

In terms of benthic impacts, assimilative capacity as the ability of sediments to accept some degree of organic enrichment without generating anoxic crises is well grounded conceptually. A comparison of benthic carbon supply via fish feed and fecal input to oxygen demand of the sediment forms the basis of some the original models of benthic impact [27] including the long-standing Norwegian MOM approach [21].



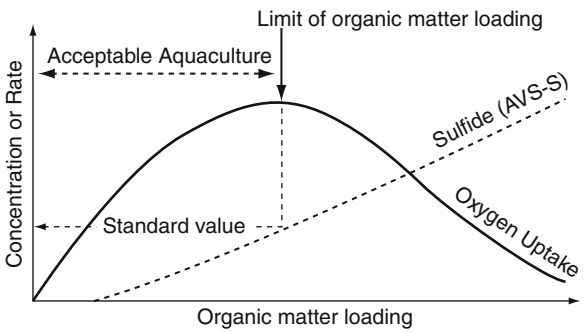
Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 6
Conceptual framework of assimilative and carrying capacities [22]

This concept was widely applied to multiple salmon sites by Morrisey et al. [28]. De Gaetano et al. [78] produced a more sophisticated version of the model for Mediterranean fish culture.

The intermediate disturbance hypothesis (e.g., [29]) provides a functional form to the model by suggesting a parabolic response of benthic diversity and activity to organic loading. Stimulation of aerobic demand occurs at low enrichment, peaking at intermediate levels and declining at high levels (Fig. 7; [30]).

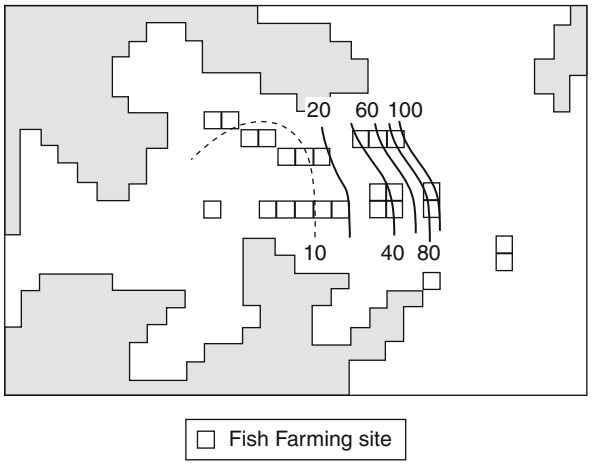
The latter authors define "acceptable aquaculture" as keeping sediment oxygen demand at or below the peak. They produced a numerical model which included a 3D circulation component, organic deposition, and decomposition based on a sediment diagenesis model using oxygen fluxes and anaerobic processes. Output is in the form of mapped values of organic loading based on fish stocking density that produces maximum aerobic oxygen concentration (Fig. 8).

The map shows that farms within the inner parts of the inlet have less permissible organic sedimentation due to reduced flushing. We suggest that this example is the appropriate approach to assimilative capacity since it models system function and its response to organic



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 7
Acceptable limits for organic matter loading from fish farms and the relationship to benthic oxygen uptake and sediment sulfide content [30]

loading based on the capacity of the benthos to absorb organic matter while maintaining oxic conditions. In addition, inclusion of a spatial diffusion–advection model allows incorporation of nonlocal processes and provides mapped output. Results are inherently inclusive of far-field effects and include interaction of multiple farms. Although the paper did not have the



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 8
Isopleths of the limit values of the organic matter loading rate ($\mu molO_2/cm^2/day$) in Gokasho Bay, estimated by a numerical model [30]

context of marine spatial planning, it is clearly applicable in terms of both the approach and the results. The comprehensive nature of this study and its faithfulness to the concept of assimilative capacity make it noteworthy in the literature.

Models of assimilative capacity, like those for shellfish carrying capacity, differ in spatial resolution. The original index models are 1-box, 0D models treating any body of water as a single basin. Physical exchange is thus averaged over the entire domain. This is often problematic since estuaries or other embayments are places with strong gradients in flushing. In addition, aquaculture sites are averaged in this scheme, so no questions regarding optimal location can be addressed.

One hybrid approach which solves some of these problems is the use of a full circulation model whose output is applied to local models. In this case, at least the physics is location specific, and subregions of the system may be considered without the 0D averaging.

Lee et al. [31] applied this approach to yellowtail culture in Hong Kong Harbour by comparing oxygen delivery to fish oxygen consumption and determining net oxygen concentration relative to water quality standards for different levels of stocked biomass. Similar schemes have been used for finfish culture in Scotland [22].

Models for Finfish, Shrimp, and Bivalve Culture

Models for Open Water Culture Models for organic loading by finfish include submodels of circulation, particle transport, and benthic response [21]. Models may also include a phytoplankton-nutrients-zooplankton (PNZ) component, simulating trophodynamics in the water column. This may be necessary due to the importance of phytoplankton and the microbial loop as nutrient processors [22]. For either finfish or shellfish, some version of a farm production or bioenergetics model is essential to estimate waste outputs from cultured animals. Benthic models applied to aquaculture are typically diagenetic, aimed at resolving sediment decomposition and nutrient regeneration [78].

For finfish, benthic deposition of farm wastes and consequent impacts are the primary emphasis; since fish are not dependent on the environment for food, trophodynamics are less relevant. Similarly, the models are usually localized since much of the waste material remains near the cage site (e.g., DEPOMOD). Nonetheless, there is concern that wastes reach the far field and produce negative benthic impacts. Despite this potential, far-field models of finfish cage culture are uncommon. Some early examples used 3D circulation models to examine waste dispersal over kilometer scales [87].

In a more recent example, Symonds [32] compared near-field models such as DEPOMOD to a far-field model based on a 3D circulation. He found that the dependence of near-field models on limited current meter observations was subject to the noise and uncertainty inherent in those measurements. In addition, the far-field model had the potential for bidirectional transport of waste, whereas the near-field model had permanent escape from its limited domain causing potential underestimation of benthic impacts. In addition, the far-field model allows consideration of multiple farms and their interaction. Skogen et al. [33] used a 3D circulation model coupled to a full ecosystem model to study the effects of fish farms on far-field oxygen, nutrients, and primary production, concluding that eutrophication in the far field of a Norwegian fjord was not enhanced by the farm.

The goal of most bivalve shellfish models is to understand seston depletion as a limiting food resource

for farmed animals. This requires a trophodynamic model which includes primary production, bivalve grazing, and advection–diffusion. Because farmed shellfish feed on phytoplankton from distant sources, recent examples involve models which include the far field. Nevertheless, there are many examples of 1-box, 0D models in which the physics and biology are averaged over the basin. Environmental impact of biodeposition to the benthos has been considered in several models including spatial examples [30, 34], but this is less frequently addressed compared to seston depletion. The opposite is true for field studies where benthic impacts are emphasized, and seston depletion is rarely addressed due to the difficulty in observing it. Because seston depletion occurs first at a farm scale, models of the depletion process have also been created at the local scale, prominently the FARM model [4]. Far-field models of seston depletion are increasingly common [20, 35].

It may be concluded that models of finfish aquaculture impacts would benefit from more spatial realism and far-field content, as well as further emphasis on assimilative capacity. Similarly, shellfish models would benefit from inclusion of benthic impact prediction in association with existing focus on seston depletion. The development of assimilative capacity models would be identical for both finfish and shellfish culture. This also places the context to that of ecosystem function. The present context of faunal indices based on carbon deposition in local models is important [36, 37], but is predicted on the basis of somewhat tenuous empirical relationships which are a departure from the more quantitative nature of the models.

Models for Land-Based Culture Models that simulate land-based culture taking place in ponds, tanks, or raceways use many of the biogeochemical features described above, but the physics is simplified to a reactor type of system and serves to determine water exchange and effluent loading to adjacent water bodies. Such models are cheaper to develop and implement than the examples given for open waters (here including lakes and estuaries, where water circulation should also be accounted for). According to the latest figures from FAO, over 70% of freshwater aquaculture in China takes place in ponds, corresponding to an area the size of New Jersey and an annual production of over 15 million tons [88].

This number is triple the total aquaculture production of America, Europe, and Africa combined, which suggests that substantial emphasis should be placed on models that can assist with site selection, optimization of carrying capacity, and evaluation of negative environmental externalities of pond culture.

Various examples of this type of approach exist (e.g., [38, 77]. Figure 9 shows mass balance results obtained with the Pond Aquaculture Management and Development (POND) model [39], which simulates the production and environmental effects of shrimp, fish, and bivalves cultivated in ponds, in monoculture or IMTA.

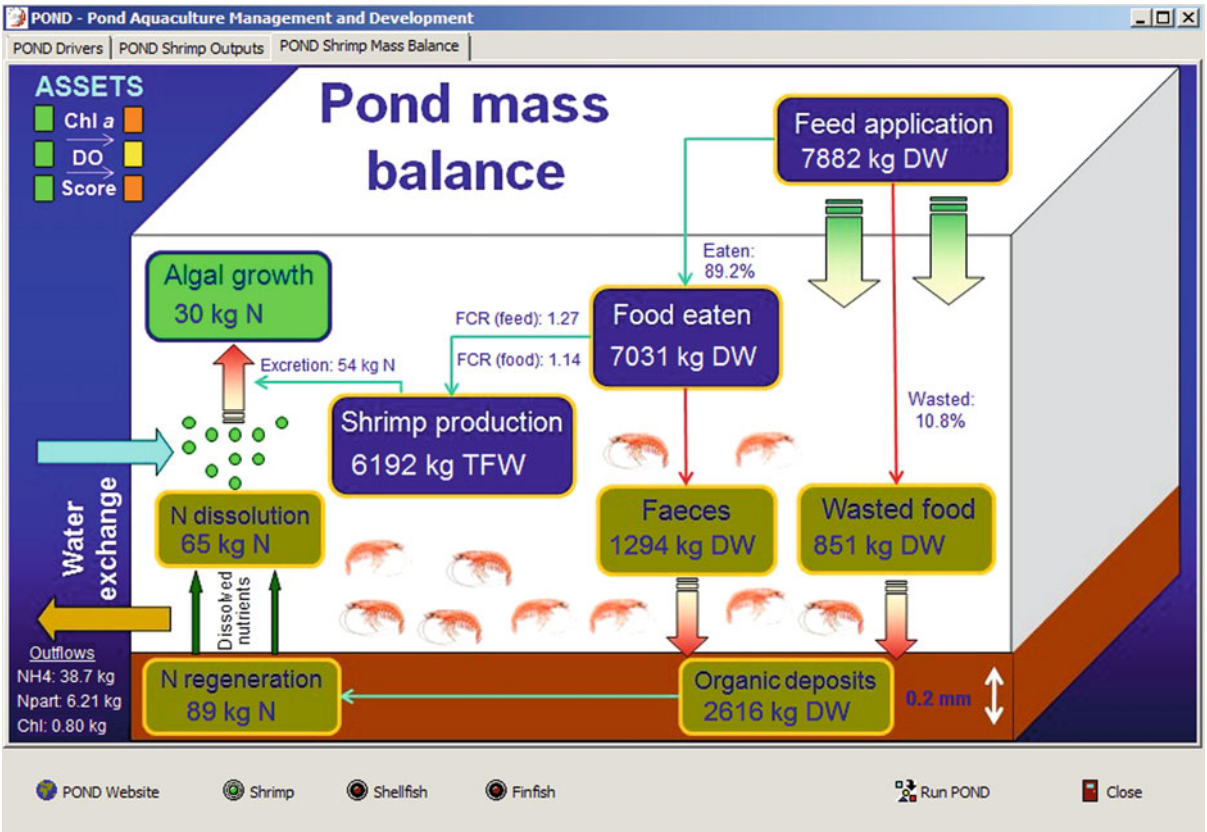
The model was run for a site at 23°N, with a daily water renewal of 3% of the pond volume, and estimates an environmental discharge of about 45 kg of nitrogen (mostly dissolved, but also as algae), roughly 14 population equivalents (PEQ) per year for the 90-day cultivation cycle. The cost of offsetting these emissions is over 500 USD [40]. In developing countries, these waste costs are not internalized but rather imposed on to the environment, contradicting the first principle of the ecosystem approach to aquaculture [8] that:

- Aquaculture should be developed in the context of ecosystem functions and services (including biodiversity) with no degradation of these beyond their resilience.

By contrast, pond production in the United States already requires a National Pollutant Discharge Elimination System (NPDES) permit [41]. Often, this means that large agro-industrial companies from developed countries price-leverage the lack of environmental regulation and/or implementation in the developing world.

Models of this kind can be coupled with a range of other models to provide a decision support framework. POND uses the well-tested Assessment of Estuarine Trophic Status (ASSETS) model [42], providing a color-coded assessment (Fig. 9) of the degradation of water quality over the culture cycle.

Models for Integrated Multi-trophic Aquaculture IMTA has been practised in Asia for thousands of



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 9
Mass balance of whiteleg shrimp (*Penaeus vannamei*) culture, including production and environmental externalities for a 1-ha pond

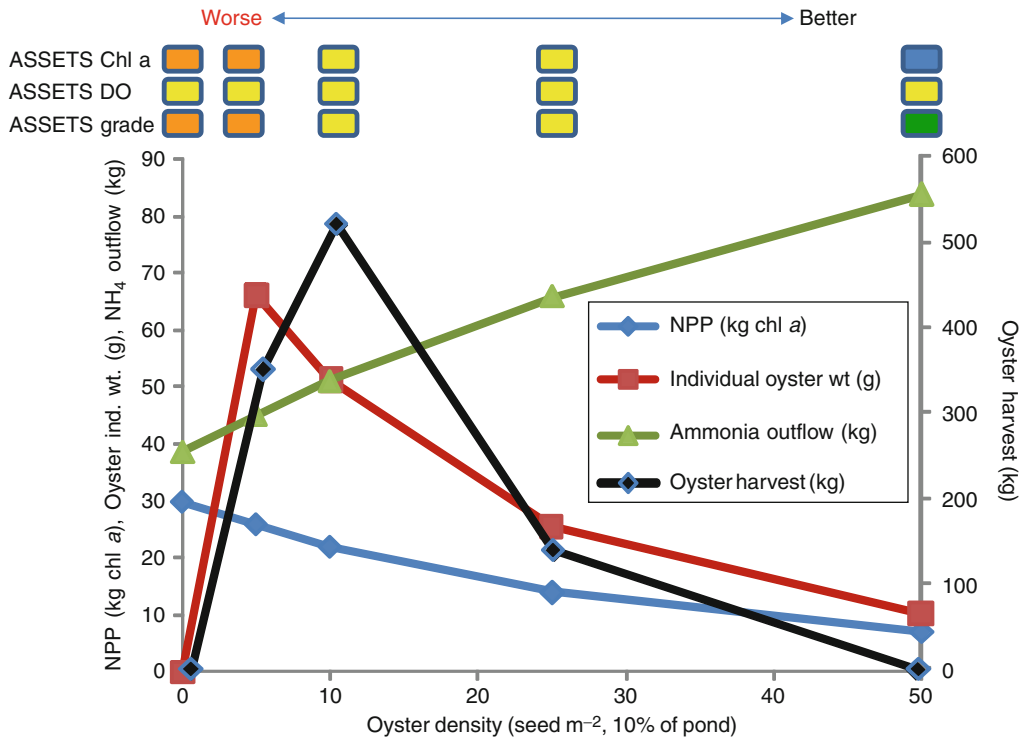
years. In the fifth century B.C., the Chinese aquaculturalist Fan Lee [81] wrote:

- You construct a pond out of six mou of land. In the pond you build nine islands. Place into the pond plenty of aquatic plants that are folded over several times. Then collect twenty gravid carp that are three chih in length and four male carp that are also three chih in length. Introduce these carp into the pond during the early part of the second moon of the year. Leave the water undisturbed, and the fish will spawn. During the fourth moon, introduce into the pond one turtle, during the sixth moon, two turtles: during the eighth moon, three turtles. The turtles are heavenly guards, guarding against the invasion of flying predators.

A substantial proportion of Asian aquaculture currently takes place in cocultivation, improving production, optimizing resources, and reducing environmental waste [14]. Despite this oriental wisdom, multi-trophic aquaculture is still rare in North America and Europe, although commercial interest is growing rapidly. This is reflected in research (e.g., [43, 44]), with the annual number of scientific publications on IMTA doubling from 2007 to 2010 (SCOPUS).

The combinations of species, relevant proportions, and culture practice are key to successful IMTA. There is traditional knowledge in China and other parts of Asia on what works best, but advances in mathematical modeling can make a substantial contribution by quantifying energy flows, production, and environmental externalities ([45], Nobre et al. [11], [16]).

Figure 10 shows a simulation of IMTA for a shrimp farm, with cocultivation of the Pacific oyster *Crassostrea gigas* at different densities, using the POND model. As the oyster density in the ponds increases,



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 10
Analysis of IMTA production and externalities by means of the POND model

the net primary production (NPP) of phytoplankton is reduced due to top-down control, although NH_4^+ increases due to bivalve excretion. The model is not simulating macroalgae or other aquatic plants, which if added would significantly reduce the output of ammonia.

The ASSETS score is best at the higher oyster density, but at a density of 10 oysters m^{-2}, the shrimp culture cycle would yield a harvestable oyster biomass of over 500 kg to provide an annualized extra income of over 10,000 USD. The removal of algae and detritus by the filter-feeding bivalves corresponds to three PEQ per year, about 15% of the discharge. At the higher densities, the bivalves are performing a bioextractive function and do not reach market size in a short cycle due to food depletion. A more detailed analysis can be made by simulating oyster growth continuously over multiple cycles of shrimp culture. Such interactions can also be modeled for other combinations, for example, tilapia and shrimp or salmon and mussels.

Models for Disease Spread and Control In determining the carrying capacity of aquaculture operations, it is important to ensure aquaculture production practices and systems within a farm, managed region, or zone are resilient to the effects of disease. Modeling provides a means of investigating the interactions occurring among the four pillars and the spread and establishment of pathogens; however, to date, most models have ignored the influence of society on aquatic disease. Many different models have been derived to investigate the spread and impact of particular pathogens: In aquatic systems, two of the most common approaches are (1) compartment-based models and (2) network models.

Compartment-based models assume that individuals transition through a series of states from susceptible (S) to infected (I), and potentially back, or through a series of other states such as resistant (R). These models are often referred to as SIR models [46] and are usually based on continuous time, and therefore use differential equations; however, stochastic and

discrete time approaches are also used. SIR models do not track individuals but assume the population follows a set of behavior rules, and that at each point in time, a proportion of the population leaves one state to enter another according to these rules. In aquatic systems, these models are generally used to track disease through a population of animals, but they have also been applied to look at spread through a population of sites. Simple implementations are often analytically tractable, allowing conditions under which thresholds and equilibria occur to be found without the need to run simulations.

One of the key pieces of information that can be derived from these models is a maximum (susceptible) host carrying capacity for which a pathogen cannot persist. Such carrying capacities used in the context of pathogens are often referred to as a critical threshold (N_T) and may be useful to wildlife and farm managers when attempting to control or prevent disease. This threshold is however largely dependent on the assumptions made regarding the way a pathogen is transmitted, and obviously does not apply if transmission is not dependent on host density, but is frequency based (as is often assumed to be the case when modeling sexually transmitted infections). It is therefore important that the correct form of transmission is used in a disease model to avoid false inference. The most commonly used form of model assumes that transmission occurs by contact between animals or sites, that the number of contacts is density dependent, and that mixing between individuals occurs equally and at random. This is often referred to as a "density-dependent transmission" or "pseudo mass action" model [47, 48].

Under these assumptions, one method by which N_T can be derived is by determining the conditions under which the basic reproductive number, R0, is equal to 1. In simple terms, R0 is the number of secondary infections that arise if an infected individual enters a wholly susceptible population. If greater than 1, the pathogen will establish; if less than 1, it will die out. R0 is governed by the total population density, the period over which an infected animal sheds pathogen, and the transmission rate β (the rate of contacts between individuals and that the probability that given a contact, infection occurs). Both environmental conditions and management/husbandry decisions can influence this number, and therefore the critical threshold for establishment. Understanding the influence of each of the four pillars of carrying capacity on R0 is therefore critical in order to predict and manipulate N_T. Omori and Adams [49] illustrate the application of compartment-based models to assess the influence of the ecology and production pillars on the dynamics of Koi herpesvirus in farmed carp populations *(Cyprinus carpio)*. Their approach showed that temperature and on-farm production processes used in conjunction with this could be used to immunize a population, preventing clinical disease expression.

The assumption of random mixing of a population is often not reasonable, as in reality some individuals will be more social or isolated than others, and individuals tend to have discrete groups of contacts that may or may not be connected to other groups within a population. Under these circumstances, the assumption of random mixing can lead to substantial overprediction of the epidemic process. Social network analysis (SNA) and modeling approaches provide a means of incorporating the contacts that occur between individuals, and therefore the consequence this may have on pathogen spread. In the case of agriculture and aquaculture, these models tend to be based at the level of the site, rather than the animal in question.

A major advantage of this modeling approach is that much epidemiologically useful information can be obtained merely by examining the network properties, without parameterizing for a particular disease. For example, in order to develop generic surveillance and control strategies, it may be useful to identify:

- Clusters of connected individuals within the network and whether it is possible to make them epidemiologically distinct through the removal of a few connections.
- Long chains of connection that join the network throughout and thus allow disease spread.
- Super-spreaders, which are individuals that contact a high number of other individuals and can thus rapidly spread pathogens.
- Super-sinks, which are individuals that receive from a lot of other individuals. Though they may not

spread a pathogen, they are most likely to receive one and may therefore be useful for surveillance purposes.

Many statistics can be generated to summarize different properties of a network. Although R0 can also be estimated, other statistics such as the degree of centrality or clustering coefficient may provide more useful means of assessing the likelihood of spread through a network. Green et al. [50] demonstrated the application of such statistics using SNA applied to the Scottish network of trout and salmon farms. The analysis showed how much transmission was likely to occur in the network as it stood but also used a variety of methods to remove the most influential connections to reduce transmission.

In addition to examining the network properties, stochastic simulations can be run over the network. In such simulations, the network is randomly seeded with infected individuals and, at each subsequent time point, connected individuals change state from susceptible to infected at a given probability and given the likelihood of a contact. This approach is often useful when evaluating rates of spread and the effectiveness of control strategies but also has the potential to be used as a real-time tool during an outbreak, if it can be parameterized for the pathogen of interest. Further useful information may be gained from these models by making network models spatially explicit, as this facilitates the designations of control zones.

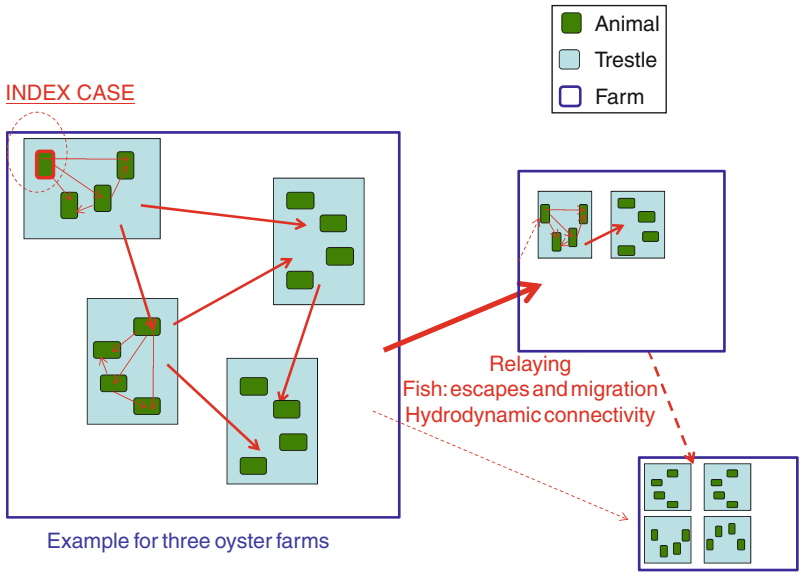
Werkman et al. [51] used stochastic network simulations to good effect to determine the efficacy of different fallowing strategies in eradicating disease from areas producing different amounts of fish. Thrush and Peeler [52] used a simulation of movements over the English and Welsh trout industry network to investigate the potential spread of *Gyrodactylus salaris*. This demonstrated that in 95% of the simulations run, less than 63 of 193 river catchments would be at risk of getting the pathogen in the 12 months following introduction. Jonkers et al. [53] developed this model further into a strategic tool that could be used to evaluate the effectiveness of different control and surveillance efforts on disease spread.

One of the major limitations of network approaches is that they rely on knowledge of the complete network of connections and assume that this remains stable over time. Missing connections or changes to the network with time could lead to misdirected efforts. For many aquatic systems, reliable network data are not available or are difficult to compile. Under these circumstances, simpler compartment-based approaches may still be of value in informing control policies. One such application was demonstrated by Taylor et al. [54] to evaluate the effectiveness of different control options in reducing the spread of Koi herpesvirus in the UK between sites holding carp.

Most models applied to aquatic systems to date tend to be based at either the level of the site or animal, with few attempting to combine the two approaches. There is, however, substantial scope to develop future models that incorporate multiple levels that account for transmission between individual animals in a unit, the influence this has on the transmission between units on a farm, and the subsequent effect on between-farm spread (Fig. 11).

Where attempts to link these two levels together have been made, useful results have been obtained. Hydrodynamic models have been applied to look at the spread of sea lice between Norwegian salmon cages in fjord systems [55]. These models track the number of lice generated and monitor their dispersal and decay over time and space to see whether they reach and infect other sites. Green [56] combined compartment-based and network approaches to incorporate the influence of within-site processes on network spread and found that differences in site biomass influenced the rate of between-site transmission if density-dependent transmission was assumed. One of the major advantages of models that link the within-site epidemic with between-site spread is that they allow for the consequences of different detection (or action) rates to be assessed, allowing more efficient resource planning.

The primary goal of all of the approaches described above is often to apply changes to the system to understand their influence on pathogen spread. In application to aquaculture systems, this may be conducted to establish how best to maximize N_T (either in the number of animals produced within a site or the number of sites in an area) and thus increase carrying capacity. Generally speaking, N_T can be increased by reducing the amount of connectivity between individuals (restrictions in movements), changing their susceptibility to disease (e.g., by vaccination) or reducing the



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 11
Network models working at different scales in time and space

period over which an individual is able to transmit a pathogen (e.g., through good surveillance and rapid culling). Other important disease management tools that have or could be evaluated by the modeling approaches described above include:

- The use of management areas in which aquaculture activities such as harvesting or treatment are coordinated between all farms in an area
- Fallowing of sites between production cycles to allow pathogens present to die out and potentially reduce the infection pressure other sites are exposed to
- Year class separation, where only one age class is present at a time causing all animals to be harvested prior to stocking new animals
- Removal of high-risk contacts between sites that are likely to spread disease widely
- Biomass limits to reduce the maximum amount of pathogen that could be discharged from a site
- Minimum distances between sites to reduce transmission via hydrographic connectivity

Building a Framework

Screening Models and Research Models The models reviewed above address different components of aquaculture carrying capacity, focusing mainly on production and environmental effects, including disease aspects. Additionally, they consider different scales in time and space and range from statistical models to spatially discrete representations, which may (e.g., hydrodynamic models) or may not (GIS) be time varying. It is useful in this context to distinguish between screening models and research models.

Screening models typically use a limited set of inputs and provide highly aggregated outputs, such as an index of suitability or an environmental scorecard. Examples include a comparison of ammonia excretion by caged salmon compared to tidal flushing [57] and the production of biodeposits compared to tidal removal for suspended mussel culture [23].

Models of this type (e.g., FARM, [4]; POND; Fig. 9) are easy to use by the management community and provide a quick diagnosis for a specific site, or a generic overview comparison for multiple areas.

Models that are complex to apply (and usually lengthy and complex to develop and implement) may be termed research models and are of limited practical use in day to day management. Partly, this is due to the knowledge required for parameterization, volumes of data involved, and substantial requirements in processing and interpreting results. This does not mean that the results of such models do not have

a clear application for management, but operating them may be beyond the scope of many institutions.

The concept of a single overarching model, able to provide answers across a range of space and time, has been shown repeatedly to be unsound. Just as software suffers from feature creep [58, 59], stand-alone models tend to become increasingly overparameterized, partly in an effort to better match reality and partly in an attempt to solve per se what should really be approached with a combination of models.

A more promising alternative is to combine GIS, dynamic models, network models, and remote sensing tools as appropriate to deal with questions that range from the impact of a HAB event on a salmon cage at the scale of days to the economic success of 10 years of mussel farming.

An increase in model complexity does not necessarily equate to increased accuracy [60], whether in physical or biological models. In both cases, the scale at which predictions may be made is limited by our incapacity to accurately predict the weather. As a consequence, model resolution and accuracy are limited by key drivers such as river flows, salinity patterns, and water and air temperature, which impact metabolic rates, algal blooms, turbidity, spawning, or larval dispersal.

Models in the Context of Integrated Coastal Zone Management Carrying capacity models for aquaculture are useful in their predictive capability and therefore in management of both cultured biomass as well as location. Spatially resolved models are particularly notable in this context. Within the catchment, this may be addressed by means of GIS (see, e.g., [61]) and can be enhanced through a combination of dynamic simulations and spatially resolved models.

Aguilar-Manjarrez and Nath [89] performed an extended analysis of the potential for fish aquaculture in Africa, based on GIS models, using a resolution of 3 arc minutes (25 km^2 at the equator). For small-scale operations, suitability was based on water requirements, soil and terrain, availability of feed inputs, and farmgate sales projections. This analysis was also carried out for larger, commercial farming and concluded that for the three species considered (Nile tilapia, African catfish, and carp), about 23% of the area of continental Africa scored very suitable for both types of farming. These authors did not explicitly consider environmental effects of fish farming, probably because that analysis is best performed regionally or locally, as part of detailed site selection, but the modeling tools for addressing these impacts are available today.

GIS models have also been combined with remote sensing to assess aquaculture opportunities, for example, for crab and shrimp in the Khulna region of Bangladesh [62]. In this case, the focus was on gross production, economic output, and employment, and discussed species suitability with respect to factors such as salinity distribution and freshwater availability. Once again, the environmental externalities of the different types of culture were not included but can be modeled to provide a more complete picture for decision support, including externality costs.

Figure 12 shows the Jaguaribe estuary, in the state of Ceará, northeast Brazil. As part of the application of the ASSETS model to determine the eutrophication status of the estuary (Eschrique and Braga, personal communication, 2011), the nitrogen loading from 1,200 ha of shrimp farms, located between the city of Aracati and the dunes fringing the beach, was determined using POND.

The contribution of shrimp farming to the nitrogen load was estimated to be of the order of 60 t year^{-1}, roughly equivalent to 20% of the total loading, or about 15,000 PEQ.

Screening models of this kind are simple to use and help water managers in developing countries to address the challenge of multiple uses and nutrient sources to the coastal zone and to make better decisions with respect to site selection and waste treatment. They can also be included in a more general catchment modeling approach, combining their outputs with hydrological models such as the Soil and Water Assessment Tool (SWAT) model (e.g., Nobre et al. [11]).

In open water, including semi-enclosed systems such as estuaries and bays, because spatially resolved models incorporate physical circulation, they are potentially useful for other aspects of coastal zone management.

There are of course a variety of possibly impactful activities in the coastal zone including eutrophication, fisheries and fish processing, contaminant input, resource extraction, and transportation. There are inevitably competing uses of the water and bottom,

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 12
The Jaguaribe estuary, in Ceará, NE Brazil. Shrimp farms occupy an area of about 12 km^2 and discharge the effluent into the estuary

and making predictions about aquaculture in isolation is insufficient. Water and sediment quality standards applied over large spatial scales help to apply objectives that may be seen as ecosystem-based management. The implementation of these objectives is achieved through marine spatial planning. The implication of planning is that predictions can be made to anticipate overlaps, conflicts, and cumulative impacts of various activities.

Although models exist for these other activities, particularly those acting through eutrophication, the models need to interact coherently, and their outputs should be tied together in a way that is useful for management decisions. Two essential features of this integration are physical models that unify the transport of materials common to almost every process in the ocean and GIS to maintain data layers. The physical model establishes the spatial domain which can be either conserved, collapsed, or expanded within the GIS. At present, models run within GIS are necessarily simple and based on averaged values. For example, the AKVAVIS tool developed in Norway [63] utilizes a GIS with calculation of shellfish growth as well as fish farm effects on water quality at any location based on an inline model using depth, temperature, and other spatially located characteristics within the GIS [63].

Tironi et al. [24] utilized an open source circulation model to predict far-field deposition for Chilean salmon culture. An essential feature of their work was a GIS interface used to depict other coastal activities in light of hydrodynamics and dispersal of farm waste. Nobre et al. [11]) combined models of watersheds, aquaculture, and eutrophication to look at mitigation strategies for improving nutrification in Chinese bays.

In marine systems with aquaculture but lacking other activities (e.g., some Norwegian fjords), the culture model may form the basis of the decision support system. In Lysefjord (southern Norway), a pump was used to transport water to depth where it enhanced diffusion of nutrient from below the thermocline to the euphotic zone [90]. The increase in primary production was used as a food source for cultured mussels. A model was employed to determine the best location for the upweller as well as the mussels in order to benefit from increased primary production, balancing the increase in new production with mussel removal to maintain chlorophyll within its natural limits. This example is of interest because the ecosystem was truly managed in terms of bottom-up nutrient supply, new production, grazing in terms of mussel culture, and marine spatial planning optimized for the extent and level of shellfish culture.

As more types of activities are added to decision support tools, one approach is to use GIS as a wrapper for model outputs produced as layers. Decision support comes from ancillary software with features such as portrayal of alternative land uses, exclusion of protected areas, weighting of valued features and habitats, and economic analyses. Several initiatives have been undertaken in this vein, with the incorporation of simulation models mostly in the initial stages of progress. Examples which have freely available ArcGIS extensions include NatureServe Vista (natureserve.org) and Marine InVEST (naturalcapitalproject.org). There are different emphases in the various projects, including conservation, community engagement, ecosystem services, and land use, in addition to water quality issues.

Among these, Marine InVEST seeks to maintain ecosystem services in the context of activities such as fisheries, aquaculture, renewable energy, and recreation [64]. Input information ranging from oceanographic data to species distribution is used in various models to consider outputs in terms of ecosystem services provided, traditional model results (e.g., water quality), and socioeconomic valuation (Fig. 13).

We note the importance of models other than those based on eutrophication and water quality. For example, some of the same spatial data used to model aquaculture impacts can be used to predict the location of sea grasses or species at risk [65, 66]. Protected areas or buffer zones may then become part of the plan. In this way, critical habitat and biodiversity are also considered along with aquaculture siting. Moreover, these decision-support frameworks can be used in public forums to incorporate community input, as has been done with protected area planning via MARXAN GIS [67]. In practice, these are few examples of models used to this extent, but it provides a very useful management shell in which to consider aquaculture submodels.

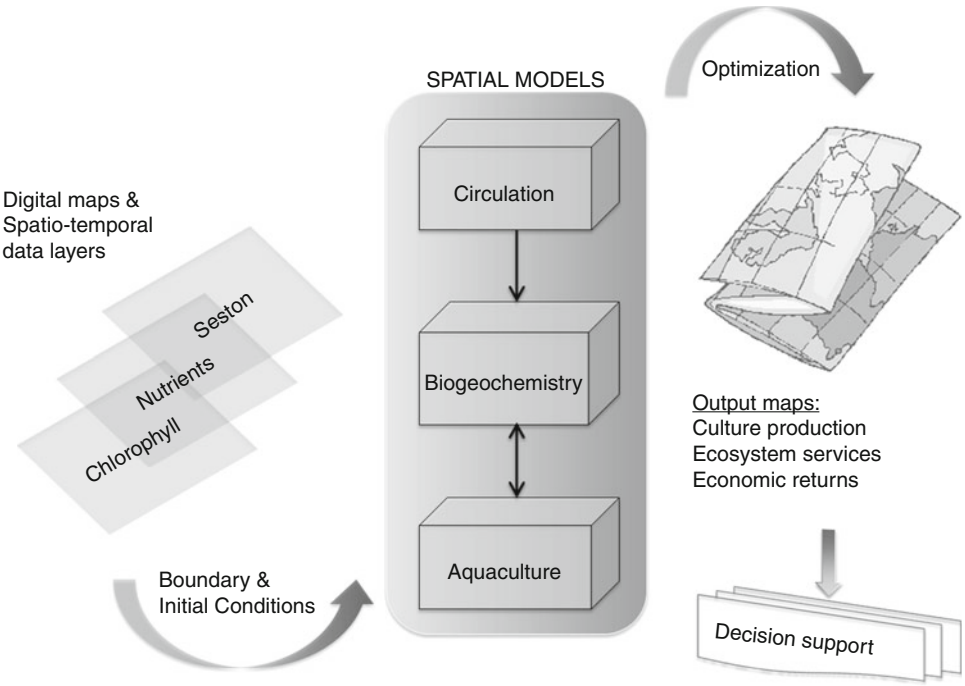
The Other 50% of the Problem At present, two major components of carrying capacity, the social and governance aspects, are not amenable to mathematical modeling (Fig. 5); they are nevertheless fundamental areas for aquaculture management.

The social pillar, from the perspective of social acceptance of aquaculture and, in particular, of the definition of limits to industry growth, has been discussed, for example, by Byron et al. [18]. Stakeholder dialogue, understanding of terms and concepts, and the simple fact that opinions can be voiced during the decision-making process are major contributors to generate consensus. The tools employed include questionnaires, meetings, and presentations of different sides of the issue. Simulation models can help inform those discussions by, for example, providing quantitative data on development scenarios but social positions often have a strong emotional component.

The governance pillar plays a major role in the assessment of carrying capacity. Table 1 shows key aspects of the human interaction in aquaculture and highlights the consequences of poor practices and governance.

The issues identified have consequences that vary in severity, from a reduction in revenue and profit to major outbreaks of disease and the loss of aquaculture resources across large areas.

Because the effects of human mismanagement can be so far reaching, it is important to discuss to what extent modeling frameworks can be used to assist the



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 13
Models for different purposes are linked through input data layers that are used to build scenarios about management actions (e.g., restoration of eelgrass, increase in aquaculture) and climate change (e.g., sea-level rise, water temperature)

farmer and the water manager. The eight topics in Table 1 fall under the heading of governance (here taken to include self-regulation by farmers and farmers' associations, as well as legal instruments, policy, and implementation), but only three (dark blue) can benefit directly from recommendations from simulation models, addressing stocking density and feeding practices.

Five of the issues identified introduce disease-related problems, and whereas procedures such as relaying, or seed import from contaminated sources, are strictly within the remit of good governance, models can to some extent assist in predicting the likelihood (i.e., risk), spread, and establishment of diseases, if present within an aquaculture area.

Regulations enacted to control spread of diseases can directly mandate what species and culture practices, stocking densities, etc., are permitted within a zone/region. Firstly, this would include planning applications assessments that would consider, among other factors, how siting a farm may potentially adversely affect other aquaculture operations located in that area, as well as potential effects on wild animal populations. In some cases, e.g., in Chile, regulations have been enacted that specify minimum distances between farms.

There is also an increasing move by some large-scale industries, as part of developing common codes of practice, to manage farms to minimize the effects of disease on production. These codes can include restricting stocking densities, production on farms, specifying minimum allowable distances between farms, and introduction of mandatory fallowing periods between production cycles. All these are measures that would theoretically constrain carrying capacity, at least in the short term, but might well increase the overall sustainability of the industry in the zone or region in the longer term. Although these may not be enforced by government regulations when first developed (e.g., they are often "voluntary codes"), those signing up may be entering legally binding agreements. These codes may then be used as the basis to develop more formal regulatory frameworks in the future at the local, national, and even supranational level.

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Table 1 Key issues in culture practice, time frames, and potential consequences (color coding reflects the extent to which models can be applied: *black* – substantial; *dark red* – reasonable; *light red* – inapplicable)

N°	Topic	Time frame	Issues/consequences	Examples
1.	Species selection	Prior to cultivation	Imported exotics, disease, proliferation	Pacific oyster, now considered invasive in the Netherlands; *Perkinsus* (dermo) and MSX, U.S. East Coast
2.	Seed purchase	Start of cultivation	Disease from imports, stable broodstock	Herpes virus spreading in oysters across Europe
3.	Relaying	Variable	Disease spread	Transmission of Herpes in oysters across Europe, ISA in Chilean salmon
4.	Stocking (farm or pond scale)	Critical periods	Overstocking can lead to: mass mortalities due to dissolved oxygen depletion, other environmental factors, and stress-related disease outbreaks	Whitespot Syndrome Virus (WSSV) in Penaeid shrimp, *Perkinsus* in clams
5.	System-wide carrying capacity	Months to years	If carrying capacity is significantly exceeded: harvest reductions, disease outbreaks, economic hardship, system collapse, long recovery cycles, long-term loss of resource	Marennes-Oléron, France, longer oyster culture cycles; Sacca di Goro, Italy, mass mortality of Manila clam; Qinshan, Fujian province, China, 50,000 fish cages (yellow croaker), mass mortality
6.	Feeding practice	Culture cycle	Ecologically damaging practices with ecosystem consequences, e.g., overfeeding of caged fish and benthic hypoxia	Fertilization of intertidal/subtidal areas in China to promote seaweed growth, increasing yields of commercial products (seaweed, gastropods); use of juvenile fish as fish meal for cage culture in parts of Asia
7.	Spatial distribution of culture	Culture cycle	If inappropriate, e.g., by combining year classes in shellfish, more labor-intensive, greater impacts on the ecosystem, e.g., through sediment reworking	Clam culture in southern Portugal
8.	Lease structure	–	Fragmented/inexistent lease structure, smallholdings governance issues, due to multiple stakeholders	Pond culture in Thailand (average freshwater pond: 0.28 ha); Clam culture in southern Portugal (average lease: 0.15–0.5 ha)

Regulation can also influence the carrying capacity of a zone or region both directly and indirectly. In particular, to help control the spread of diseases between countries, the OIE aquatic animal code [91] lists a number of infectious aquatic animal diseases that are generally considered untreatable, pose a significant threat to aquaculture and/or wild fish populations, and are not widely distributed (e.g., are exotic to most countries where the species they affect are farmed). Countries and regions also have laws and regulations to prevent the entry and, where pathogens do gain entry, specify measures for their control and eradication. For instance, Directive 2006/88/EC lays down, for European Member States, the required minimum animal health requirements, disease prevention, and control measures for aquaculture animals and products.

Eradication of a disease from a country or zone may involve extensive depopulation of affected farms and not restocking them until it is confirmed that the risk from disease is significantly reduced. This has obvious short- and long-term consequences on the carrying capacity of the affected species in the affected areas.

For example, following detection of the notifiable viral disease Infectious Salmon Anemia (ISA) on an Atlantic salmon farm in Chile in 2007, the disease spread throughout the salmon industry [92]. The effects were dramatic. It is estimated that salmon production in 2010 was little more than 98,000 t, down from 386,000 t in 2006 [93]. With the 1.5–2.5-year production cycle for salmon, these effects will be felt for years to come, with estimated smolt release in 2009 only 10% what it was in 2007 [94]. It is estimated that, compared to production in 2007, levels will be reduced cumulatively by at least 700,000 tons during the period 2009–2011, with their value reduced by more than two billion USD.

Disease controls can restrict the types of aquaculture activity that are permitted, or are practically possible, within a zone or region. Here, the constraints to carrying capacity are often opportunity costs, with fish not reared in a particular area because of potential disease risks. The effects can be quite subtle. For instance, in the UK, controls in place to restrict the spread of bacterial kidney disease are considered by some sectors of the rainbow trout farming industry to adversely affect their operations at the expense of the larger Atlantic salmon industry that has generally supported the maintenance of these controls. This would also include the effects of restrictions placed on the movements of live fish and ova between countries that limit the opportunities to farm those species in other countries.

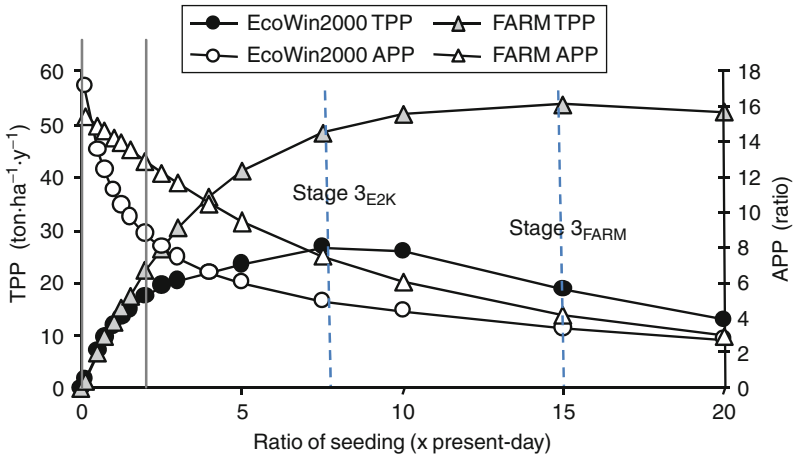
Finally, societal acceptance of aquaculture activities may be influenced by disease risk concerns, particularly to wild fish stocks. This is perhaps best illustrated by sea lice spread to wild fish. There is additional public concern as to the effects on the environment of use of chemical treatments. In particular, there is strong resistance to fish farming in many remote and pristine environments due to fears they may adversely affect local ecosystems. Increased public awareness of welfare issues in aquaculture may also lead to practices that reduce the incidence of disease as a consequence.

Blueprint for a Model System

There are considerable challenges in selecting and combining different types of models, each of which plays a particular role, for carrying capacity assessment. A number of models are available, many of which reviewed above, but the level of usability varies widely, as does the capacity of one model to "speak" to another. This comes about through the "one tool" syndrome: If your only tool is a hammer, all your problems are nails. One tool, i.e., one model, may be fine for a particular level of analysis, but it will be incapable of doing all the work.

For instance, a local-scale model such as DEPOMOD [36], MOM [68], or FARM [4] will need current velocity fields supplied either by field measurements or by other types of models. Equally, it will not provide a robust answer with respect to system-scale carrying capacity. Even at the farm scale, such models do not consider the interactions among farms, although the siting of a new farm in a region will inevitably affect environmental conditions and production in existing farms. These changes will then feed back, so that the original farm-scale assessment will change because the model input data will change.

Figure 14 illustrates this difference for mussel culture in Killary Harbour, Ireland, by comparing two models at differing scales [35]. EcoWin2000 (E2K) is an ecological model applied at the system scale, whereas FARM simulates production and environmental carrying capacity at the local scale. Both models can be used to perform a marginal analysis [4] to determine stocking densities that lead to optimal profitability. This is extremely useful for licensing purposes since farms often maximize income rather than profit (i.e., aim for the highest TPP). It can be seen that for a coastal or semi-enclosed system, there appear to be significant differences between the results for the system-scale model, where the dotted line indicating highest TPP (which exceeds the seeding density of maximum profit) occurs at a density 7–8 times greater than the present situation. FARM, however, which deals only with the local scale, determines the end of stage 2, i.e., highest TPP, as around X15 density. This is because (1) E2K runs multiple production cycles, typically for periods of 10–20 years, i.e., the ecosystem model reports what is actually harvested, whereas FARM reports what is harvestable over one cycle, and (2) FARM does not account for interactions among farms, whereas E2K considers the farms in the whole water body.



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 14
Impacts of different mussel-stocking densities on total physical product (TPP, harvestable biomass) and average physical product (APP, the ratio between seeded and harvested biomass) as simulated by EcoWin2000 (system scale, per unit of cultivated area) and FARM (single farm) for Killary Harbour, Ireland (Adapted from [35])

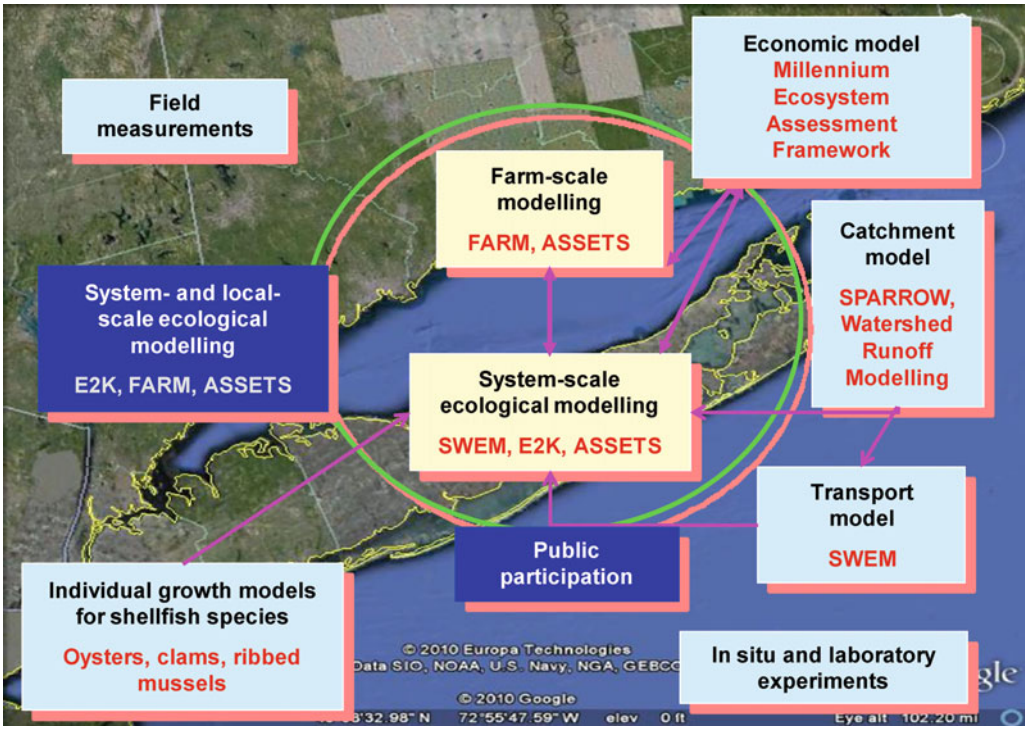
The following set of properties is desirable when assembling a modeling system to address aquaculture carrying capacity:

1. No single model solves all problems. Overparameterization and code bloat only make matters worse.
2. Models should only be as complex as the problem requires. In other words, as simple as possible. Increased computational power is no excuse for unnecessary complexity.
3. Models should be able to work independently, present value in doing so, and add further value when working in conjunction with other models.
4. Models should define exactly what problems they can address, as part of the overall questions for an ecosystem, rather than the opposite.
5. Any model in the system must be able to receive input from data or from other models and be able to supply outputs in a form that can be easily used by other models.
6. Different models are appropriate for different scales in space and time. Carrying capacity assessment may require scales as short as a tidal cycle (e.g., for intertidal culture of clams) and as long as a decade (e.g., for coupling ecological models with economic models).
7. Models share a challenge with field sampling with respect to the conversion of data (measured or modeled) into information that is useful for managers; the use of screening models, or other approaches that help to distil data into meaningful information, is a vital component of any system.

Figure 15 shows an example of such a multi-model framework for investigating the role of bioextraction by oysters, clams, and ribbed mussels as part of a nutrient control strategy for Long Island Sound. The various models chosen conform to the seven properties described above.

Previous applications of this kind of framework include the SMILE project in Northern Ireland [19] and the SPEAR project in China ([69], Nobre et al. [11]).

A different kind of multi-model framework has been developed by Kapetsky et al. [70], through the application of various types of remotely sensed data in a GIS system, incorporating variables such as sea surface temperature, current speed, and chlorophyll *a*. In the example shown in Fig. 16, current speeds of 0.1–1 m s^{-1} are combined with suitable depth ranges for fish cages and shellfish longlines. High current speeds are problematic for both culture structures, due to excessive hydrodynamism, and finfish production, due to higher metabolic costs and lower yields; very low current speeds may cause sediment organic enrichment and quality loss for certain species.



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 15
Modeling framework: the REServ shellfish bioextraction project, Long Island Sound, USA (NOAA/EPA REServ Project)

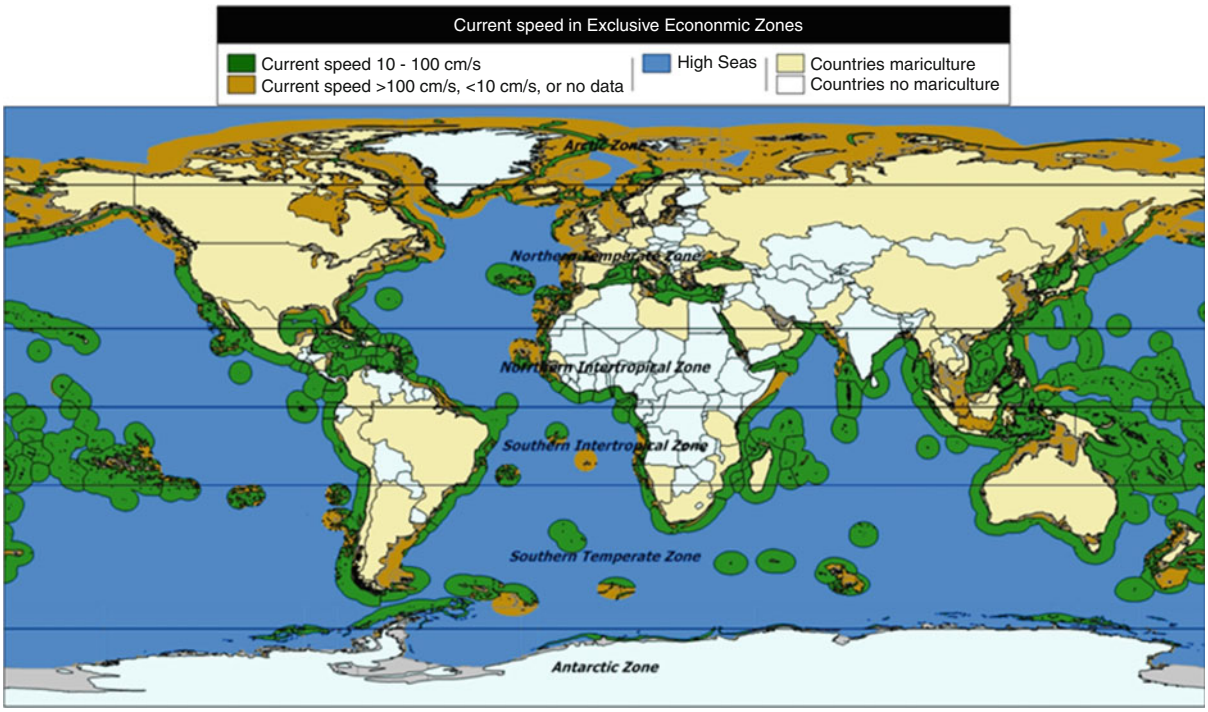
Quantification of suitable areas indicates that 123 countries have at least 100 km^2 within their exclusive economic zone that meet these criteria.

For an annual production of 1 kg m^{-2} $year^{-1}$, this corresponds to 10^7 t $year^{-1}$ of aquatic products. This analysis has been further refined by considering distance to port, a key economic consideration for offshore aquaculture, which will considerably reduce this estimate. The same applies to other factors, such as marine protected areas. An assessment of spatial potential (a component of *production* carrying capacity) was executed at a finer scale for selected areas in Canada, Chile, Ireland, and Norway (Fig. 17). For a number of specific locations (farms) in these four countries, the time required for growing an adult salmon was then determined using a dynamic model [71], forced by remotely sensed sea surface temperature. In this case, the model end point was a particular fish biomass, rather than a specific cultivation period.

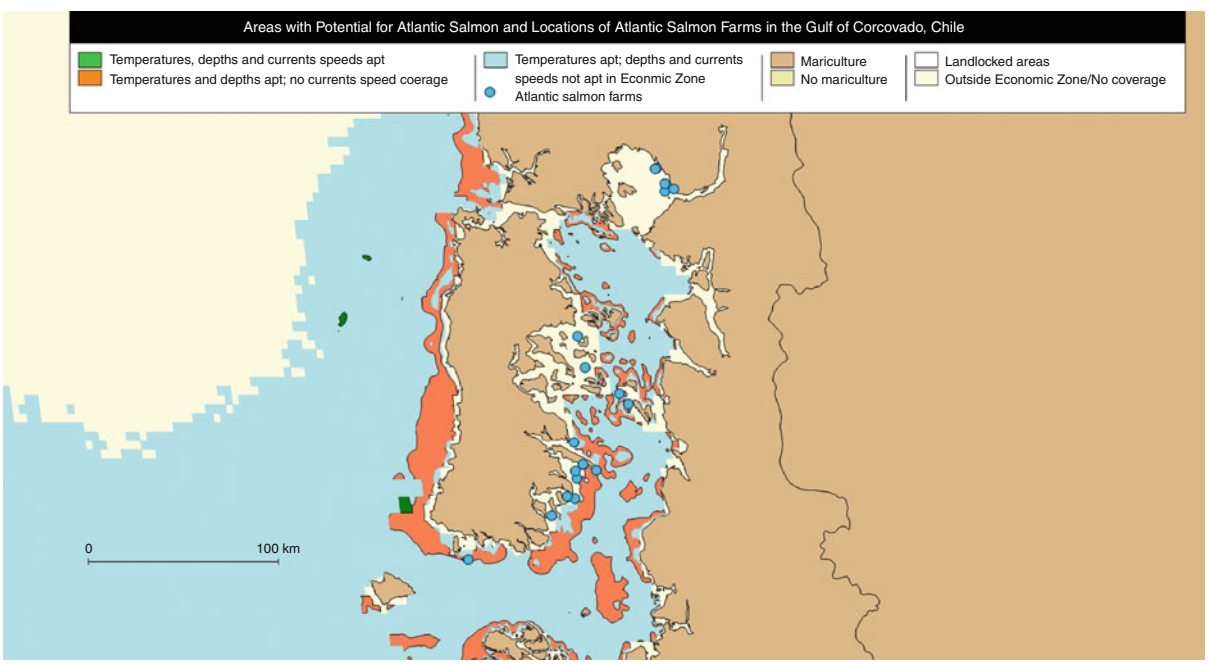
It is easy to see how this kind of coupling can be leveraged, for example, by introducing the effect of current speeds on metabolic requirements, using economic models to analyze the trade-offs between biomass and market price (lower yield, higher product quality), and using models such as FARM to quantify environmental effects, considering factors such as current speed and depth of site, both of which are readily available.

It is clear from this discussion that different modeling products and services are required for different markets. Water managers or planners may for instance be concerned with bay-scale carrying capacity, taking into account the multi-sectorial context of aquaculture, i.e., integrating ecological models with marine spatial planning. Those types of models will help inform decisions on the conservation of wild populations and biodiversity and also provide support for licensing, legislative compliance, and source apportionment for managing pressures.

On the other hand, a farmer will probably be more focused on optimization of production, improvement of feed conversion ratios, and business profits.



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 16
World ocean areas suitable for offshore aquaculture (From [70])



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 17
Areas in Chiloé Island, Chile, with temperature apt for Atlantic salmon mariculture (22–32°C) and depths (25–100 m) and current speeds (10–100 cm/s) apt for submerged cages (From [70])

But increasingly, the environmental footprint of the farm, together with potential positive impacts, such as the role of shellfish in reducing eutrophication symptoms, will be of interest. In the European Union, with legislative requirements for Good Ecological Status by 2015 in all EU water bodies and Good Environmental Status in all marine waters by 2020, some of the modeling approaches and tools described above will undoubtedly be in demand to perform self-assessments and to resolve conflicts.

As models of disease risks evolve, and become better integrated with other types of models, these too will be in demand for siting decisions.

Some of these models will be products, some will be services, and some will be a combination of both. The ways in which those markets develop and the future directions in which the science and technology may evolve will be discussed in the final section of this entry.

Future Directions

Support for an Ecosystem Approach to Aquaculture

Application of the EAA on a worldwide scale requires the harmonization of (1) environmental, (2) social, and (3) multi-sectorial planning objectives [8]. These three principles and their relative weights differ substantially across world regions, and it is not feasible from a social and political standpoint to establish uniform compliance with respect to limits and thresholds.

The only solution is to define appropriate approaches, which within particular world regions define a gradient in relative terms, assessing EAA in terms of the principles stated above. The three principles of EAA can be mapped onto the four pillars of carrying capacity and illustrated as the overlap of these [16]. The importance of each theme represented will vary regionally and will evolve through time based on societal cues.

It is clear that aquaculture in Europe and North America is more closely aligned with EAA, and the present effort in marine spatial planning will only reinforce that. It is also clear that aquaculture production in developed countries will grow little, and that consumer demand in these regions is satisfied in large part through imports (Fig. 18).

In the United States, 84% of aquatic products are presently imported, of which 50% are from aquaculture. This has resulted in a nine-billion-USD seafood trade deficit [82]. Table 2 presents a summary of the main issues that are presently considered in aquaculture carrying capacity and site selection, together with what may constitute future components for assessment. Models play a key role.

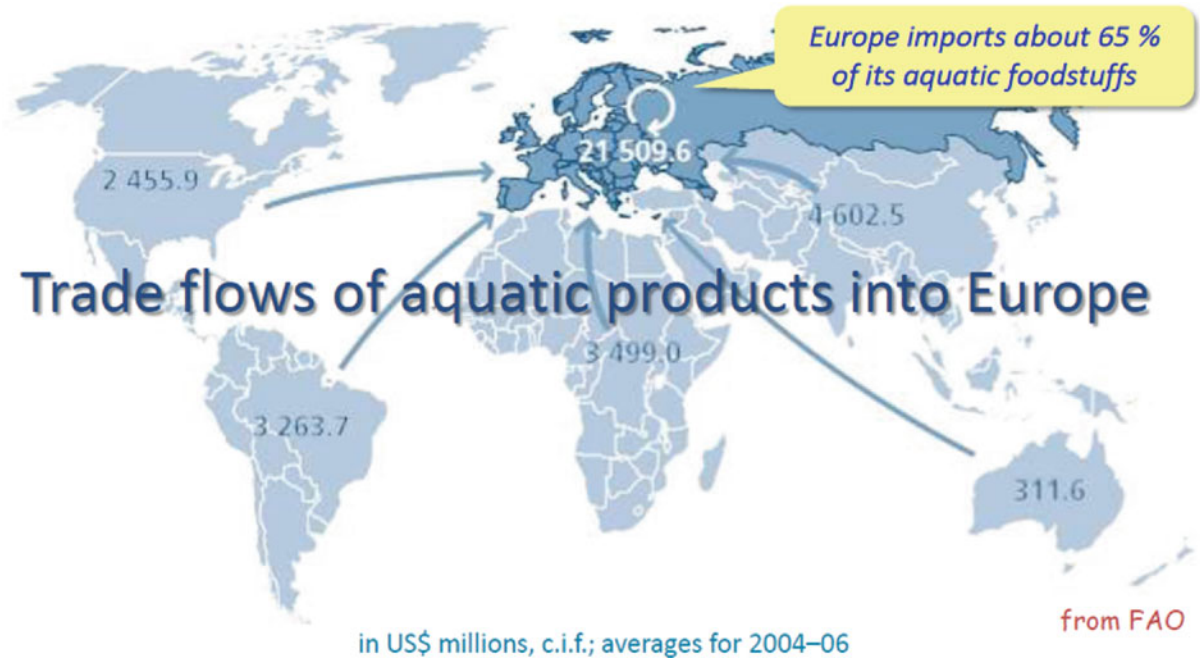
Since developing nations supply the bulk of farmed aquatic products, will continue to do so, and will probably increase their contribution, it is critical that state-of-the-art tools are available to ensure sustainability in the countries where they are most needed.

Which developing countries and which types of culture should be priorities for the application of such models? From an EAA perspective, those that have the highest impact on the environment are the most promising candidates. Kapetsky et al. [73] used FAO production statistics at country environment level (freshwater, brackish water and marine) to estimate the intensity at which aquaculture was practised in each of those environments. A knowledge of the species being cultured can reveal the production systems and their associated impacts in a very general way. The approach outlined above can be refined to focus more closely on modeling needs by considering the potential impacts by species and culture systems in countries in which production data by species are reported.

Dissemination of models can be passive (e.g., packages freely accessible via the Internet) or active (training courses and workshops by region or by country). In both cases, it is essential to establish the technical capacity, level of interest, and financial commitment of the audience and the status of the Internet as a communications and data pipeline for technical support in each country. The focus should not be on developing countries alone because (1) virtual technology specialists in developed countries may be in a position to aid dissemination and (2) companies established in developed countries often have aquaculture operations in developing countries, and could therefore also help to bridge the gap.

Looking into the Crystal Ball

The Thematic Review "Virtual Tools for Aquaculture," presented at the FAO Global Aquaculture Conference



Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 18
Trade flows of aquatic products into Europe [72]

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Table 2 Novel management approaches (Adapted from [10])

Topic	Now	Tomorrow
Feed based (cage, pond)	Site selection based on holding capacity (cages), wastewater minimization (ponds)	Integrated model systems, risks, welfare, and disease. Holistic indicators
		LCA: inefficiencies and eco-labeling
		Mechanistic and statistical models
		Data assimilation models
Shellfish farming	Large areas	Economic sustainability, ecology and economics, disease
	Focus on production and social carrying capacity NIMBY, (Not in my backyard) NIMTO (Not in my term in office)	Coupled GIS expert systems including xenobiotics, HAB, etc.
		Model uncertainties in yield
		Early warning
Integrated multi-trophic aquaculture	Optimize production	Integrated coastal zone management
	Reduce negative externalities	Simulate species combinations
		Full economic assessment
		Combine GIS, remote sensing, and modeling

in 2010 [10], encapsulates much of our thought on the future of models in aquaculture. The final points in this entry are drawn from that review.

The aquaculture industry is going to be affected by many different issues and trends over the coming years, often operating concurrently, sometimes in unexpected ways, and producing changes in the industry that may be very rapid indeed. Mathematical models will play an important role in addressing many of these, particularly in the following areas:

1. Information exchange and networking are going to accelerate the use of virtual technology and decision-making for problem solving to support industry growth. Web-based access to real-time information will further accelerate this growth.
2. Links between industry and research centers will become more effective responding to objective-led demand for virtual technology-driven RTD.
3. Strategic alliances will need to be reinforced or created for the implementation of virtual technology for aquaculture in developing countries, for example, FAO and WorldFish Center are working in many of the same target countries, and this could facilitate the transfer of research outcomes on virtual technology to end users. The same applies to collaborative research with third countries mediated, for example, by the European Union, the United States of America, and Canada.
4. Many virtual technology tools will need to be more production and management oriented. And even if attractive and promising, these tools will have to be adapted to local realities and conditions to really become useful (and used) in the future. This requires a compromise with respect to ease of use, data requirements, and scientific complexity. Many such tools will evolve from service to product, requiring academic developers to accept a loss of control in conditions of application, as a natural trade-off (and inherent risk) of product maturity.

A number of key thematic and technical areas where models for aquaculture are currently incipient, and expected to develop strongly in the next decade or so, have been identified below. In all the examples, such models will contribute to an overall modeling framework, by integrating and complementing existing tools.

Disease Disease in cultivated aquatic species is a major source of concern, yet disease modeling is still relatively underutilized in these systems though its worth has clearly been demonstrated in several studies. However, while epidemiological theory predicts that host density thresholds may be an important part of host–parasite dynamics, clear empirical examples are rare, as much in aquaculture as in other studied agricultural systems. It is suggested that this is not evidence that host thresholds do not exist, rather that statistical difficulties arise with confounding factors or inadequate data. Evidence here is afforded from much better-understood and more data-rich systems, for example, human measles [95–97], where there is compelling evidence on the effects of host density on the likelihood of disease outbreaks taking place within populations.

One of the major limitations in aquatic systems, however, is that the routes by which pathogens are transmitted and their relative contributions to an epidemic are often not understood. If transmission is incorrectly specified, there can be substantial consequences to estimates of N_T. Additional experimental and field data are required to identify and quantify transmission routes for many aquatic animal pathogens, if prediction of the effects of host density on disease incidence is to be made through modeling. To facilitate this, increasing emphasis is required on the use of real-time data acquisition combined with models for real-time analysis and short-term prediction of animal welfare. It is expected that such systems will become cheaper and more generalized, and that some of the indicators and trends will find application at longer timescales, albeit by means of a probabilistic approach.

A further potential limitation of the models currently used is their inability to predict across and link scales from the processes occurring within a tank to the transmission across and then between sites. Methods of investigating disease processes through these metapopulation approaches should be investigated further.

To date, only a few models have been developed to simulate pathogenic infections of shellfish with respect to physiology, for example, Powell et al. (1996) for the American oyster *C. virginica*, but with widespread concerns about relaying, susceptibility, and mortality,

models focusing on a more mechanistic approach will undoubtedly appear over the next decade.

Harmful Algal Blooms This is another area where little predictive capacity exists, except in the short term through the use of operational oceanography, relying on bloom identification and tracking. Management is at present reactive, and modeling of appearance and development of such blooms is in its infancy, due to the lack of an appropriate paradigm. Sensors such as targeted RNA probes (e.g., [74]), integrating handheld devices, or potentially deployed in situ and used in a networked framework will both help in early detection and management and contribute to the understanding of the underlying triggers. Considerable developments are also expected in remote sensing algorithms able to discriminate (at least) between HAB and nontoxic blooms (S. Bernard, personal communication, 2010).

Certification and Traceability The arrays of sensors that can be deployed at the farm scale to enable coupled monitoring and modeling are important for both product certification and traceability. The number, reliability, and accuracy of underwater sensors will increase, and the cost will decrease, both with technological developments and market growth.

Real-time data acquisition and interpretation will make it possible for consumers to visualize the whole "cradle to grave" cycle of an aquaculture product. For instance, a batch of oysters may be "bar coded" to reveal the origin of seed and the entire environmental interaction over the culture period, including metadata and measured data on water quality, HAB events, condition (meat ratio) of the animals, and impact on their environment, for example, in terms of reduction of eutrophication symptoms through the indirect removal of nitrogen and phosphorus, or conversely the addition of particulate organic material due to biodeposition. Such sensors will typically be queried at a sub-hourly frequency, particularly if they are also used for welfare monitoring; this will easily allow importers, health inspectors, or consumers to perform verification and certification, and will provide an important contribution to both food safety and environmental awareness.

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 19
QR code for farmed shellfish, readable on any smart phone

For the farmer, the existence of this kind of integrated coding will also help improve various aspects of culture practice and increase attractiveness of the business model to the key sector of insurance. For the consumer, such data will need to be presented in a comprehensible format, for example, in the form of a few indicators (Fig. 19).

Modeling with Data Scarcity Good data are required to support acceptable model predictions. The acquisition of high-quality data, with appropriate spatial and temporal resolution, is expensive and often beyond the scope of developing countries, except on a fairly limited scale. This, together with an often fragmented approach to the study of interacting ecosystems, in many cases driven by institutional barriers, presents a challenge to the application of models.

Improved mechanisms for data access, particularly for remotely sensed data, together with models that deal with uncertainty and risk, will contribute to conversion of sparse data into more meaningful information – although such an approach may be considered unsuitable in parts of the developed world, in many countries it will be a much better basis for decisions

than the options that are presently used. In addition, it will promote a "virtuous cycle" toward more informed decision support and promote the use of better data and more sophisticated models, as the data become available to drive them.

A wide variety of models will benefit from this development because many models developed and tested for temperate systems need adjustment with respect to parameters, thresholds, and equations, when applied to tropical and subtropical systems.

Information Technology The last 5 years have seen a huge leap in various areas of distributed computing, all of which are expected to develop significantly in the coming years.

Three examples are presented here:

1. Web 2.0 now provides a large diversity of community- and corporation-based resources. Currently, over 7,000 items exist for aquaculture on YouTube – at the time of presentation of the VITAL Thematic Review [10] there were 1,800. For aquaculture modeling, that number has also quadrupled since 2010 and now stands at 75.
2. There is a strong trend toward the development and use of *Software as a Service* (SAAS), deployed on the web and competing with traditional desktop applications. This is incipient in the aquaculture world but can be seen, for example, in the WinShell application (http://longline.co.uk/winshell), which allows users to simulate individual shellfish growth on line. Central to the development of this kind of application are Rich Internet Applications (RIA), which provide a full user experience and are an area of rapid growth [75]
3. Mobile computing is increasingly ubiquitous, and it is now possible to use models on many handheld devices, as illustrated in Fig. 20, which shows a tide prediction model for Mumbai, India. This kind of model might be used to help predict the yield in intertidal culture of bivalve shellfish.

The trend toward the increasing use of such devices, including for various real-time applications in aquaculture management, will increase. In parallel, the stand-alone server is rapidly being replaced by cloud computing, which will tend to make the circulation of data both easier and cheaper. Both elements will contribute to bridge the information divide between richer and poorer nations.

Computer access, literacy, and internet connectivity are significant barriers to entry for the population in the rural areas in developing countries, where aquaculture takes place. Over the coming decade, many aquaculture farmers will have their first contact with the World Wide Web by means of smart phones.

Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of. Figure 20
Tidal prediction for the port of Mumbai using the Oceanus21 smart phone app (http://longline.co.uk/oceanus21). Ports can be automatically selected based on GPS coordinates and phone location

Cell phones are ubiquitous even in remote areas and are much better adapted to local language and alphabet, which constitute other barriers in accessing technology.

The future of aquaculture is promising. It needs to be, given the world population growth expected in the next decades. Simulation models, particularly when used within appropriate frameworks, show enormous potential to inform and guide the future development of aquaculture, toward a world which is more socially responsible, more equitable, and more sustainable.

Acknowledgments

We are grateful to the EU FP7 COEXIST project, and to FAO, for the opportunity to develop many of the ideas presented here. We would like to thank B. Costa-Pierce for the opportunity to contribute to this volume, all our coauthors in the Virtual Tools for Aquaculture FAO report presented at the FAO Global Aquaculture Conference 2010, E.S. Braga and S. Eschrique for data on the Jaguaribe estuary, Ceará, Brazil, and S.B. Bricker and L. Ramos for comments on the draft. We thank H. Mildmay-White for design work on figures.

Bibliography

1. Frei M, Razzak MA, Hossain MM, Oehme M, Dewan S, Becker K (2007) Performance of common carp, *Cyprinus carpio* L. and *Nile tilapia*, *Oreochromis niloticus* (L.) in integrated rice–fish culture in Bangladesh. Aquaculture 262(2–4):250–259
2. Bacher C, Duarte P, Ferreira JG, Héral M, Raillard O (1998) Assessment and comparison of the Marennes–Oléron Bay (France) and Carlingford Lough (Ireland) carrying capacity with ecosystem models. Aquat Ecol 31(4):379–394
3. Smaal AC, Prins TC, Dankers N, Ball B (1998) Minimum requirements for modelling bivalve carrying capacity. Aquat Ecol 31:423–428
4. Ferreira JG, Hawkins AJS, Bricker SB (2007) Management of productivity, environmental effects and profitability of shellfish aquaculture – the farm aquaculture resource management (FARM) model. Aquaculture 264:160–174
5. Jolly CM, Clonts HA (1993) Economics of aquaculture. Food Products, New York, 319 pp
6. Inglis GJ, Hayden BJ, Ross AH (2000) An overview of factors affecting the carrying capacity of coastal embayments for mussel culture. NIWA Client Report CHC00/69, Christchurch, New Zealand
7. McKindsey CW, Thetmeyer H, Landry T, Silvert W (2006) Review of recent carrying capacity models for bivalve culture and recommendations for research and management. Aquaculture 261(2):451–462
8. Soto D, Aguilar-Manjarrez J, Hishamunda N (eds) (2008) Building an ecosystem approach to aquaculture. In: FAO/Universitat de les Illes Balears Expert Workshop. 7–11 May 2007, Palma de Mallorca, Spain. FAO Fisheries and Aquaculture Proceedings, No 14, Rome, FAO, 2008, 221 p
9. Dillon PJ, Rigler FH (1974) A test of a simple nutrient budget model, predicting the phosphorus concentration in lake water. J Fish Res Can 31:1771–1778
10. Ferreira JG, Aguilar-Manjarrez J, Bacher C, Black K, Dong SL, Grant J, Hofmann E, Kapetsky J, Leung PS, Pastres R, Strand O, Zhu CB (2010a) Expert panel presentation V.3. Progressing aquaculture through virtual technology and decision-making tools for novel management. In: Book of abstracts, global conference on aquaculture 2010, 22–25 Sept 2010. FAO/NACA/Thailand Department of Fisheries, Bangkok, Thailand, pp 91–93
11. Nobre AM, Ferreira JG, Nunes JP, Yan X, Bricker S, Corner R, Groom S, Gu H, Hawkins A, Hutson R, Lan D, Lencart e Silva JD, Pascoe P, Telfer T, Zhang X, Zhu M (2010) Assessment of coastal management options by means of multilayered ecosystem models. Estuarine, Coastal Shelf Sci 87:43–62
12. Ferreira JG, Sequeira A, Hawkins AJS, Newton A, Nickell TD, Pastres R, Forte J, Bodoy A, Bricker SB (2009) Analysis of coastal and offshore aquaculture: application of the FARM model to multiple systems and shellfish species. Aquaculture 289:32–41
13. Silva C, Ferreira JG, Bricker SB, DelValls TA, Martín-Díaz ML, Yañez E (2011) Site selection for shellfish aquaculture by means of GIS and farm-scale models, with an emphasis on data-poor environments. Aquaculture 318:444–457
14. Sorgeloos P (2010) Resources, technologies and services for future aquaculture: a needs assessment for sustainable development. In: Book of abstracts, global conference on aquaculture 2010, 22–25 Sept 2010. FAO/NACA/Thailand Department of Fisheries, Bangkok, Thailand, pp 29–31
15. Swaminathan MS (2010) Aquaculture and nutritional security in a warming planet. In: Book of abstracts, global conference on aquaculture 2010, FAO/NACA/Thailand Department of Fisheries, Bangkok, Thailand, 22–25 Sept 2010, pp 4–6
16. Ferreira JG, Ramos L, Costa-Pierce BA (2010b) Key drivers and issues surrounding carrying capacity and site selection, with emphasis on environmental components. In: FAO expert workshop on aquaculture site selection and carrying capacity estimates for inland and coastal waterbodies. Institute of Aquaculture, University of Stirling, Stirling, UK, 6–8 Dec 2010
17. Byron C, Costa-Pierce BA (2010) Carrying capacity tools for use in the implementation of an ecosystems approach to aquaculture. In: FAO expert workshop on aquaculture site selection and carrying capacity estimates for inland and coastal waterbodies. Institute of Aquaculture, University of Stirling, Stirling, UK, 6–8 Dec 2010
18. Byron C, Bengtson D, Costa-Pierce B, Calanni J (2011) Integrating science into management: ecological carrying capacity of bivalve shellfish aquaculture. Marine Policy 35:363–370
19. Ferreira JG, Hawkins AJS, Monteiro P, Moore H, Service M, Pascoe PL, Ramos L, Sequeira A (2008) Integrated assessment

of ecosystem-scale carrying capacity in shellfish growing areas. Aquaculture 275:138–151
20. Filgueira R, Grant J (2009) A box model for ecosystem-level management of mussel culture carrying capacity in a coastal bay. Ecosystems 12:1212–1233
21. Stigebrandt A (2011) Carrying capacity: general principles of model construction. Aquac Res 42:41–50
22. Tett P, Portilla E, Gillibrand P, Inall M (2011) Carrying and assimilative capacities: the acexr-lesv model for sea-loch aquaculture. Aquac Res 42:51–67
23. Grant J, Cranford P, Hargrave B, Carreau M, Schofield B, Armsworthy S, Burdett-Coutts V, Ibarra D (2005) A model of aquaculture biodeposition for multiple estuaries and field validation at blue mussel (*Mytilus edulis*) culture sites in eastern Canada. Can J Fish Aquat Sci 62:1271–1285
24. Tironi A, Marin VH, Campuzano J (2010) A management tool for assessing aquaculture environmental impacts in chilean patagonian fjords: integrating hydrodynamic and pellets dispersion models. Environ Mgmnt 45:953–962
25. Hargrave B (2010) Empirical relationships describing benthic impacts of salmon aquaculture. Aquacult Environ Interact 1:33–46
26. Brigolin D, Pastres R, Nickell TD (2009) Modelling the impact of aquaculture on early diagenetic processes in sea loch sediments. Marine Ecol Prog Ser 388:63–80
27. Findlay R, Watling L (1997) Prediction of benthic impact for salmon net-pens based on the balance of benthic oxygen supply and demand. Mar Ecol Prog Ser 155:147–157
28. Morrisey D, Gibbs M, Pickmere S, Cole R (2000) Predicting impacts and recovery of marine-farm sites in Stewart Island, New Zealand, from the Findlay-Watling model. Aquaculture 185:257–271
29. Probert PK (1984) Disturbance, sediment stability, and trophic structure of soft-bottom communities. J Mar Res 42:893–921
30. Abo K, Yokoyama H (2007) Assimilative capacity of fish farm environments as determined by the benthic oxygen uptake rate: studies using a numerical model. Bull Fish Res Agen 19:79–87
31. Lee J, Choi K, Arega F (2003) Environmental management of marine fish culture in Hong Kong. Mar Pollut Bull 47:202–210
32. Symonds A (2011) A comparison between far-field and near-field dispersion modelling of fish farm particulate wastes. Aquacult Res 42:73–85
33. Skogen MD, Eknes M, Asplin LC, Sandvik AD (2009) Modelling the environmental effects of fish farming in a Norwegian fjord. Aquaculture 298:70–75
34. Chapelle A, Lazure P, Souchu P (2001) Modelling anoxia in the Thau Lagoon(France). Oceanol Acta 25:79–86
35. Nunes JP, Ferreira JG, Bricker SB, O'Loan B, Dabrowski T, Dallaghan B, Hawkins AJS, O'Connor B, O'Carroll T (2011) Towards an ecosystem approach to aquaculture: assessment of sustainable shellfish cultivation at different scales of space, time and complexity. Aquaculture 315:369–383
36. Cromey CJ, Nickell TD, Black KD (2002) DEPOMOD – modelling the deposition and biological effects of waste solids from marine cage farms. Aquaculture 214:211–239
37. Cromey CJ, Nickell TD, Black KD, Provost PG, Griffiths CR (2002) Validation of a fish farm waste resuspension model by use of a particulate tracer discharged from a point source in a coastal environment. Estuaries 25:916–929
38. Funge-Smith SJ, Briggs MRP (1998) Nutrient budgets in intensive shrimp ponds: implications for sustainability. Aquaculture 164:117–133
39. Ferreira JG, Zhu CB, Saurel C, Bricker SB, Vale C (2011) Modelling the role of bivalve shellfish in IMTA. Examples for shrimp ponds and offshore finfish cage culture. In: 103rd Meeting of the National Shellfish Association, Baltimore, March 2011
40. Lindahl O, Hart R, Hernroth B, Kollberg S, Loo L, Olrog L, Rehnstam-Holm A (2005) Improving marine water quality by mussel farming: a profitable solution for Swedish Society. Ambio 34(2):131–138
41. Boyd CE (2009) Advances in technologies and practice for land-based aquaculture systems: ponds for finfish production. In: Burnell G, Allan G (eds) New technologies in aquaculture: improving production efficiency, quality and environmental management, Chapter 32. CRC/Woodhead, Oxford, pp 984–1009
42. Bricker SB, Ferreira JG, Simas T (2003) An integrated methodology for assessment of estuarine trophic status. Ecol Model 169(1):39–60
43. Chopin T, Troell M, Reid G, Knowler D, Robinson S, Neori A, Buschmann A, Pang S, Fang JG (2010) Integrated-multi-trophic aquaculture (IMTA) – a responsible practice providing diversified seafood products while rendering biomitigative services through its extractive components. Aquaculture Europe 2010, Porto, October 2010
44. Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Shpigel M, Yarish C (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361–391
45. Ferreira JG, Zhu CB, Bricker SB (2009b) Eutrophication and aquaculture: la vie en rose? Eutrophication, integrated multi-trophic aquaculture, and top-down control. In: ASLO Aquatic Sciences Meeting, Nice
46. May RL, Anderson RM (1991) Infectious diseases of humans: dynamics and control. Oxford University Press, Oxford [Oxfordshire]. ISBN 0-19-854040-X
47. Begon M, Bennett M, Bowers RG, French NP, Hazel SM, Turner J (2002) A clarification of transmission terms in host-microparasite models: numbers, densities and areas. Epidemiol Inf 129:147–153
48. McCallum H, Barlow N, Hone J (2001) How should pathogen transmission be modelled? Trends Ecol Evol 16:295–300
49. Omori R, Adams B (2011) Disrupting seasonality to control disease outbreaks: the case of koi herpes virus. J Theor Biol 271:159–165

50. Green DM, Gregory A, Munro LA (2009) Small- and large-scale network structure of live fish movements in Scotland. Prev Vet Med 91:261–269
51. Werkman M, Green DM, Murray AG, Turnbull JF (2011) The effectiveness of fallowing strategies in disease control in salmon aquaculture assessed with an SIS model. Prev Vet Med 98:64–73
52. Thrush M, Peeler E (2006) Stochastic simulation of live salmonid movement in England and Wales to predict potential spread of exotic pathogens. Dis Aquat Organ 72:115–123
53. Jonkers ART, Sharkey KJ, Thrush MA, Turnbull JF, Morgan KL (2010) Epidemics and control strategies for diseases of farmed salmonids: a parameter study. Epidemics 2:195–206
54. Taylor NGH, Norman RA, Way K, Peeler EP (2011) Modelling the koi herpesvirus (KHV) epidemic highlights the importance of active surveillance within a national control policy. J Appl Ecol 48:348–355
55. Asplin L, Boxaspen K, Sandvik AD (2004) Modelled distribution of salmon lice in a Norwegian fjord. ICES CM/P:11,12 pp
56. Green DM (2010) A strategic model for epidemic control in aquaculture. Prev Vet Med 94:119–127
57. Gillibrand P, Turrell W (1997) The use of simple models in the regulation of the impact of fish farms on water quality in Scottish sea lochs. Aquaculture 159:33–46
58. Brooks FP (1995) The mythical man-month. Essays on software engineering. Addison-Wesley, London, 322 pp
59. McConnell S (1995) Achieving leaner software. IEEE Software, Nov/Dec 1995, 127–128
60. Uncles RJ, Peterson DH (1996) The long-term salinity field in San Francisco Bay. Cont Shelf Res 16(15):2005–2039
61. Nath SS, Bolte JP, Ross LG, Aguilar-Manjarrez J (2000) Applications of geographical information systems (GIS) for spatial decision support in aquaculture. Aquac Engin 23:233–278
62. Salam MA, Ross LG, Beveridge M (2003) A comparison of development opportunities for crab and shrimp aquaculture in southwestern Bangladesh, using GIS modelling. Aquaculture 220:477–494
63. Ervik A, Agnalt A-L, Hansen AA, Aure J, Bekkvik T, Døskeland I, Hageberg A, Hansen T, Karlsen Ø, Oppedal F, Strand Ø (2008) AkvaVIS–dynamic GIS-tool for sitting of fish farms for new aquaculture species. Environmental quality requirements for new aquaculture species and Atlantic salmon. Tech Rep 10/2008. Institute of Marine Research, Bergen, Norway, in Norwegian, 90 pp
64. Lester SE, Mcleod KL, Tallis H, Ruckelshaus M et al (2010) Science in support of ecosystem-based management for the US west coast and beyond. Biol Conserv 143:576–587
65. Fonseca M, Whitfield PE, Kelly NM, Bell SS (2002) Modeling seagrass landscape pattern and associated ecological attributes. Ecolog Applic 12:218–237
66. Franklin J (2010) Mapping species distributions: spatial inference and prediction (Ecology biodiversity and conservation). Cambridge University Press, Cambridge, 338 pp
67. Watts ME, Ball IR, Stewart RR, Klein CJ, Wilson K, Steinback C, Lourival R, Kircher L, Possingham HP (2009) Marxan with Zones: software for optimal conservation based land- and sea-use zoning. Environ Modell Software 24:1513–1521
68. Ervik A, Kupka-Hansen P, Aure J, Stigebrandt A, Johannessen P, Jahnsen T (1997) Regulating the local environmental impact of intensive marine fish farming. I. The concept of the MOM system (Modelling-ongrowing fish farms-monitoring). Aquaculture 158:85–94
69. Ferreira JG, Andersson HC, Corner RA, Desmit X, Fang Q, de Goede ED, Groom SB, Gu H, Gustafsson BG, Hawkins AJS, Hutson R, Jiao H, Lan D, Lencart-Silva J, Li R, Liu X, Luo Q, Musango JK, Nobre AM, Nunes JP, Pascoe PL, Smits JGC, Stigebrandt A, Telfer TC, de Wit MP, Yan X, Zhang XL, Zhang Z, Zhu MY, Zhu CB, Bricker SB, Xiao Y, Xu S, Nauen CE, Scalet M (2008b) Sustainable options for people, catchment and aquatic resources. In: IMAR (ed) The SPEAR project, an international collaboration on integrated coastal zone management, Institute of Marine Research/European Commission, 180 pp. http://www.biaoqiang.org/documents/SPEAR book.pdf
70. Kapetsky J, Aguilar-Manjarrez J, Jenness J, Ferreira JG (2010a) Spatial analysis for the sustainable development of off-the-coast and offshore aquaculture from a global perspective. FAO Rome, (in press)
71. Stigebrandt A (1999) Turnover of energy and matter by fish – a general model with application to salmon. Fisken and Havet No. 5, Institute of Marine Research, Norway, 26 pp
72. FAO (2009) The state of world fisheries and aquaculture 2008. Food and Agriculture Organization of the United Nations, Rome
73. Kapetsky JM, Aguilar-Manjarrez J, Soto D (2010b) Status and potential of spatial planning tools, decision-making and modelling in implementing the ecosystem approach to aquaculture. In: Aguilar-Manjarrez J, Kapetsky JM, Soto D (eds) The potential of spatial planning tools to support the ecosystem approach to aquaculture. FAO/Rome. Expert Workshop. 19–21 Nov 2008, Rome, Italy. FAO Fisheries and Aquaculture Proceedings, No 17, Rome, FAO. pp 19–176. www.fao.org/docrep/012/i1359e/i1359e00.htm
74. Greenfield DI, Marin R III, Jensen S, Massion E, Roman B, Feldman J, Scholin C (2006) Application of the environmental sample processor (ESP) methodology for quantifying pseudonitzschia australis using ribosomal RNA-targeted probes in sandwich and fluorescent in situ hybridization. Limnol Oceanogr Meth 4:426–435
75. Anderson J, McRee J, Wilson R (2010) Effective UI – the art of building great user experience in software. O'Reilly Media, USA, 294 pp
76. Aguilar MJ, Nath SS (1998) A strategic reassessment of fish farming potential in Africa. Food and Agriculture Organization, Rome

77. Burford MA, Lorenzen K (2004) Modeling nitrogen dynamics in intensive shrimp ponds: the role of sediment remineralization. Aquaculture 229(1–4):129–145
78. De Gaetano P, Doglioli A, Magaldi M, Vassallo P, Fabiano M (2008) Foam, a new simple benthic degradative module for the lamp 3d model: an application to a mediterranean fish farm. Aquacult Res 39:1229–1242
79. European Commission (2000) Directive 2000/60/EC of the European Parliament and of the council of 23 October 2000 establishing a framework for Community actions in the field of water policy. Off J Eur Commun L327:1, 22 Dec 2000
80. European Commission (2008) Directive 2008/56/EC of the European parliament and of the council of 23 october 2000 establishing a framework for community action in the field of marine environmental policy (Marine strategy framework directive). Off J Eur Communs L164:19, 17 June 2008
81. Moo TSY, Undated. Excerpts from Chinese fish culture, by Fan Lee. Contribution No. 459, Chesapeake Biological Laboratory. University of Maryland, Solomons, MD, USA
82. National Oceanic and Atmospheric Administration (2011) NOAA aquaculture program: aquaculture in the U.S. http://aquaculture.noaa.gov/us/
83. United Nations Environment Programme, undated. Remote sensing images of mangrove destruction. http://www.grid.unep.ch/activities/global_change/atlas/images/honduras_s.jpg
84. Krkošek M (2010) Host density thresholds and disease control for fisheries and aquaculture. Aquac Environ Interact 1:21–32
85. Verner-Jeffreys DW, Pond MJ, Peeler EJ, Rimmer G, Oidtmann B, Way K, Mewett J, Jeffrey K, Bateman K, Reese RA, Feist SW (2008) Emergence of coldwater strawberry disease of rainbow trout, Oncorynchus mykiss Walbaum in England and Wales: outbreak investigations and transmission studies. Dis Aquat Org 79:207–218
86. Johansen LH, Jensen I, Mikkelsen H, Bjørn PA, Jansen PA, Bergh Ø (2011) Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway. Aquaculture 315:167–186
87. Dudley RW, Panchange VG, Newell CR (2000) Application of a comprehensive modeling strategy for the management of net-pen aquaculture waste transport. Aquaculture 187: 319–349
88. Food and Agriculture Organization (2011) The state of world fisheries and aquaculture 2010. Fisheries Department, Food and Agriculture Organization of the United Nations, Rome, 218 pp
89. Aguilar-Manjarrez J, Nath SS (1998) A strategic reassessment of fish farming potential in Africa. CIFA Technical Paper 32, FAO, Rome, 196 pp
90. Filgueira R, Grant J, Strand O, Asplin L, Aure J (2010) A simulation model of carrying capacity for mussel culture in a Norwegian fjord: role of induced upwelling. Aquaculture 308:20–27
91. OIE World Organisation for Animal Health (2011) Aquatic animal health code. http://www.oie.int/international-standard-setting/aquatic-code/access-online/
92. Mardones FO, Perez AM, Carpenter TE (2009) Epidemiologic investigation of the re-emergence of infectious salmon anemia virus in Chile. Dis Aquat Org 84:105–114
93. Asche F, Hansen H, Tveteras R, Tveteras S (2010) The salmon disease crisis in Chile. Mar Resour Econ 24:405–411
94. Carvajal P (2009) ISA and the reshaping of Chile's Salmon Industry. Intrafish Industry Report, May. Intrafish, Bergen
95. Keeling MJ, Grenfell BT (1997) Disease extinction and community size: modeling the persistence of measles. Science 275:65–67
96. Grenfell BT, Bjornstad ON, Finkenstadt BF (2002) Dynamics of measles epidemics: scaling noise, determinism, and predictability with the TSIR model. Ecol Monogr 72:185–202
97. Bjørnstad ON, Finkenstädt BF, Grenfell BT (2002) Dynamics of measles epidemics: estimating scaling of transmission rates using a time series sir model. Ecol Monogr 72:169–184

Carrying Capacity for Sustainable Bivalve Aquaculture

Christopher W. McKindsey
Ocean and Environmental Sciences Division, Fisheries and Oceans Canada, Institut Maurice-Lamontagne Fisheries and Oceans Canada, Mont-Joli, Québec, Canada

Article Outline

Glossary

Benthic Pertaining to the sea floor.

Carrying capacity The intensity of a practice that a given environment can sustain indefinitely given the availability of various necessities in that environment and the various pressures on them.

Ecosystem approach to aquaculture (EAA) A strategy to integrate aquaculture into context of the wider ecosystem such that it promotes sustainable development, equity, and resilience of interlinked social and ecological systems.

Ecosystem-based management (EBM) A process that highlights the need to use the best available knowledge about the marine ecosystem to manage marine resources, with an emphasis on maintaining ecosystem service functions.

Integrated coastal zone management (ICZM) A process for managing coastal zones that uses an integrated approach and considers all aspects of the coastal zones, including biological, geographical, and political boundaries, to achieve sustainability.

Model A simplified description, conceptual or mathematical, of a system or process, to assist in calculations, predictions, and understanding.

Pelagic Pertaining to the water column.

Definition of the Subject

Bivalve aquaculture is one of the fastest growing sectors of the food industry, raising concerns about the influence of the activity on the environment. This is true at two levels: First, farmers must make sure that the bivalves that they raise do not deplete resources in a given area to such an extent that bivalve production is decreased. Second, society in general wishes that such activities have an acceptable impact on the environment and are sustainable. There is also intense competition for space and its use in many coastal zones, making siting of many farms contentious. Thus, many organizations have stressed the importance of determining the carrying capacity of different areas for bivalve culture. There are a number of ways that "carrying capacity" may be defined, including physical, production, ecological, and social, and the first three categories are to lesser or greater degrees related to social expectations and standards. A number of methods have been developed to calculate these different categories of carrying capacity for bivalve culture. This entry outlines some advances to estimate the different categories of carrying capacity and suggests a framework that may be followed to encourage the development of a sustainable bivalve aquaculture industry. An emphasis is placed on the latter two categories as these are the ones for which knowledge is the most lacking, are arguably the most complex, and for which advances are the most pressing.

Introduction

Aquaculture is the fastest growing sector of the food industry. Since the 1970s, production in the sector has increased at a rate of about 7% per year and by 2008 accounted for 43% of the total annual fisheries production of 160 million tons and projections by the UN [1] suggest that this production will increase greatly in the future. This increase in production has raised concerns about the impacts of the activity on local environments, (e.g., [2]) and much work has been focused on understanding the interactions between aquaculture and the ecosystem, (e.g., [3, 4]). Although concerns were initially largely directed at the influence of finfish cage culture on the environment (e.g., nutrient

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

loading, disease issues, and interactions due to escapes), concerns have also been raised about the influence of farmed bivalves.

As for aquaculture in general, production of farmed bivalves has increased greatly over the past few years and as of 2008, 11.7 million tons of the total 13.5 million tons of worldwide bivalve captures was from aquaculture production. This includes the culture of over 4 million tons each of clams and oysters and about 1.5 million tons each of scallops and mussels. The greatest concerns relating to farmed bivalves include enhanced localized biodeposition [5], food depletion in the water column due to bivalve grazing [6], alteration of nutrient and oxygen fluxes [7], and the transfer of disease and hitchhiking species [8]. Recent reviews on the environmental interactions associated with bivalve culture have been done for oysters [9], clams [10], and mussels [11].

Increasingly, regulators and other groups are looking to science to determine whether sites have reached their carrying capacity for bivalve culture. Moreover, a number of standards – performance standards, best management practices, certification standards, etc. – have been or are being developed to ensure that bivalve production is being done in a manner that is consistent with a global environmental ethic [12–14]. Some of these codes have been developed by industry groups and are voluntary in nature, some have been developed and are used by public authorities and regulatory agencies, and others are being developed by a variety of organizations (e.g., buyers, nongovernmental organizations, marketing groups) as a means of informing consumers about a product with the goal of influencing farm practices through consumer choice and market forces [15]. In all cases, the idea is that such criteria will encourage the development of a sustainable bivalve aquaculture industry such that the carrying capacity of a given area has not been exceeded and impacts are minimized. Recently, the National Research Council [15] suggested that performance standards based on the carrying capacity of sites be developed and implemented at the ecosystem level. Similarly, the FAO [16] has suggested that an Ecosystem Approach to Aquaculture (EAA) be followed to ensure the "integration of the activity within the wider ecosystem in such a way that it promotes sustainable development, equity, and resilience of interlinked social and ecological systems." Many advances have been made in the estimation of carrying capacity for bivalve culture over the past few years. The broad aim of this entry is thus to update an earlier paper on the subject [17] and to discuss a number of ideas that have been developed in the interim. It focuses on the outgrowing stage of bivalve culture as this stage is arguably the most important in terms of its interactions with the environment. Many other activities related to bivalve culture exist (see list below) and these are discussed by McKindsey et al. [17].

Abbreviated selection of activities related to bivalve culture that may influence the ecological carrying capacity of coastal areas. (Modified from McKindsey et al. [17].)

1. Seed collection
   - (a) Dredging
     - (i) Disturbance of benthic communities, especially the removal of long-living species
     - (ii) Removal of juveniles from wild populations of target species
     - (iii) Collection of non-target species
     - (iv) Suspension of sediments
     - (v) Depletion of food resources for other species
     - (vi) Release of H_2S and reduction of dissolved oxygen in the water due to oxygen-consuming substances, release of nutrients
   - (b) Artificial collectors
     - (i) Removal of juveniles from wild population of target species
     - (ii) Increasing target and non-target species recruitment success
     - (iii) Alteration of the hydrodynamic regimes
     - (iv) Acting as FAD
     - (v) Risk of entanglement for large vertebrates (e.g., marine mammals, sea birds, turtles, sharks)
     - (vi) Foci for nuisance species
   - (c) Hatcheries
     - (i) Chemical pollution (e.g., pharmaceuticals)
     - (ii) Genetic selection
     - (iii) Spread of diseases

(d) Importation
  (i) Introduction of alien species
  (ii) Genetic pollution
  (iii) Spread of diseases
2. Ongrowing
  (a) Effects common to all techniques
    (i) Organic enrichment of seafloor
    (ii) Providing reef-like structures
    (iii) Alteration of hydrodynamic regime (current speed, turbulence)
    (iv) Food web effects: competition with other filter feeders, increasing recycling speed of nutrients, removal of eggs and larvae of fish and benthic organisms
    (v) Spawning: release of mussel larvae
    (vi) Providing food for predators of bivalves
    (vii) Control of predators and pests
  (b) Bottom culture
    (i) Activities to prepare the culture plots, e.g., dredging for predator removal
    (ii) Placement of protective structures (netting, pipes)
    (iii) Removal of associated organisms by dredging and relaying
    (iv) Competition for space with wild benthos organisms
  (c) Artificial structures for suspended and off-bottom culture (trestles, poles, rafts, longlines)
    (i) Acting as artificial reef or FAD (attraction/displacement or enhancement of animals)
    (ii) Risk of entanglement for large vertebrates (e.g., marine mammals, sea birds, turtles, sharks)
    (iii) Foci for nuisance species
3. Harvesting
  (a) Effects common to all techniques
    (i) Removal of biomass, nutrients
    (ii) Removal of filtration capacity
    (iii) Removal of non-target species
    (iv) Competition with predators
  (b) Dredging
    (i) Disturbance of benthic communities, especially removal of long-living species
    (ii) Suspension of sediments
    (iii) Release of H_2S and decrease of dissolved oxygen in the water due to oxygen-consuming substances, release of nutrients
  (c) Collection of off-bottom structures
4. Processing
  (a) Dumping of by-catch
  (b) Relaying near auction houses
  (c) Depurating
  (d) Dumping of shells
  (e) Effluents from processing plant
  (f) Spread of alien species or diseases

As a first step, it is important to understand what is meant by "carrying capacity." A number of definitions have been suggested that consider a variety of physical, biological, and social criteria. In this entry, the four functional categories of "carrying capacity" as outlined by Inglis et al. [18] and McKindsey et al. [17] are used:

1. *Physical carrying capacity* – the total area of marine farms that can be accommodated in the available physical space.
2. *Production carrying capacity* – the stocking density of bivalves at which harvests are maximized.
3. *Ecological carrying capacity* – the stocking or farm density above which unacceptable ecological impacts become apparent.
4. *Social carrying capacity* – the level of farm development above which unacceptable social impacts are manifested.

The specific aims of this entry are to (1) provide an overview and update of different categories of carrying capacity, (2) give a more in-depth review of factors that could be considered for the determination of *ecological* and *social carrying capacity* as these categories are the least developed conceptually and in terms of modeling, (3) outline a decision framework for incorporating all four categories of bivalve carrying capacity into the determination of the overall carrying capacity of a given area for bivalve culture, and (4) outline research to address knowledge gaps for carrying capacity studies.

Functional Categories of "Carrying Capacity"

Physical Carrying Capacity

The physical carrying capacity of a site (embayment, inlet, offshore area, etc.) is simply the geographic area

in which conditions are suitable for the production of a given species using a given method. It is a function of the overlap of the requirements of the species being harvested and the physical resources (e.g., depth, substrate type, salinity, hydrodynamics) in the site. The physical carrying capacity of a site may differ greatly between species and culture methods being employed. For example, an embayment may have an extensive area that is suitable for on-bottom clam culture but relatively little area that is appropriate for suspended oyster culture. Likewise, modification of an area (e.g., by the addition of a species-specific appropriate substrate) may extend the physical carrying capacity of an area.

Traditionally, the physical carrying capacity of sites was determined by building knowledge of an area using hydrographic charts as the base layer, adding other appropriate layers (e.g., temperature and salinity) as available, and analyzing the layers using formal or *ad hoc* Geographic Information Systems (GIS), (e.g., [19, 20]). With the expansion of culture sites in more remote regions where such information may not be readily available, the use of remote sensing methods to estimate various physical parameters (e.g., depth, salinity, temperature, as well as chlorophyll levels and related biological parameters that are needed to estimate production carrying capacity – see further) may be used in combination with GIS to facilitate site selection [21]. Although more of a social factor (see further), an additional advantage of using GIS for aquaculture site selection is that it can be used within a coastal management framework to include other activities so as to avoid user conflicts [22, 23].

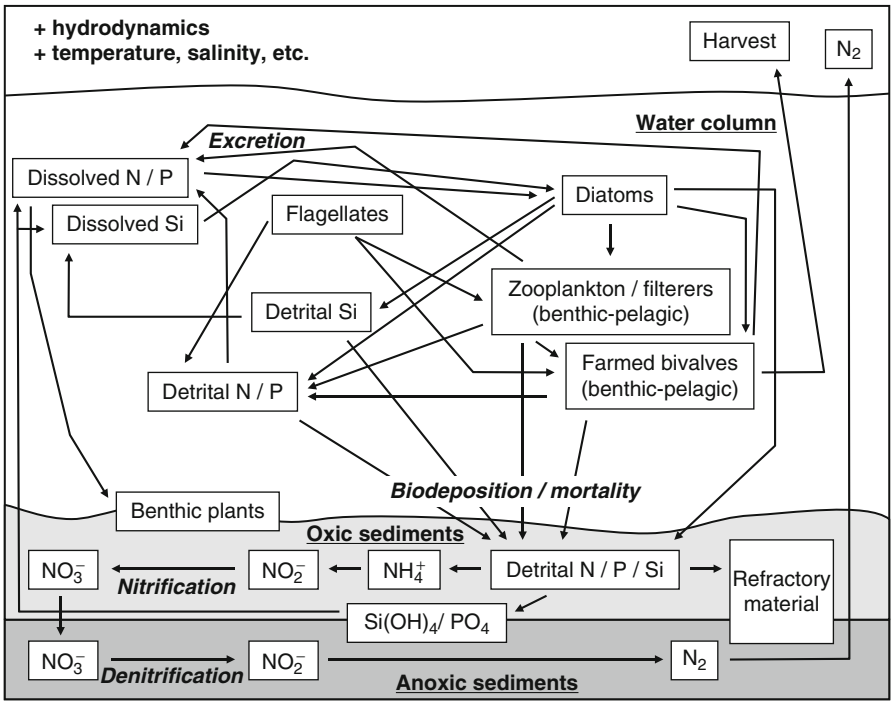
Production Carrying Capacity

The production carrying capacity of a site is the stocking density at which harvests are maximized. Given that trade-offs between bivalve growth rates, market tastes, and economic returns, etc., would likely encourage selection of a specific bivalve size and type (see further), production carrying capacity is often not necessarily the greatest biomass. This category of carrying capacity is what most people think about when they consider "carrying capacity" and is the best studied. The production carrying capacity of a given site is strongly related to hydrodynamic and food regimes and its physical carrying capacity. Indeed, various "habitat suitability models" have been developed that combine both categories of data to predict the best areas for bivalve production, (e.g., [24]). Models to predict production carrying capacity have three main components: (1) a hydrodynamic model that transports food, nutrients, and other wastes; (2) a biogeochemical component that describes processes that influence food production and consumption; and (3) a physiological component that determines the rate of food consumption and growth of the farmed bivalves [25].

Bivalve culture systems are hierarchical, with individual bivalves and their associated fouling organisms nested within culture units (socks, cages and stacks of cages, pearl nets and strings of pearl nets, etc.), these being nested within culture gear (longlines or rafts), which are nested within farms, and so on [26–28]. An understanding of processes operating at each scale as well as the relations between these scales is needed to predict hydrodynamics and how this influences biogeochemical and physiological processes within culture systems and to understand cascading effects on the greater ecosystem.

In its simplest form, the production carrying capacity of a given location is a function of the available food resources and the rate at which they are renewed via in situ production and/or flushing relative to their rate of removal (filtration) by the farmed bivalves. This type of approach has been developed for mussel culture in eastern Canada [29]. However, such models commonly under- or overestimate flushing and a number of authors have suggested that their use be avoided for production carrying capacity studies, (e.g., [30]). Despite this, a number of groups have advocated calculating the ratio of clearance time by farmed bivalves to flushing rate or water residence times (as calculated by such methods) to evaluate the sustainability of sites for bivalve culture [31] and as a rough first step in evaluating whether carrying capacity is potentially exceeded for ecological certification standards [32]. In the latter case, if the ratio between clearance and renewal times is less than unity, then further analyses based on primary productivity are required.

More encompassing approaches to calculating production carrying capacity based on hydrodynamic and mathematical models that include spatially and



Carrying Capacity for Sustainable Bivalve Aquaculture. Figure 1
Conceptual diagram of bivalve aquaculture interactions in coastal ecosystems considered in production carrying capacity estimations. Only main pathways are indicated

temporally explicit feedbacks between the farmed bivalves, phytoplankton, zooplankton, detritus, and physical (e.g., temperature, nutrients, see Fig. 1) parameters are becoming the standard. Models vary greatly in complexity. Although a simple box model for a location may be sufficient for some situations, it is likely unsuitable for embayments with some degree of environmental heterogeneity [33]. The next level of complexity is a simple 1-D hydrodynamic transport model, (e.g., [34]), which may be expanded upon to include vertical transport for suspended culture, (e.g., [35]). More complex models include multiple coupled boxes within an area driven by a 2-D hydrodynamic model (with tidal and meteorological forcings). The most complex models, fully refined 3-D finite-element hydrodynamic models, generally provide a much more accurate estimation of hydrodynamics and may be used to drive the biological models. Historically, computational power has limited the broad application of complex models [36] but this is no longer the case as powerful computers are now readily available [25]. However, increased development time and sampling requirements to validate more complex models make the use of simpler box models a good approximation for understanding the hydrodynamics of an area and slow ecological processes [37] and for carrying capacity modeling [38].

Production carrying capacity models require data on biogeochemical components which include a large number of variables related to the flux of nutrients and primary production and interactions between different food sources for the farmed bivalves. This includes nutrient fluxes due to excretion by the farmed bivalves and associated organisms and organic matter in trapped sediments in culture structures [39–41], nutrient uptake by and production of phytoplankton [42–44], zooplankton and detritus components, and a benthic component that includes biological and chemical processes [45–47]. This latter submodel is of particular importance in shallow areas [33]. Figure 1 gives an example of the types of processes that are included within such models.

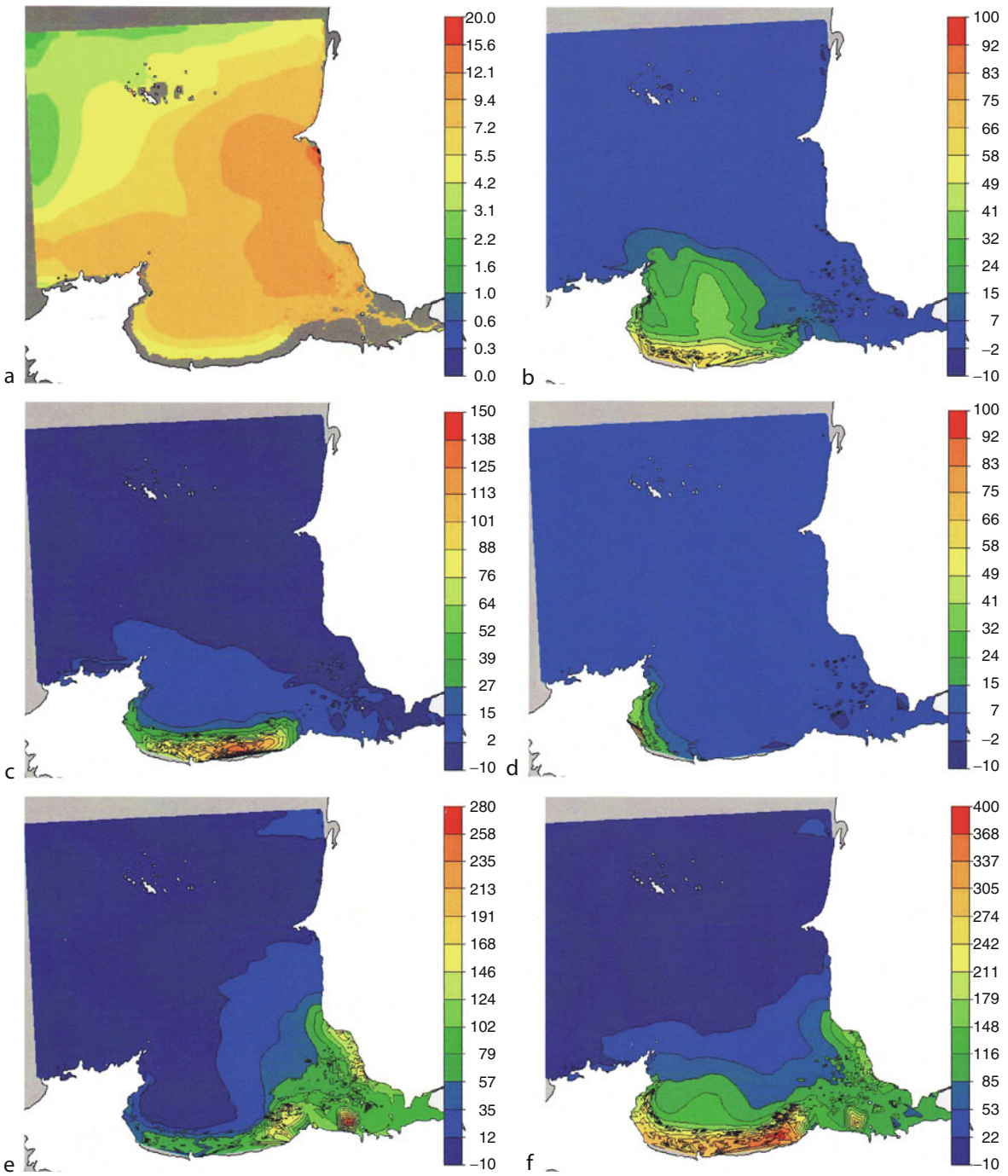
Bivalves graze on and may impact plankton and other suspended components in the water column [48]. These components and environmental factors (e.g., temperature) in turn greatly influence the growth of the farmed bivalves. Models to predict the physiological response and growth of bivalves vary greatly in their complexity. Models may be divided into statistical and more mechanistic approaches that include bioenergetics [49, 50]. Today, many studies use an approach based on dynamic energy budget (DEB) models that were developed by Kooijman, (e.g., [51–53]). In these models, energy budgets are partitioned into core processes: structural volume, reserves, and a reproductive buffer, and forced by food and physical parameters. Examples include work on a variety of bivalves, (e.g., [25, 54–56]) and have been incorporated into general models for bivalve production carrying capacity [35, 57]. A generalized set of DEB models for a few of the most commonly farmed bivalve species has been assembled and is available (http://www.shellsim.com). Production carrying capacity models based on this latter suite of components have been successfully used in a number of studies, (e.g., [35]). Although these types of models are attractive in that they include logical mechanistic processes, their complexity makes them difficult to apply at times and requires substantial ground truthing and simpler models will often yield acceptable results.

Ultimately, the different sub-models must be put together to estimate production carrying capacity. An important advance in modeling production carrying capacity is the development of Graphical User Interface (GUI) modeling environments that allow users to link different sub-models fairly easily through a visual environment rather than with text commands. An example of this is the use of object-oriented modeling environment, as promoted for the FARM model developed by Ferreira et al. [35, 57]. This model links together various components forced by a 1-D hydrodynamic model, or 1-D with horizontal mixing for off-bottom bivalve culture, to estimate production carrying capacity and eutrophication assessment (see further) at the farm-scale based on a limited number of parameters. It may also be used to guide the selection of growing sites, culture layouts, production densities, farmed species, and to maximize production or economic returns. The model has been shown to be useful in a number of locations and for a number of species [57] and is available as a client–server application (www.farmscale.org; www.longline.co.uk/winshell). Simile [58] is an object-based GUI modeling environment that has been used extensively by Grant and colleagues [25, 33, 38, 54, 59]. This environment is well suited to constructing carrying capacity models for bivalve culture because of its inherent ability to represent spatial elements and specify hydrodynamic connections between them and, because of its GUI environment, it is transferable to nonexperts [38].

Models for production carrying capacity usually consider only the farmed bivalves as consumers of plankton. However, in some situations, particularly shallow sites with large densities of natural bivalves, grazing by natural bivalves may also exert a large pressure on plankton communities [60–62]. Thus Sequeira et al. [63] have recently developed a model that includes natural benthic communities as a forcing function on plankton communities and suggest that these populations may significantly reduce production carrying capacity. Similarly, Cugier et al. [64] found that wild native and exotic filter-feeding organism had a greater effect on the control of primary productivity than did the farmed bivalves in Mont Saint Michel Bay, France (Fig. 2). The biomass of filter-feeding fouling organisms, particularly tunicates, on farmed bivalves and infrastructure may be considerable, at times greater than that of the farmed bivalves [65]. Given that they may have similar grazing rates to that of farmed bivalves [66], these should be included within production carrying capacity models where fouling is great. This has been attempted for the Thau Lagoon [67] and the Oosterschelde Estuary [68].

Ecological Carrying Capacity

The definition the "ecological carrying capacity" of a system is greatly dependent on social values as what is considered to be an "unacceptable" impact is dependent on the values of a given society. A society or their representatives must select which components (e.g., species or habitats) of a given area are important and set acceptable limits of change for each. Often, information on specific parameters may not be available and managers may choose to consider components



Carrying Capacity for Sustainable Bivalve Aquaculture. Figure 2

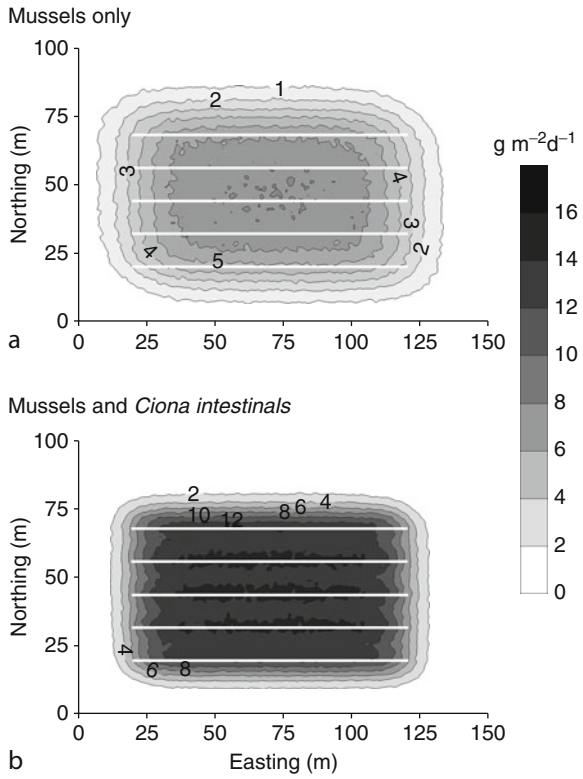
Simulated annual maximum chlorophyll a for the reference case (A – current situation with all filter feeders included) and differences with the reference case (in%) for five scenarios of with different filter feeders removed, (B – without exotic slipper-limpets, C – without farmed mussels, D – without farmed oysters, E – without native filter feeders, F – with no filter feeders in the bay). Because of the wide range of differences between the scenarios, the scale bar is not the same for each sub-figure (From Cugier et al. [64])

for which information is available or more easily obtained. As pointed out by McKindsey et al. [17], this may be a logical choice given that components that have a high societal value are also likely to have been studied.

While production carrying capacity focuses on the farmed bivalves themselves and the organisms that support their production, ecological carrying capacity also includes other organisms and habitats in the ecosystem. There are three main categories of ecological carrying capacity: (1) that related to the pelagic habitat; (2) that related to the benthic habitat; and (3) that which employs ecosystem function approaches. The first category is largely related to plankton depletion due to grazing by farmed bivalves. The second category is related to increased sedimentation within culture sites due to biodeposition by the farmed bivalves. The third category considers both these issues and changes in biomass and energy flow in a system. Ecological carrying capacity is strongly related to both the physical carrying capacity and the production carrying capacity of a site, particularly with respect to that related to the pelagic habitat.

Models to estimate the production carrying capacity of a site contribute to determining the ecological carrying capacity of a site for organisms that are dependent on the delivery of food from the water column. Presumably, if the farmed filter-feeding bivalves impact themselves by overgrazing the available resources, then they are also likely impacting other organisms that feed in a similar manner [69]. Indeed, some recent production carrying capacity models explicitly included benthic filter-feeding organisms [63, 64] and some work has suggested that overgrazing by farmed bivalves may impact other suspension feeders in the surrounding ecosystem [70]. The calculation of carrying capacity for other planktivores in the water column may be similarly evaluated. Sedimentation rates to the bottom are a function of the plankton communities in an area (e.g., [71]). Thus, as has been shown for natural systems with bivalves [72, 73], depletion of the water column via grazing by farmed bivalves may influence sedimentation rates within a given area and thereby benthic communities, potentially enhancing differences between benthic communities within and outside of culture sites [11].

With respect to the benthic habitat, research has largely focused on the impact of increased sedimentation due to biodeposit production by farmed bivalves on infaunal communities [9–11]. Research on the ecological carrying capacity of benthic habitats has likewise been focused on predicting how increased sedimentation due to biodeposit production by farmed bivalves influences infaunal communities. Grant et al. [74] predicted benthic loading rates from biodeposits from farmed mussels (*Mytilus edulis*) at a bay scale as a balance between biodeposit production, as calculated using a simple physiological model, and flushing, as calculated based on a simple tidal prism model (validated using a finite-element 2-D hydrodynamic model and field work). Although there was some variation between modeled and observed biodeposition for both studied bays, the authors suggest that the approach is valuable as a screening tool to develop relative ranking of different systems and identify potential issues. A number of approaches have also been evaluated that couple spatially explicit hydrodynamic-dependent particle tracking models to predict flux of biodeposits from farmed bivalves to the bottom to predict benthic loading footprints and associated benthic community changes. A simple approach is to modify the existing DEPOMOD model, which was developed to this end for finfish cage culture but for which individual bivalve culture structures (e.g., mussel longlines, see Fig. 3) or groups of structures in a system may be modeled [75]. However, this approach has a number of limitations. First, the model assumes a homogenous flow field, an assumption that is unlikely to be true for extensive culture sites. Second, the module for resuspension of sedimented biodeposits is not fully developed and remains one of the greatest sources of uncertainty for biodeposition/impact models [74]. Although resuspension may be negligible in areas with weak currents [75], Giles [76] used a more advanced hydrodynamic model to drive a biodeposit dispersion model and stressed the importance of this aspect for predicting the dispersal of biodeposits in areas with strong currents. A number of recent studies have started to address this issue and supply data to better parameterize this module, (e.g., [75, 77, 78]). Although general relationships exist for benthic organic loading and infaunal community structure [79], predictive dose-dependent relationships between organic loading from bivalve biodeposits and faunal responses are



Carrying Capacity for Sustainable Bivalve Aquaculture. Figure 3

Modeled biodeposition footprint (g m^{-2} d^{-1}) within an idealized suspended mussel farm with (**a**) mussels only and (**b**) mussels fouled with *Ciona intestinalis*. Biodeposition rates were modeled for five backlines (white lines in figure) measuring 100 m each based on currents measured during a 24 h period in St. Marys Bay, eastern Canada (Modified from McKindsey et al. [65])

largely lacking, making predictions difficult [80]. Similarly, there is no universal metric available to describe benthic responses although a large number of indices have been developed, some showing good relationships with aquaculture [81]. As noted above, the biomass of tunicates and other fouling organisms on farmed bivalves and infrastructure may be considerable, and these likely contribute greatly to organic loading to the sea bottom in some cases [65, see Fig. 3] and thus must be considered when determining the biodeposition-related ecological carrying capacity of an area for the benthic habitat.

Tenore et al. [82] used a mass-balance approach to examine the influence of bivalve culture as a part of a coastal ecosystem but specifically to predict ecological carrying capacity. Gibbs [83] used food web analysis to estimate the level of bivalve culture that could develop before it dominated the energy flow in a marine system and impacted fisheries resources. More recent work has used Ecopath with Ecosim [84] to determine the trophic functioning of various areas that include bivalve culture [85–88]. These models differ considerably in their complexity (i.e., number of trophic groups included) and have generally found that bivalve culture promotes short energy pathways with high trophic efficiency and energy cycling. However, the aim of these studies was not specifically to determine the ecological carrying capacities of the studied areas. Research [69] to evaluate the carrying capacity of an area for mussel culture in New Zealand using Ecopath found that the ecological carrying capacity of the area (defined as the level of culture that would not significantly change the major energy fluxes or structure of the food web) was much less than the production carrying capacity (defined as the level of production at which the ecosystem collapses down to a nutrient–phytoplankton–culture–detritus-dominated system) of the area. More recent research in the eastern United States based on the same approach but for coastal lagoons with oysters also found that the stocking density calculated for ecological carrying capacity was less than that calculated for production carrying capacity [89]. Although the original version of Ecopath was limited in its applicability because it could not be used to simulate changes to flows with time, the new version does not assume a steady state. Rather, it bases the parameterization on an assumption of mass balance over an arbitrary period, often a whole year, and biomasses of any given trophic group need not be at equilibrium. A component of Ecopath, Ecosim, accepts time series data for different trophic groups and may be appropriate to evaluate different management (i.e., stocking and harvesting) options. These approaches have some limitations. Perhaps the most important for bivalve culture is that the models are not spatially explicit. Thus, the model may not be used to identify near-field and far-field effects and exchanges between areas are assumed to be instantaneous. This is not logical in

complex coastal areas where most bivalve culture is done. This issue has been addressed to some extent with the development of Ecospace, a dynamic, spatial version of Ecopath that incorporates the key elements of Ecosim [84]. This review found no studies that use either Ecosim or Ecospace to estimate ecological carrying capacity for bivalve culture; information to do this is usually not available and when such spatially explicit temporal data are not available, Ecopath provides a standardized methodology for developing a model [89]. As it is, data on many specific biological variables (life history values, interactions, etc.) are lacking for most systems. If default values are used, these must not be simply accepted without critical evaluation [90]. Even when data is available to guide the determination of variable values, many must be adjusted to make the model balance [69]. Perhaps most importantly, Ecopath uses a largely top-down mass-balance approach and thus poorly represents bottom-up effects [91], a situation that will be problematic for bivalve culture given that it largely impacts nutrients and lower trophic levels.

In sum, most potential measures of ecological carrying capacity consider only a single or a restrained number of ecosystem components [92]. Little research has been directed at understanding the impact of biodeposition (of biodeposits and of farmed bivalves and associated organisms) on the productivity or sustainability of benthic infaunal communities or the communities of larger invertebrates and fishes that may prefer to associate with culture sites to profit from the biodeposition. Likewise, little research has addressed the impact of the modification/addition of physical structure associated with bivalve culture (i.e., both the infrastructure and the farmed product, as well as removal of seagrass, etc.) [11]. The addition of structure may attract and create suitable habitat for a large variety of organisms, such as fish and decapods, but also of fouling organisms that may otherwise not have appropriate habitat in a given area. In contrast, the removal of some features for bivalve culture may repel other organisms. Thus, flexible approaches to evaluating ecological carrying capacity need to be developed to incorporate our evolving understanding of the functioning of marine ecosystems and their interactions with bivalve culture [17, 89].

Social Carrying Capacity

The social carrying capacity of a site is the most complex of all types of carrying capacity to determine. It depends on not only the three above categories of carrying capacity but also on trade-offs between stakeholders to meet the demands of both the population (socioeconomic factors such as traditional fisheries, employment in other sectors, and recreational use) and the environment (protected habitats, species, etc.) [22, 31, 83, 93–95]. Moreover, social issues often inform other categories of carrying capacity (see further in "Decision Framework" section). This category is at the heart of Marine Ecosystem-Based Management (EBM) and Integrated Coastal Zone Management (ICZM) and must be fully developed so that responsible management decisions may be made [96]. That being said, the techniques for determining the social carrying capacity of a site are the least developed of all carrying capacity categories [97].

Despite these limitations, a number of criteria are common to each of the different methods: representativeness, independence, and involvement/buy-in of the groups involved in the process, and transparency [98].

One method that has been developed to manage natural resources is the concept of "limits of acceptable change," or LAC. Although originally developed to define the limit of recreational activities in terrestrial wilderness areas, it may also be applicable to a wider range of natural resource management issues [99, 100]. More recently, it has been applied to bivalve aquaculture in New Zealand [101]. It was recognized that LAC was not a tool to determine the level of bivalve culture that was ecologically sustainable. Rather, it provided an adaptive management framework to prevent significant negative effects due to the activity. Through a collaborative process, LAC provides a framework to identify indicators of change, setting levels of changes of indicators that are acceptable, and identifying management responses that are to be undertaken if these levels are exceeded. The selected indicators must be relevant and practical to measure. Although in the New Zealand example ecological indicators were selected (spatial extent of phytoplankton depletion, also done recently in eastern Canada [25]), the approach could also be used for other types of

indicators derived from other categories of carrying capacity evaluations. The important aspect is that the selected indicators are chosen in a collaborative way [102], including input and discussions with members of the public, environmental managers, scientists, members of the industry, groups with conflicting interests, etc. In addition to the process being representative, it must also be transparent and adaptive so that new information may be easily included in the decision-making process. Indeed, much work has shown that collaborative efforts between all stakeholders are essential to ensuring satisfaction in the consultation process and developing policy [103].

A recent example of this is the evaluation of the ecological carrying capacity of lagoons in Rhode Island, eastern United States, for oyster culture [97]. This involved the development of the Working Group on Aquaculture Regulations (WGAR), which guided and oversaw the development of a mass-balance modeling approach to calculate both the ecological and social carrying capacities of the area for oyster culture. Whereas it is usually left up to the modelers to determine what constitutes an acceptable or an unacceptable impact [97], it was considered that the process would be much more transparent and inclusive by including input from all stakeholders, following the criteria outlined by Soto et al. [104] stating that an ecosystem approach to aquaculture (EAA) should:

1. Be developed in the context of ecosystem functions and services with no degradation of these beyond their resilience capacity
2. Improve human well-being and equity for all relevant stakeholders
3. Be developed in the context of (and integrated to) other relevant sectors

The above strategies to evaluate the social carrying capacity of locations may be considered as social-ecological systems (SESs) where all of the potential stakeholders participate. Walker et al. [105] outline four general steps in the process:

1. Stakeholder-led development of a conceptual model of the system, including its history and preliminary assessments of the drivers of key ecosystem goods and services.
2. Identification of the range of drivers of the system, stakeholder visions for the future, and contrasting possible future policies, to identify a limited set of future scenarios.
3. Identify the resilience of the system by examining the results from i and ii. This is generally done through the development of models of the system's dynamics to identify important attributes that affect resilience.
4. Stakeholder evaluation of the process and outcomes in terms of policy and management implications.

Ostrom [106] recently developed a framework to evaluate the sustainability of management strategies for SESs. It is a nested approach with four first-level core subsystems. In bivalve culture systems, these may include (1) resources systems – a given embayment or other logical management area; (2) resource units – such as phytoplankton biomass or physical space; (3) governance systems – the specific rules related to culture activities that manage resource use; and (4) users – the various groups that use the resource for sustenance, recreation, etc. Each core subsystem is made up of a series of secondary subsystems and interacts with other core subsystems. One of the main findings was that, although many researchers have predicted that users of a system will not self-organize to create a sustainable SES and thus regulation by governments is needed, this prediction was not supported when stakeholders were enabled to discuss management options. Monitoring and enforcement were other key components that determined the success of the studied SESs.

A further method to determine the social carrying capacity of a site is by attaching a monetary value to the different categories of impact due to aquaculture [15]. However, this is a complex undertaking and must include both positive and negative effects of the operations [107]. Moreover, acceptability of different effects is quite variable among locations and groups, further complicating the goal of attaining consensus for different variables.

The use of fuzzy expert systems has also been advocated to determine the social carrying capacity of an area for bivalve culture [17]. This approach has been shown to be useful when data types are disparate and

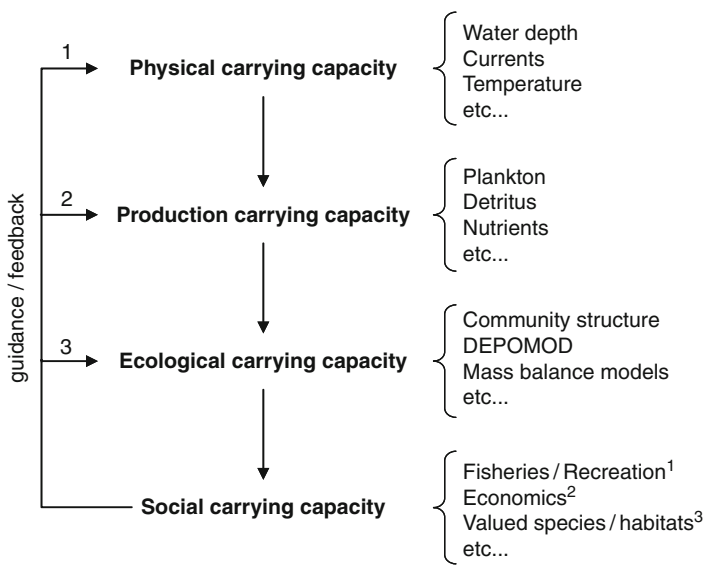
uncertainties are great or simply unknown. In short, instead of stating that a given level of production is acceptable or unacceptable, a given level is treated as being, say, 50% acceptable. It is rather more like dealing with varying shades of gray rather than cases that are black and white. This has the important effect of inducing less conflict between stakeholders on issues that may be contentious.

In sum, methods to determine social carrying capacity for bivalve culture remain poorly developed. In general, few methods have been developed specifically for aquaculture although methods developed for other sectors may be adapted. Analysis of the attempts to use SESs in other systems has shown that some approaches work; such approaches may be looked to for determining social carrying capacity for bivalve culture.

Decision Framework to Determine Carrying Capacity

A hierarchical approach to determine the carrying capacity of an area for bivalve culture is proposed (Fig. 4). The first level, the physical carrying capacity, relates to the available natural conditions and the needs of the operation and bivalves to be cultured. The second level, the production carrying capacity, is a function of the supply of food to the farmed bivalve in an area and is determined using modeling efforts. At the third level, the ecological carrying capacity of an area is estimated using modeling or by following a logical framework to evaluate the range of possible outcomes for production estimates varying between none (and/or the current level) and the maximum calculated production carrying capacity; there is little use to go beyond this point as it is assumed that this level will not be surpassed knowingly. Finally, managers weigh and balance the different scenarios based on the outcomes from each of the preceding categories of carrying capacity and make a decision as to what level of productivity is acceptable – the social carrying capacity.

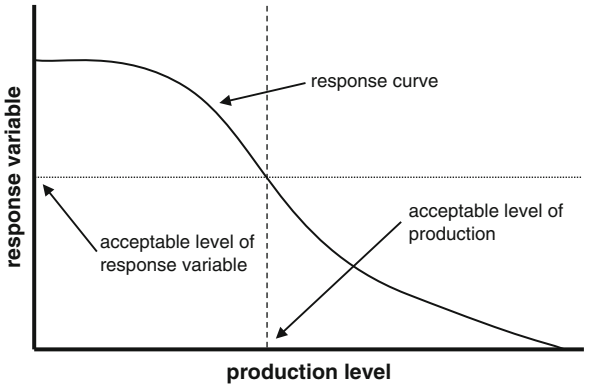
It must be understood that the three first categories of carrying capacity are a function of social values. For example, a given coastal area in which bivalve culture may be done is often valued for myriad competing activities, such as water quality, visual aesthetics,



Carrying Capacity for Sustainable Bivalve Aquaculture. Figure 4
Hierarchical structure to determine carrying capacity of a given area. Note that social carrying capacity provides guidance to choosing pertinent response variables and on establishing limits for these. Superscripts indicate examples of the type of information that informs the selection of response variables for other carrying capacity categories (Modified from McKindsey et al. [17])

competition for limited space, other species, etc. For the first category, physical carrying capacity, competition for physical space may be direct or indirect. For example, direct competition may result as there may be multiple demands to use the same area for different purposes, such as bivalve culture as well as fishing or recreational boating. Indirect competition may arise because of visual aesthetics or the not-in-my-backyard factor where waterfront owners or users do not want their views affected by aquaculture installations. Production carrying capacity is likely rarely maximized because of economic costs related to production. Thus, Nobre et al. [108] developed a modeling approach that considers both ecological and economic aspects and their interactions to maximize profit and EAA as focusing aquaculture management on maximizing output is likely economically inefficient and carries unnecessary ecological risks. In fact, this approach addresses all four categories of carrying capacity with an emphasis on maximizing economic returns. The ecological carrying capacity of an area is also clearly a function of social values as what is valued varies among populations (e.g., specific bird and fish populations, water clarity, eelgrass, or specific rare habitats).

Consultation with stakeholders throughout the process of defining the carrying capacity for a given area will identify the appropriate (given the social values of the population) response variables or indices to be examined [97]. Ideally, the scientists should then be able to select suitable tools from a toolbox (e.g., models, GIS, and comparisons with previous studies) to predict the form of the response curves of the selected response variables to a range of production-level scenarios. It is important to note that although it is the role of scientists to describe the form of the responses to a range of production-level scenarios (see Fig. 5), their role ends once they have done this. It is then up to managers, in consultation with the various stakeholders, to use all available scientific information to inform regulations and policy [17, 97]. Again, this should be done within the context of EBM and ICZM, given that there is likely a paucity of information about a number of factors that are needed to make informed judgments [96]. Thus managers will likely have to rely on instinct, local knowledge, extrapolation from studies done elsewhere, contributions from various stakeholders, etc., to make management and policy decisions. Even faced with an absence of some types of information, this does not remove the logic of the hierarchical nature of the decision framework outlined above. Indeed, it could be argued that this is precisely when the process is most useful and should be followed using all available information – so that an unbiased view of the situation may be formed and thus promoting appropriate management decisions. As pointed out by McKindsey et al. [17], failure to follow the process (e.g., by stating at the outset of the process that certain types of development or developments in certain areas are not permitted) will likely result in otherwise feasible bivalve culture installations not being initiated. It is also contrary to the notion of effective and transparent processes in the establishment of ICZM or EBM.



Carrying Capacity for Sustainable Bivalve Aquaculture. Figure 5

Hypothetical response curve of an environmental variable under the influence of varying levels of bivalve culture production. The dotted line indicates the level of the indicator that has been determined to be acceptable and the dashed line the corresponding level of production (Modified from McKindsey et al. [17])

Knowledge Gaps and Future Directions

This entry concludes with a brief discussion of knowledge gaps and directions for future research to better estimate the carrying capacity of areas for sustainable bivalve culture, except for the estimation of physical carrying capacity for which methods are fairly well developed and are currently used extensively to this end.

While it is generally true that "existing models must be made spatially explicit" [17], this is currently being done. However, models to this end require much information that is often not easily available. So, perhaps a better knowledge gap to address is to determine when such models are needed and when can general models be used.

Similarly to the first point, although temporal variation must be included in models to consider seeding and harvesting activities, some generalized models to this end have already been developed [35, 57, 108, 109]. These should be expanded upon and evaluated in several locations to fully assess their generality.

Far-field impacts for the benthic environment need to be evaluated. For example, is sedimentation outside of culture sites decreased due to grazing and, if so, what is the influence of this on the benthic environment? How does this influence our interpretation of "impacts" on the benthic environment within farm sites?

Work is needed on how nonlinear or unexpected results may be incorporated into carrying capacity models. For example, how can the provision of bivalve culture-related structure in the environment – which may increase the abundance of fouling organisms and thus filtration capacity or attract various other organisms – be included in carrying capacity estimates?

Methods need to be developed to include both "positive" and "negative" effects into the decision-making process. For example, while suspended bivalve culture may increase organic loading locally, it may also mitigate the effects of eutrophication (and models have been developed to assess this, e.g., 35) or increase the abundance of commercially important species locally [11].

Despite the growing prevalence of certification standards and ecolabeling for aquaculture products, it is not clear that such processes are improving conditions in the field [14]. Although such standards are often elaborated within a multi-stakeholder framework, it is not clear under which conditions they are necessary and how the three previous points are considered in their development or application.

Given that many groups promote including the notions of reversibility or resilience in the definition of carrying capacity, (e.g., [16]), surprisingly little work has actually addressed this for the benthic environment [but see 110]. Work on evaluating the resilience of benthic systems to bivalve culture is needed.

Appropriate management tools, such as methods for combining disparate data types, need to be developed to incorporate the information needed to estimate various aspects of carrying capacity to aid in decision making and policy development.

Bibliography

Primary Literature

1. FAO Fisheries and Aquaculture Department (2010) Global datasets, FishStat Plus – Universal software for fishery statistical time series. http://www.fao.org/fishery/statistics/software/fishstat/en. Accessed 6 Feb 2011
2. Black KDE (2001) Environmental impacts of aquaculture. CRC Press, Boca Raton
3. Holmer M, Black K, Duarte CM, Marbà N, Karakassis I (2008) Aquaculture in the ecosystem. Springer, New York
4. Davenport J, Black K, Burnell G, Cross T, Culloty S, Ekarante S, Furness B, Mulcahy M, Thetmeyer H (2003) Aquaculture: the ecological issues. Blackwell, Oxford
5. Mattsson J, Lindén O (1983) Benthic macrofauna succession under mussels, *Mytilus edulis* L. (Bivalvia), cultured on hanging long-lines. Sarsia 68:97–102
6. Troost K, Gelderman E, Kamermans P, Smaal AC, Wolff WJ (2009) Effects of an increasing filter feeder stock on larval abundance in the Oosterschelde estuary (SW Netherlands). J Sea Res 61:153–164
7. Prins TC, Smaal AC, Dame RF (1998) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquat Ecol 31:349–359
8. McKindsey CW, Landry T, O'Beirn FX, Davies IM (2007) Bivalve aquaculture and exotic species: a review of ecological considerations and management issues. J Shellfish Res 26: 281–294
9. Forrest BM, Keeley NB, Hopkins GA, Webb SC, Clement DM (2009) Bivalve aquaculture in estuaries: review and synthesis of oyster cultivation effects. Aquaculture 298:1–15
10. Dumbauld BR, Ruesink JL, Rumrill SS (2009) The ecological role of bivalve shellfish aquaculture in the estuarine environment: a review with application to oyster and clam culture in West Coast (USA) estuaries. Aquaculture 290:196–223
11. McKindsey CW, Archambault P, Callier MD, Olivier F (in press) Influence of suspended and off-bottom mussel culture on the sea bottom and benthic habitats: a review. Can J Zool
12. Young JA, Brugere C, Muir JF (1999) Green grow the fishes-Oh? Environmental attributes in marketing of aquaculture products. Aquacult Econom Manage 3:7–17
13. Lee D (2008) Aquaculture certification. In: Ward T, Phillips B (eds) Seafood ecolabelling: principles and practice. Wiley-Blackwell, Oxford, pp 106–133
14. Tlusty MF (2011) Environmental improvement of seafood through certification and ecolabelling: theory and analysis. Fish Fisher. doi: 10.1111/j.1467-2979.2011.00404.x

15. National Research Council (2009) Ecosystem concepts for sustainable bivalve mariculture. National Academies Press, Washington
16. Soto D, Aguilar-Manjarrez J, Hishamunda N (2008) Building an ecosystem approach to aquaculture. In: FAO/Universitat De Les Illes Balears expert workshop, 7–11 May 2007, Palma De Mallorca, Spain. FAO Fisheries and Aquaculture proceedings, vol 14. Rome
17. McKindsey CW, Thetmeyer H, Landry T, Silvert W (2006) Review of recent carrying capacity models for bivalve culture and recommendations for research and management. Aquaculture 261:451–462
18. Inglis GJ, Hayden BJ, Ross AH (2000) An overview of factors affecting the carrying capacity of coastal embayments for mussel culture. NIWA Client Report CHC00/69, National Institute of Water & Atmospheric Research Ltd, National Institute of Water & Atmospheric Research Ltd, pp vi + 31
19. Nath SS, Bolte JP, Ross LG, Aguilar-Manjarrez J (2000) Applications of geographical information systems (GIS) for spatial decision support in aquaculture. Aquacult Eng 23:233–278
20. Longdill PC, Healy TR, Black KP (2008) An integrated GIS approach for sustainable aquaculture management area site selection. Ocean Coast Manage 51:612–624
21. Meaden GJ, Kapetsky JM (1991) Geographical information systems and remote sensing in inland fisheries and aquaculture. FAO Fish. Tech. paper no. 318, FAO, Rome, pp 262
22. Dempster T, Sanchez-Jerez P (2008) Aquaculture and coastal space management in Europe: an ecological perspective. In: Holmer M, Black K, Duarte CM, Marbà N, Karakassis I (eds) Aquaculture in the ecosystem. Springer, The Netherlands, pp 87–116
23. Goulletquer P, Le Moine O (2003) Shellfish farming and coastal zone management (CZM) development in the Marennes-Oléron Bay and Charentais Sounds (Charente Maritime, France): a review of recent developments. Aquacult Int 10:507–525
24. Vincenzi S, Caramori G, Rossi R, De Leo GA (2007) A comparative analysis of three habitat suitability models for commercial yield estimation of *Tapes philippinarum* in a North Adriatic coastal lagoon (Sacca di Goro, Italy). Mar Pollut Bull 55:579–590
25. Guyondet T, Roy S, Koutitonsky VG, Grant J, Tita G (2010) Integrating multiple spatial scales in the carrying capacity assessment of a coastal ecosystem for bivalve aquaculture. J Sea Res 64:341–359
26. Fréchette M (2008) Hierarchical structure of bivalve culture systems and optimal stocking density. Aquacult Int. doi:10.1007/s10499-008-9198-2
27. Stevens C, Plew D, Hartstein N, Fredriksson D (2008) The physics of open-water shellfish aquaculture. Aquacult Eng 38:145–160
28. Plew DR, Stevens CL, Spigel RH, Hartstein ND (2005) Hydrodynamic implications of large offshore mussel farms. IEEE J Oceanic Eng 30:95–108
29. Carver CEA, Mallet AL (1990) Estimating the carrying capacity of a coastal inlet for mussel culture. Aquaculture 88:39–53
30. Guyondet T, Koutitonsky VG, Roy S (2005) Effects of water renewal estimates on the oyster aquaculture potential of an inshore area. J Mar Syst 58:35–51
31. Gibbs MT (2007) Sustainability performance indicators for suspended bivalve aquaculture activities. Ecol Indic 7:94–107
32. WWF (2009) Bivalve aquaculture dialogue: environmental and social standards for bivalve aquaculture. http://www.worldwildlife.org/what/globalmarkets/aquaculture/WWFBinaryitem13945.pdf. Accessed 3 May 2011, pp 22
33. Grant J, Bacher C, Cranford PJ, Guyondet T, Carreau M (2008) A spatially explicit ecosystem model of seston depletion in dense mussel culture. J Mar Syst 73:155–168
34. Bacher C, Grant J, Hawkins AJS, Fang J, Zhu M, Besnard M (2003) Modelling the effect of food depletion on scallop growth in Sungo Bay (China). Aquat Living Resour 16:10–24
35. Ferreira JG, Hawkins AJS, Bricker SB (2007) Management of productivity, environmental effects and profitability of shellfish aquaculture – the farm aquaculture resource management (FARM) model. Aquaculture 264:160–174
36. Duarte P, Meneses R, Hawkins AJS, Zhu M, Fang J, Grant J (2003) Mathematical modelling to assess the carrying capacity for multi-species culture within coastal waters. Ecol Eng 168:109–143
37. Ménesguen A, Cugier P, Loyer S, Vanhoutte-Brunier A, Hoch T, Guillaud J-F, Gohin F (2007) Two- or three-layered box-models versus fine 3D models for coastal ecological modelling? A comparative study in the English Channel (Western Europe). J Mar Syst 64:47–65
38. Grant J, Curran KJ, Guyondet TL, Tita G, Bacher C, Koutitonsky V, Dowd M (2007) A box model of carrying capacity for suspended mussel aquaculture in Lagune de la Grande-Entrée, Iles-de-la-Madeleine, Québec. Ecol Eng 200:193–206
39. Mazouni N, Gaertner JC, Deslous-Paoli JM (2001) Composition of biofouling communities on suspended oyster cultures: an in situ study of their interactions with the water column. Mar Ecol Prog Ser 214:93–102
40. Richard M, Archambault P, Thouzeau G, Desrosiers G (2006) Influence of suspended mussel lines on the biogeochemical fluxes in adjacent water in the Îles-de-la-Madeleine (Quebec, Canada). Can J Fish Aquat Sci 63:1198–1213
41. Nizzoli D, Bartoli M, Viaroli P (2007) Oxygen and ammonium dynamics during a farming cycle of the bivalve *Tapes philippinarum*. Hydrobiologia 587:25–36
42. Souchu P, Vaquer A, Collos Y, Landrein S, Deslous-Paoli JM, Bertrand Bibent B (2001) Influence of shellfish farming activities on the biogeochemical composition of the water column in Thau lagoon. Mar Ecol Prog Ser 218:141–152
43. Cugier P, Ménesguen A, Guillaud JF (2005) Three-dimensional (3D) ecological modelling of the Bay of Seine (English Channel, France). J Sea Res 54:104–124
44. Cranford PJ, Strain PM, Dowd M, Hargrave BT, Grant J, Archambault MC (2007) Influence of mussel aquaculture on nitrogen dynamics in a nutrient enriched coastal embayment. Mar Ecol Prog Ser 347:61–78

45. Alonso-Pérez F, Ysebaert T, Castro CG (2010) Effects of suspended mussel culture on benthic-pelagic coupling in a coastal upwelling system (Ría de Vigo, NW Iberian Peninsula). J Exp Mar Biol Ecol 382:96–107
46. Nizzoli D, Welsh DT, Fano EA, Viaroli P (2006) Impact of clam and mussel farming on benthic metabolism and nitrogen cycling, with emphasis on nitrate reduction pathways. Mar Ecol Prog Ser 315:151–165
47. Richard M, Archambault P, Thouzeau G, McKindsey CW, Desrosiers G (2007) Influence of suspended scallop cages and mussel lines on pelagic and benthic biogeochemical fluxes in Havre-aux-Maisons Lagoon, Îles-de-la-Madeleine (Quebec, Canada). Can J Fish Aquat Sci 64: 1491–1505
48. Dame RF, Prins TC (1998) Bivalve carrying capacity in coastal ecosystems. Aquat Ecol 31:409–421
49. Beadman HA, Willows RI, Kaiser MJ (2002) Potential applications of mussel modelling. Helgol Mar Res 56:76–85
50. Grant J, Bacher C (1998) Comparative models of mussel bioenergetics and their validation at field culture sites. J Exp Mar Biol Ecol 219:21–44
51. Kooijman SALM (2010) Dynamic energy budget theory for metabolic organisms, 3rd edn. Cambridge University Press, Cambridge
52. Sousa T, Domingos T, Poggiale J-C, Kooijman SALM (2010) Dynamic energy budget theory restores coherence in biology. Philos Trans R Soc B 365:3413–3428
53. Saraiva S, Van Der Meer J, Kooijman SALM, Sousa T (2011) Modelling feeding processes in bivalves: a mechanistic approach. Ecol Eng 222:514–523
54. Filgueira R, Grant J, Strand Ø, Asplin L, Aure J (2010) A simulation model of carrying capacity for mussel culture in a Norwegian fjord: role of induced upwelling. Aquaculture 308:20–27
55. Bacher C, Gangnery A (2006) Use of dynamic energy budget and individual based models to simulate the dynamics of cultivated oyster populations. J Sea Res 56:140–155
56. Rosland R, Strand Ø, Bacher C, Alunno-Bruscia M, Strohmeier T (2009) Applying dynamic energy budget (DEB) theory to predict growth and bio-energetics of blue mussels under low seston conditions. J Sea Res 62:49–61
57. Ferreira JG, Sequeira A, Hawkins AJS, Newton A, Nickell TD, Pastres R, Forte J, Bodoy A, Bricker SB (2009) Analysis of coastal and offshore aquaculture: application of the FARM model to multiple systems and shellfish species. Aquaculture 289:32–41
58. Muetzelfeldt R, Massheder J (2003) The simile visual modelling environment. Eur J Agron 18:345–358
59. Filgueira R, Grant J (2009) A box model for ecosystem-level management of mussel culture carrying capacity in a coastal bay. Ecosystems 12:1222–1233
60. Cloern JE (1982) Does the benthos control phytoplankton biomass in south San Francisco Bay? Mar Ecol Prog Ser 9:191–202
61. Officer CB, Smayda TA, Mann R (1982) Benthic filter feeding: a natural eutrophication control. Mar Ecol Prog Ser 9:203–210
62. Klepper O, van der Tol MWM, Scholten H, Herman PMJ (1994) SMOES: a simulation model for the Oosterschelde ecosystem. Part 1: Description and uncertainty analysis. Hydrobiologia 282–283:437–451
63. Sequeira A, Ferreira JG, Hawkins AJS, Nobre A, Lourenço P, Zhang XL, Yan X, Nickell T (2008) Trade-offs between shellfish aquaculture and benthic biodiversity: a modelling approach for sustainable management. Aquaculture 274: 313–328
64. Cugier P, Struski C, Blanchard M, Mazurié J, Pouvreau S, Olivier F, Trigui JR, Thiébaut E (2010) Assessing the role of benthic filter feeders on phytoplankton production in a shellfish farming site: Mont Saint Michel Bay, France. J Mar Syst 82:21–34
65. McKindsey CW, Lecuona M, Huot M, Weise AM (2009) Biodeposit production and benthic loading by farmed mussels and associated tunicate epifauna in Prince Edward Island. Aquaculture 295:44–51
66. Petersen JK (2007) Ascidian suspension feeding. J Exp Mar Biol Ecol 342:127–137
67. Gangnery A (2003) Etude et modélisation de la dynamique des populations de bivalves en élevage (*Crassostrea gigas* et *Mytilus galloprovincialis*) dans le bassin de Thau (Méditerranée, France) et des ascidies solitaires associées. PhD thesis. Universite de Montpellier II - Sciences et Techniques du Languedoc, Montpellier, France
68. van der Tol MWM, Scholten H (1998) A model analysis on the effect of decreasing nutrient loads on the biomass of benthic suspension feeders in the Oosterschelde ecosystem (SW Netherlands). Aquat Ecol 31:395–408
69. Jiang WM, Gibbs MT (2005) Predicting the carrying capacity of bivalve shellfish culture using a steady, linear food web model. Aquaculture 244:171–185
70. van Stralen MR, Dijkema RD (1994) Mussel culture in a changing environment: the effects of a coastal engineering project on mussel culture (*Mytilus edulis* L.) in the Oosterschelde estuary (SW Netherlands). Hydrobiologia 282(283):359–379
71. Wassmann P (1998) Retention versus export food chains: processes controlling sinking loss from marine pelagic systems. Hydrobiologia 363:29–57
72. Strayer DL, Smith LC, Hunter DC (1998) Effects of the zebra mussel (*Dreissena polymorpha*) invasion on the macrobenthos of the freshwater tidal Hudson River. Can J Zool 76:419–425
73. Connell SD (2005) Assembly and maintenance of subtidal habitat heterogeneity: synergistic effects of light penetration and sedimentation. Mar Ecol Prog Ser 289:53–61
74. Grant J, Cranford P, Hargrave B, Carreau M, Schofield B, Armsworthy S, Burdett-Coutts V, Ibarra D (2005) A model of aquaculture biodeposition for multiple estuaries and field validation at blue mussel (*Mytilus edulis*) culture sites in eastern Canada. Can J Fish Aquat Sci 62:1271–1285
75. Weise AM, Cromey CJ, Callier MD, Archambault P, Chamberlain J, McKindsey CW (2009) Shellfish-DEPOMOD: modelling the biodeposition from suspended shellfish aquaculture and assessing benthic effects. Aquaculture 288:239–253

76. Giles H (2009) Modelling the dispersal of biodeposits from mussel farms: the importance of simulating biodeposit erosion and decay. Aquaculture 291:168–178
77. Giles H, Pilditch CA (2004) Effects of diet on sinking rates and erosion thresholds of mussel *Perna canaliculus* biodeposits. Mar Ecol Prog Ser 282:205–219
78. Walker TR, Grant J (2009) Quantifying erosion rates and stability of bottom sediments at mussel aquaculture sites in Prince Edward Island, Canada. J Mar Syst 75:46–55
79. Pearson TH, Rosenberg R (1978) Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. Oceanogr Mar Biol Annu Rev 16:229–311
80. Callier MD, Richard M, McKindsey CW, Archambault P, Desrosiers G (2009) Responses of benthic macrofauna and biogeochemical fluxes to various levels of mussel biodeposition: an in situ "benthocosm" experiment. Mar Pollut Bull 58:1544–1553
81. Borja Á, Rodríguez JG, Black K, Bodoy A, Emblow C, Fernandes TF, Forte J, Karakassis I, Muxika I, Nickell TD, Papageorgiou N, Pranovi F, Sevastou K, Tomasetti P, Angel D (2009) Assessing the suitability of a range of benthic indices in the evaluation of environmental impact of fin and shellfish aquaculture located in sites across Europe. Aquaculture 293:231–240
82. Tenore KR, Boyer LF, Cal RM, Corral J, Garcia-Fernandez C, Gonzalez N, Gonzalez-Gurriaran E, Hanson RB, Iglesias J, Krom M, Lopez-Jamar E, McClain J, Pamatmat MM, Perez A, Rhoads DC, de Santiago G, Tietjen J, Westrich J, Windom HL (1982) Coastal upwelling in the Rias Bajas, NW Spain: contrasting the benthic regimes of the Rias de Arosa and de Muros. J Mar Res 40:701–772
83. Gibbs MT (2004) Interactions between bivalve shellfish farms and fishery resources. Aquaculture 240:267–296
84. Christensen V, Walters CJ, Pauly D (2005) Ecopath with Ecosim: a user's guide. Fisheries Centre, University of British Columbia, Vancouver
85. Wolff M (1994) A trophic model of Tongoy Bay – a system exposed to suspended scallop culture (Northern Chile). J Exp Mar Biol Ecol 182:149–168
86. Lin HJ, Shao KT, Kuo SR, Hsieh HL, Wong SL, Chen IM, Lo WT, Hung JJ (1999) A trophic model of a sandy barrier lagoon at Chiku in southwestern Taiwan. Estuar Coast Shelf Sci 48:575–588
87. Wolff M, Koch V, Isaac V (2000) A trophic flow model of the Caeté Mangrove Estuary (North Brazil) with considerations for the sustainable use of its resources. Estuar Coast Shelf Sci 50:789–803
88. Brando VE, Ceccarelli R, Libralato S, Ravagnan G (2004) Assessment of environmental management effects in a shallow water basin using mass-balance models. Ecol Eng 172: 213–232
89. Byron C, Link J, Costa-Pierce B, Bengtson D (2011) Modeling ecological carrying capacity of shellfish aquaculture in highly flushed temperate lagoons. Aquaculture 314:87–99
90. Plaganyi EE, Butterworth DS (2004) A critical look at the potential of ECOPATH with ECOSIM to assist in practical fisheries management. S Afr J Mar Sci 26:261–287
91. Steele JH (2009) Assessment of some linear food web methods. J Mar Syst 76:186–194
92. Broekhuizen N, Zeldis J, Stephens SA, Oldman JW, Ross AH, Ren J, James MR (2002) Factors related to the sustainability of shellfish aquaculture operations in the Firth of Thames: a preliminary analysis. NIWA client report EVW02243. NIWA, New Zealand, pp vi + 106
93. Hoagland P, Jin D, Kite-Powell H (2003) The optimal allocation of ocean space: aquaculture and wild-harvest fisheries. Mar Resour Econ 18:129–147
94. Dolmer P, Frandsen RP (2002) Evaluation of the Danish mussel fishery: suggestions for an ecosystem management approach. Helgol Mar Res 56:13–20
95. Stead SM, Burnell G, Goulletquer P (2003) Aquaculture and its role in integrated coastal zone management. Aquac Int 10:447–468
96. Nobre AM, Ferreira JG, Nunes JP, Yan X, Bricker S, Corner R, Groom S, Gu H, Hawkins AJS, Hutson R, Lan D, Lencart e Silva JD, Pascoe P, Telfer T, Zhang X, Zhu M (2010) Assessment of coastal management options by means of multilayered ecosystem models. Estuar Coast Shelf Sci 87:43–62
97. Byron C, Bengtson D, Costa-Pierce B, Calanni J (2011) Integrating science into management: ecological carrying capacity of bivalve shellfish aquaculture. Mar Policy 35:363–370
98. Norton BG (2005) Sustainability: a philosophy of adaptive ecosystem management. University of Chicago Press, Chicago
99. Cole DN, Stankey GH (1997) Historical development of limits of acceptable change: conceptual clarifications and possible extensions. In: McCool SF, Cole DN (eds) Proceedings – limits of acceptable change and related planning processes: progress and future directions: from a workshop held at the University of Montana's Lubrecht Experimental Forest. Gen. Tech. Rep. INT-GTR-371. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Ogden, UT, pp 5–9
100. McCool SF (1996) Limits of acceptable change: a framework for managing national protected areas: experience from the United States. In: Workshop on impact management in marine parks, Maritime Institute of Malaysia, August 13–14. Kuala Lumpur, Malaysia, pp 1–15
101. Zeldis J, Felsing M, Wilson J (2006) Limits of acceptable change: a framework for managing marine farming. Water Atmosphere 14:16–17
102. Krumpe E, McCool SF (1997) Role of public involvement in the limits of acceptable change wilderness planning system. In: McCool SF, Cole DN (eds) Proceedings – limits of acceptable change and related planning processes: progress and future directions: from a workshop held at the University of Montana's Lubrecht Experimental Forest. Gen. Tech. Rep. INT-GTR-371. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Ogden, UT, pp 16–20
103. Weible CM, Pattison A, Sabatier PA (2010) Harnessing expert-based information for learning and the sustainable management of complex socio-ecological systems. Environ Sci Policy 13:522–534

104. Soto D, Aguilar-Manjarrez J, Brugère C, Angel D, Bailey C, Black K, Edwards P, Costa-Pierce B, Chopin T, Deudero S, Freeman S, Hambrey J, Hishamunda N, Knowler D, Silvert W, Marba N, Mathe S, Norambuena R, Simard F, Tett P, Troell M, Wainberg A (2008) Applying an ecosystem-based approach to aquaculture: principles, scales and some management measures. In: Soto D, Aguilar-Manjarrez J, Hishamunda N (eds) Building an ecosystem approach to aquaculture. FAO/ Universitat De Les Illes Balears expert workshop, 7–11 May 2007. Palma De Mallorca, Spain; FAO Fisheries and Aquaculture proceedings, vol 14. Rome, pp 15–35
105. Walker B, Carpenter S, Anderies J, Abel N, Cumming G, Janssen M, Lebel L, Norberg J, Peterson GD, Pritchard R (2003) Resilience management in social-ecological systems: a working hypothesis for a participatory approach. Conserv Ecol 6:1–14
106. Ostrom E (2009) A general framework for analyzing sustainability of social-ecological systems. Science 325:419–422
107. Whitmarsh D, Palmieri MG (2009) Social acceptability of marine aquaculture: the use of survey-based methods for eliciting public and stakeholder preferences. Mar Policy 33:452–457
108. Nobre AM, Musango JK, de Wit MP, Ferreira JG (2009) A dynamic ecological-economic modeling approach for aquaculture management. Ecol Econ 68:3007–3017
109. Ferreira JG, Hawkins AJS, Monteiro P, Moore H, Service M, Pascoe PL, Ramos L, Sequeira A (2008) Integrated assessment of ecosystem-scale carrying capacity in shellfish growing areas. Aquaculture 275:138–151
110. Lu L, Grant J (2008) Recolonization of intertidal infauna in relation to organic deposition at an oyster farm in Atlantic Canada – A field experiment. Estuar Coasts 31:767–775

Books and Reviews

Department of Marine and Natural Resources (2001) Guidelines for landscape and visual impact assessment of marine aquaculture. Department of Marine and Natural Resources, Dublin, pp 1–65

GESAMP (2008) Assessment and communication of environmental risks in coastal aquaculture. GESAMP reports and studies no 76. FAO, Rome, pp 198

Hewitt CL, Campbell ML, Gollasch S (2006). Alien species in aquaculture. Considerations for responsible use. IUCN, Gland and Cambridge, UK, pp viii + 32

Marques JC, Salas F, Patrício J, Teixeira H, Neto JM (2009) Ecological indicators for coastal and estuarine environmental assessment. WIT Press, Southampton

Ward T, Phillips B (2008) Ecolabelling of seafood: the basic concepts. Wiley-Blackwell, Oxford, p 472

WWF (2010) Bivalve aquaculture dialogue standards. http://www.worldwildlife.org/what/globalmarkets/aquaculture/WWFBinaryitem17872.pdf. Accessed 3 May 2011, pp 22

Commercialisation of GM Crops: Comparison of Regulatory Frameworks

Wendy Craig[1], Siva Reddy Vanga[2], Jorge Cabrera Medaglia[3,4]
[1]Biosafety Unit, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy
[2]ICGEB, Plant Biology: Plant Transformation Group, New Delhi, India
[3]Centre for International Sustainable Development Law, Lead Counsel on Biodiversity and Biosafety, Montreal, Canada
[4]University of Costa Rica, San Rafael de Heredia, Costa Rica

Article Outline

Glossary

Cartagena protocol on biosafety (CPB) The Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international agreement which aims to ensure the safe handling, transport, and use of living modified organisms resulting from modern biotechnology that may have adverse effects on biological diversity, taking also into account risks to human health. It was adopted on 29 January 2000 and entered into force on 11 September 2003 (for full text, see http://bch.cbd.int/protocol/text/).

Convention on biological diversity (CBD) The objectives of this Convention are the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources, including by appropriate access to genetic resources and by appropriate transfer of relevant technologies, taking into account all rights over those resources and to technologies, and by appropriate funding (for full text, see http://www.cbd.int/convention/text/).

Convention/protocol/treaty A treaty is an agreement in written form between nation-states (or international agencies, such as the United Nations, that have been given treaty-making capacity by the states that created them) that is intended to establish a relationship governed by International Law. It may be contained in a single instrument or in two or more related instruments such as an exchange of diplomatic notes. Various terms have been used for such an agreement, including treaty, convention, protocol, declaration, charter, covenant, pact, act, statute, exchange of notes, agreement, modus vivendi ("manner of living" or practical compromise), and understanding. The particular designation does not affect the agreement's legal character.

Genetically modified/genetically engineered/transgenic organisms Organisms, such as plants, animals, and microorganisms (with the exception of human beings), in which the genetic material (DNA) has been altered in such a way that does not occur naturally by mating and/or natural recombination (The terms "genetically modified" (GM), "transgenic," "genetically engineered" (GE), and "living modified" (LM) are used in different legal instruments around the world. It is useful (and deliberate) in this document to essentially use them interchangeably).

Living modified organism (LMO) Any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology (according to the CPB).

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Modern biotechnology The application of [1] in vitro nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct introduction of nucleic acid into cells or organelles, or [2] fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection (according to the CPB).

Definition of the Subject

This contribution describes and compares the regulation of GMOs and the underpinning legislative frameworks in selected countries from around the world. It also includes a description of the relevant international agreements related to biosafety and a description of the main characteristics and attributes of a modern biosafety regulatory framework in this area.

Introduction

The rapid development and deployment of modern biotechnology in the last decades have made biosafety a critical issue. Although modern biotechnology has the potential of benefiting agricultural interests in developing countries as well as overall human welfare, living modified organisms (LMOs) resulting from modern biotechnology or genetically modified organisms (GMOs) remain a source of concern with regard to the conservation and sustainable use of biodiversity, as well as to human health. The perceived risks, which relate to the release of GMOs into the environment as well as the placement of GMOs onto the market, have as much to do with social values as scientific concerns. For example, social concerns may require the labeling of genetically modified (GM) food and feed, the mitigation of socioeconomic impacts, and the demonstrated potential for the co-existence of organic, conventional, and GM farming [1].

Global use of GM crops is growing rapidly, increasing approximately 87-fold from 1996 to 2010 to 148 million hectares under cultivation by 15.4 million farmers in 29 different countries [2]. Furthermore, the estimated value of the global GM crop market in 2010 grew to US$11.2 billion, while the value of harvested products was estimated at US$150 billion [2]. Although the majority (52%) of GM crops are still grown in industrialized countries, developing countries are rapidly approaching parity and, due to their high rates of adoption, are soon expected to grow the majority of GM crops [2]. By 2009, the major biotech crops had achieved high levels of market penetration: 77% of soybean, 49% of cotton, 26% of maize, and 21% of oilseed rape grown globally in 2009 were GM varieties [3].

Despite expected high benefit-cost ratios from biotechnology, only a few developing countries, such as Brazil and Argentina, have had high uptake rates (over 20 million hectares) [2] of GM crops, with uptake typically concentrated in crops that are exported to developed country markets. Few others (mainly India and China) have started exploring their own national research capability in biotechnology. In the vast majority of developing countries, both investment in biotechnology research and development and the transfer to farmers of transgenic crops already being marketed have been generally low. This in part reflects a lack of transparent regulatory capacity necessary in dealing with risks associated with biotechnology as well as in addressing the issues of property rights development and protection that are essential to promoting innovative research. This is particularly important because of the high cost of undertaking the initial research and development in biotechnology [4].

As GM technologies are very recent and fast developing, most governments are trying to keep pace by developing regulatory policies that reflect consumer demands and preferences affecting GM agricultural products. Almost all developed countries require products derived from GM sources to be assessed both for their safety as foods and for their environmental impacts. However, there are considerable differences in the approaches taken by different countries. In the United States, analysis and approval mechanisms for GM foods have been subsumed into existing regulations governing the release of new foods, plants, and pesticides, whereas in the European Union, regulation of GM products requires considerable separate scrutiny (see chapter "► Commercialisation of GM Crops: Comparison of Regulatory Frameworks" by Devos et al.). Countries worldwide are in different stages of policy development, with the majority of the developing countries still in the infant stage [4]. In setting up

domestic legislation, developing countries seem to be paying increasing attention to international trade concerns [5].

India along with Argentina, South Africa, and others constitute a group of developing countries that aspire to develop domestic biotechnology through national public R&D and/or by creating incentives for the participation of multinationals as sources of technology. They each have relatively liberal regulations as well as more explicit regulatory institutional arrangements [6]. Those developing countries with well-developed public agricultural research and extension systems (such as India) are well placed to benefit promptly from the new biotechnology by working in partnership or in parallel with private biotechnology and seed companies. Approving investments in those activities by the private sector – and the overall investment climate – will allow the process of adaptation and adoption to move forward. The experiences in India, China, and South Africa all indicate that rapid and widespread adoption is then possible, including by small farmers [7].

The biosafety frameworks of the described countries were selected based, primarily, on considering the following criteria: level of production and commercialization of GMOs, investments made and political commitment with the research and development particularly in the field of GMOs, regional leadership in the adoption of GMOs and in the elaboration and implementation of biosafety legal frameworks, and the existence of a functional biosafety framework in place.

Argentina

Argentina is a major producer of agricultural products and the third largest producer of soybeans. Initially, the harvested area of soybean was 36,000 ha (59,000 mt) in 1970, increasing to 5.98 million hectares in 1995/1996 (12.43 mmt). The country has historically been the earliest and most aggressive adopter of GM crops in Latin America, first planting glyphosate-tolerant soybeans in 1996, which sparked a further expansion of soy production and which is now in excess of 14 million hectares, of which at least 98% is GM [8]. This rate of adoption is far higher, and much faster, than that in the USA, which was the first country to introduce this technology [9]. In addition, Argentina also grows significant quantities of GM corn (Bt and glufosinate ammonium tolerant) and Bt cotton, comprising 40% and 20% of overall production in 2009 for these two crops, respectively [3, 10]. Available estimates place accumulated benefits (extra income which would have not been generated in the absence of the technology) until the year 2001/2002 at approximately US$5.2 billion in the case of soybeans, about US$400 million for Bt maize, and approximately US$40 million for Bt cotton [7]. Argentina is now third only to the United States and Brazil in terms of the area planted with transgenic crops (22.9 million hectares) [2] and is thus a very important player in the international arena. Notably, Argentina signed, but has yet to ratify, the Cartagena Protocol on Biosafety (CPB, hereinafter referred to as "the Protocol") of the Convention of Biological Diversity in May 2000. Argentina is currently undergoing a consultation process, analyzing and debating with all the involved sectors the position the country will take in this respect [8].

Regulatory Oversight in Argentina

Argentina was one of the first countries to establish a system of regulatory oversight for GMOs [11]. It has instituted regulatory measures for the safe development and application of biotechnology in general and GMOs in particular. It chose to develop the policy on biosafety within the context of trade-related issues [12] and as such has policies, procedures, and institutional arrangements to regulate the development, importation, and export of GMO products. The Argentine biosafety system is based on guidelines, not on legislation. Argentina's legislative framework for regulating GMOs is based on the existing agricultural regulatory system (e.g., for plant protection chemicals) supplemented with GM crop-specific regulations established to specify conditions for environmental release or to assess food safety. The non-statutory guidelines include standards for facilities and practices designed to prevent the unintended release of a GMO, conditions of isolation, monitoring field trials, and standards for risk assessment for conducting the environmental release. This approach gives the system flexibility and allows for changes needed to keep up with scientific advances. One disadvantage, however, is that compliance with guidelines is

not legally enforceable; there is no way to prosecute offenders in the rare cases of non-compliance that have occurred [13].

Similar to the USA and Canada (see below), Argentine biosafety regulation follows a product-based approach which results in several agencies, all within the Agriculture Directorate of the Secretariat of Agriculture, Livestock, Fisheries and Food (SAGPyA), mandated to regulate GM crops and products. The National Advisory Commission on Agricultural Biotechnology (CONABIA) is the lead agency in charge of regulating GM crops and was established in 1991 by Resolution 124/91 (later expanded by Resolution 669/93) of the SAGPyA to provide advice and oversee the implementation of biosafety regulations [14]. CONABIA's jurisdiction and procedures were established in Resolutions 656/92, 837/93, and 289/97 (later replaced with 39/03) [13]. Resolution 39/03 is part of the general regulatory system governing the existing agricultural regulations in Argentina related to plant protection (Decree-Law of Agricultural Production Health Defense 6704/66 and its amendments), seed and phytogenetic creations (Seed and Phytogenetic Creations Law 20.247/73 and its regulatory decree), and animal health (Law of Veterinarian Products, and Supervision of Creation and Commercialisation 13.636/49).

CONABIA is a multidisciplinary and interinstitutional organization with advisory duties and comprises representatives from the public sector, academia, and private sector organizations related to agricultural biotechnology. Its main responsibility is to assess the potential environmental impact of the introduction of GMOs in Argentine agriculture [8]. The Commission handles applications for laboratory and greenhouse testing, field trials, and governs the "flexibility status" of release conditions (unconfined release, usually large-scale, for regulatory purposes or off-season seed multiplication) of GM plants [13]. Resolution 60/2007 provides a differentiated treatment for the authorization of the breeding of parental material which contains transgenic events already approved for commercialization. Furthermore, it advises SAGPyA on the issuance of necessary licenses and authorizations for experimentation and/or environmental release of GM microorganisms, as well as GMO-derived or GMO-containing products (although the final decision is made by SAGPyA) [6]. In order to obtain the appropriate marketing license, varieties must also comply with requirements stipulated by the National Service of Health and Agrofood Quality (SENASA) [15]. SENASA's jurisdiction concerning the oversight of GMO-derived food was established in Resolution 289/87, while Resolution 511/98 including Annexes established the food-safety review criteria [13]. The latter was based on FAO and WHO documents, as well as on relevant regulations from Australia, Canada, the EU, Japan, and the US, but has since been replaced by Resolution 412/2002.

A key part of the GMO regulatory process consists of verifying that the commercial approval will not have a negative impact on Argentina's foreign trade. This specific assessment is carried out under Resolution 39/03 by the National Bureau of Agrifood Markets (NBMA), and it includes an analysis of the current status of regulatory systems and public acceptance in the importing countries. The National Seed Institute (ex-INASE) is responsible for ensuring that all the necessary requirements for registration in the National Registry of Cultivars have been established. Ex-INASE plays a further role in the biosafety system by receiving and logging applications for GMO field trials. Applications containing confidential business information are kept secure at INASE's offices. Agency personnel perform field test site inspections, checking for compliance with the biosafety requirements set by CONABIA [13].

During 2001, the SAGPyA actively cooperated with members of the Argentine Congress in drafting a biosafety law. This draft represented a major improvement on the current situation, since it clearly set forth a conceptual framework, as well as issues and instances to be considered as participants in risk analysis procedures. But due to the institutional and economic crisis that broke out on December 2001, the draft was never discussed in Congress, and there is no evidence that it will be discussed in the near future [8].

Commercialization of GM Crops: Argentine Approval Process

When an organization intends to obtain an authorization for commercialization of a GM crop in Argentina, it has to pass reviews by the three regulatory agencies. Briefly, CONABIA should determine that the environmental impact of the large-scale release of the GM crop

will not significantly differ from that of its non-modified counterpart; SENASA's Technical Advisory Committee on the Use of GMOs should determine that the derived foods are safe for human and animal consumption, and the DNMA should determine that the release will not have an undesired impact on the country's international trade [16].

The prerequisite for entering the commercial evaluation process is that authorizations for experimentation and/or release into the environment of the specific GM crop have been previously granted [16]. After at least one release into the environment has been approved and the safety of the GM crop has been demonstrated, the applicant can apply for a "flexibilization" permit which allows future releases by simply providing notification of the location, area, sowing date, and intended harvest date [11]. Flexibility status conditions are granted for the following purposes [13]:

- For providing testing material
- For export
- For off-season seed multiplication (not for use in Argentina)
- For tests, which need to be presented at later stage (e.g., variety registration)
- For pre-commercial seed multiplication for a pending variety registration

The deregulation of field testing conditions is dependent on the results of the biosafety assessment conducted by CONABIA with regard to the criteria laid down in Resolution 131/98. These include the characterization of the GMO (recipient organism, genetic modification, insert, donor organisms, phenotypic characterization, potential environmental interactions of GMO) and the impacts expected from the production of the GM crop at commercial scale (environmental effects and impact on human health) [16]. If SAGPyA, on the recommendation of CONABIA, authorizes "flexibility status" release conditions for the GM crop in question, the applicant only needs to submit information on the area to be sown, the date of sowing, the site of release and the harvest date [16]. The flexibility status of a GM crop allows large-scale planting, but not planting for commercial purpose.

The second step to commercialization is the evaluation of the safety of the GM crop for human consumption and feed. This evaluation is carried out by SENASA under Resolution 412/2002 [17]. In the third step of the commercialization process, the NBMA assesses the impact of the GM crop in question on export market security. It does this by analyzing the status (if any) of the specific event in the destination markets and, as a result, whether the addition of this event to Argentina's export supply might represent a potential barrier to the access to these markets.

After completion of all of the steps mentioned above, CONABIA's Office of Technical Coordination compiles all pertinent information and prepares a "Project of Resolution" on the basis of its own, SENASA's, and DNMA's assessments and submits it to the SAGPyA, which takes the final decision on approval or denial of the commercialization request [13]. Should the GM crop be authorized for open cultivation, it must also be registered in the National Registry of Cultivars, a process overseen by ex-INASE. For those GM crops expressing either herbicide tolerance or insect resistance, they require a pesticide approval from SENASA prior to their commercial use [13].

Canada

Regulatory Oversight in Canada

In line with a similar approach adopted in the United States (see below), the regulatory framework established in Canada is based on the extension of the existing regulations to GMOs [18]. However, in contrast to all other countries, Canada relies on the concept of novelty to trigger regulatory oversight, thereby enabling the regulation of a wider array of novel seeds or food [19], and includes those produced by conventional breeding, mutagenesis, or rDNA techniques. Directive 94–08, first published in 1994, defines these as "plants containing traits not present in plants of the same species already existing as a stable population" [20].

The Canadian Environmental Protection Act (CEPA) of 1988 formally recognizes biotechnology as a manufacturing process for products potentially posing environmental risks, and therefore, the act requires environmental assessments. CEPA, however, embraces a product-based approach to biotechnology, an approach explicitly defined in the 1993 Regulatory Framework for Biotechnology [21]. In accordance

with this framework, policy-makers proceeded to the amendment of a series of regulations (below) contiguous to the laws governing the products of biotechnology. These amendments were mostly aimed at inscribing a trigger (the novelty of the trait) launching the risk evaluation process for the products of biotechnology.

The Canadian approach is based on an agreement between the Canadian Federal agencies in 1993 that was renewed in 1998. The responsibility for regulating plants with novel traits (PNTs), including GM plants, is shared between the Canadian Food Inspection Agency (CFIA) and Health Canada [18]. The CFIA operates under the authority of the Seed Act, the Plant Protection Act, the Feeds Act, the Fertilizer Act, and the Health of Animals Act. It also shares some responsibilities with Environment Canada under the Canadian Environmental Protection Act, and with Health Canada under the Pest Control Products Act, and the Food and Drugs Act. The Canadian Environmental Protection Act is an umbrella legislation intended to serve as a regulatory "safety net" for any biotechnological products not currently regulated by another federal act. The Department of Fisheries and Oceans regulates aquatic organisms under the Fisheries Act.

In 1997, the CFIA took over the risk management of novel seeds and feeds from Agriculture and Agri-food Canada. The agency regulates novel plants following assessment criteria provided by Directive 94–08. In particular, the CFIA is responsible for the regulations and guidelines dealing with cultivating PNTs, assessing their impact on the environment and biodiversity. Canadian authorities state that "all plants derived through genetic engineering have been considered novel, and as such have undergone a full, comprehensive, and rigorous safety assessment prior to release into the environment" [22]. In addition, the agency is also in charge of ensuring livestock feed safety, along with the responsibility for the regulation of seeds, veterinary biologics, and fertilizers. Furthermore, the CFIA develops standards related to the packaging, labeling, and advertising of foods and handles all inspection and enforcement duties [23].

Many GM crops are destined, in whole or in specific parts, for the human food supply system. For this reason, they must not only obtain CFIA approval but must also be assessed by Health Canada. It is within the jurisdiction of Health Canada to regulate GM foods according to the Food and Drugs Act under Division 28 of Part B of Food and Drug Regulations (Novel Food). As with seeds, the trigger for pre-market safety assessments is novelty, and as such, Health Canada treats as novel food all those derived from GMOs "whether it is a micro-organism, a plant or an animal, such that it exhibits characteristics that were not previously observed, no longer fall within the anticipated range or no longer exhibits characteristics that were previously observed, for that plant, animal or micro-organism" [23].

Environment Canada is only responsible for the environmental assessment of GMOs used in industrial processes. Although the Canadian Environmental Act requires environmental risk assessments for GMOs, the responsibility for conducting assessments relevant to novel plants, feeds, and food rests with the CFIA, PMRA, and Health Canada. Notably, CFIA, as the agency most involved in the environmental risk assessment of GMOs, is the responsibility of the agriculture minister whose mandate is to promote agricultural development.

Commercialization of GM Crops: Canadian Approval Process

Before crops with novel traits may be authorized for unconfined release, they must be fully assessed for environmental safety by the CFIA. In meeting the extensive information requirements for these applications to the CFIA, applicants will have conducted experiments at the earlier confined release stage. These experiments are expected to contribute scientifically robust data to address the key criteria of environmental safety assessments. The applicant is required to provide the Plant Biosafety Office (PBO) at the CFIA with extensive high-quality, statistically sound data and/or valid scientific rationale to demonstrate the environmental safety of the PNT. This information initiates a review and decision for authorization of the release. CFIA officials also use pertinent information generated from the Agency's own research, either conducted in-house or contracted out, on specific key environmental areas [18].

The unconfined release assessment by the PBO focuses on real or potential interactions of the PNTs

with the wider agricultural and ecological environment, using "substantial equivalence" as the basis for these assessments. Evaluations consider the unique combination of species and traits, using standard descriptions of each species known as biology documents as a baseline for comparison. If the PBO concludes that there is minimal potential for significant negative environmental impact of the PNT relative to its unmodified counterpart, an unconfined environmental release may be authorized. In some cases, the PBO may authorize an unconfined release with conditions, such as a requirement that the applicant ensures that users of an insect-resistant PNT deploy methods to delay development of resistance among insect populations. Note that for species that may be used for food or feed, developers of PNTs must also seek approvals from Health Canada for human food use and from the CFIA Feed Section for livestock feed use [19]. The starting point for the safety assessment of novel foods is also based on "substantial equivalence," where the novel food is evaluated relative to conventional counterparts that have a history of safe use [23]. Health Canada has 45 days to decide whether the product is safe or to request additional information to pursue the risk analysis, even to the extent of involving experiments [23].

All imported PNTs (or products derived from them) require a prior import permit, being subject to the CFIA regulatory review under the Plant Protection Act and Regulations. Pest risk assessments (PRAs) are conducted by the Plant Health Division in order to evaluate the potential capability of PNTs to pose a pest risk to the agricultural and forestry environment. Those commodities determined not to pose a plant pest risk are now no longer required to have an import permit. Additional exempted commodities include: PNTs with prior approval; PNTs (or products derived from them) that are incapable of sexual or asexual propagation, i.e., have been processed in some way to render them non-viable, such as by grinding or freezing; and plants further developed from exempted PNTs, or considered substantially equivalent to them provided that the intended use is similar, and that the plants do not display any additional novel traits, do not contain novel genetic elements, and have not been subject to inter-specific breeding [24].

China

China has become one of the Asian leaders in biotechnology and has dedicated substantial economic, scientific, and technological resources to R&D. Since the 1980s, ministries and relevant government agencies in China have been investing significantly in agro-biotechnology research and have established more than 150 laboratories, resulting in the largest plant biotechnology capacity outside of North America [25]. The government has allocated research budgets to biosafety and management, and nearly all biotechnology research programs have expanded their scope into biosafety issues [5]. The commitment to sustain biosafety after project closure is demonstrated by its growing budget to support agricultural research in biosafety over the last few years. From an initial budget of slightly over US$ 80,000 in 1999, China now spends about US$ 3 million annually on agricultural biosafety-related activities [10].

A wide variety of crops and traits has passed through China's biosafety system and are now planted commercially, while many others remain at the field trial stage, including many varieties with adaptation-related traits developed by Chinese institutes and companies [26]. In contrast to the other countries described in this chapter, most of these crops have been developed predominantly by public sector laboratories in China. The biosafety regulatory system in China has also reviewed a large number of applications since it was formally set up in the late 1990s. The government received 1,044 applications for field trials or commercial release, and 777 of these were approved [27, 28]. These applications predominantly covered 60 crops, as well as several animals and a large number of microorganisms [28]. Varieties of cotton, tomatoes, phytase maize, insect-resistant rice, and sweet and chili peppers have all been approved for commercial planting [3].

Regulatory Oversight in China

China has adopted a policy that promotes research and development of biotechnology, while at the same time, retaining control over research in genetic engineering. In the early 1990s, China had already implemented a very pragmatic approach to GM crop regulation. Regulations were basically product-based with special attention given to the economic interest of a given

application. By 1993, China had already established its first biosafety regulation, namely the "Safety Administration Regulation on Genetic Engineering" issued by the Ministry of Science and Technology (MOST). This instrument required relevant ministries to draft and issue corresponding biosafety regulations on biological engineering (i.e., the Ministry of Agriculture (MOA) for agriculture and the Ministry of Public Health for food safety) and established general principles, safety categories, risk assessment and risk management procedures, application and approval mechanisms, and legal responsibilities [29]. It was followed in 1996 with the "Safety Administration Implementation Regulation on Agricultural Biological Genetic Engineering" by the MOA [12]. This was an explicit regulatory regime for the risk assessment and management of agricultural products of genetic engineering. Labeling was not part of this regulation, nor was any restriction imposed on imports or exports of GM products. The regulation did control GMOs for research and commercial production, as well as establishing the National Agricultural GMO Biosafety Committee (Biosafety Committee) to provide the MOA with expert advice on biosafety regulations.

Criticism of GM crops on environmental, food-safety, and ethical grounds, however, led to some significant changes in the Chinese legal framework on agro-biotechnology [5]. In 2001, the State Council decreed a new and general rule on biosafety, with the aim of protecting human, animal, and plant health and the environment. This new "Regulations on Safety of Agricultural Genetically Modified Organisms" replaced the 1993 Regulation issued by MOST. The 2001 regulations provide the MOA with overall national authority to oversee the use of GM crops, whereas the 31 provincial biosafety management offices are responsible for the supervision and administration of biosafety in their respective areas [30]. The 2001 regulations meet the generally accepted risk assessment procedures outlined in the relevant international instruments and also stipulate a comparative risk assessment approach, in which a GM crop is compared with the corresponding non-transgenic crop for environmental/ecological safety and food safety.

To implement this Regulation, the MOA issued three implementation regulations including Implementation Regulations on Safety Assessment of Agricultural Genetically Modified Organisms, which provided the legal basis and technical guidelines in GM crops risk assessment in China [31]. These new regulations primarily concerned Biosafety Evaluation, Import Safety, and Labeling and included several important changes to existing procedures and details of regulatory responsibilities after commercialization. The changes included an extra pre-production trial stage prior to commercial approval, new processing regulations for GM products, labeling requirements for marketing, new export and import regulations for GMOs and GMO products, and local and provincial-level GMO monitoring guidelines [29]. Specifically, the Regulation on Biosafety Evaluation establishes procedures for handling applications for GM cultivation and sets up an advisory body, the National Biosafety Committee (NBC), and a decision-making body, the Office of Agricultural Genetic Engineering Biosafety Administration (OGEBA), under the MOA to handle applications. Applicants must provide information on risk assessment, and GMOs are classified into four classes depending on their potential danger to human and animal health and to the environment. The Regulation establishes the requirements that should be met to obtain authorization to import GMOs and will vary according to the intended purposes of the imports, i.e., research, release into the environment, or processing. In response to representations from GM-producing countries, China agreed to allow trade to continue as normal until the new Regulation on Safety of Imports entered into force on 20 April 2004 [32, 34]. The Ministry of Public Health (MPH) is responsible for food-safety management of biotechnology products (processed products based on GMOs) and promulgated its first regulation on GMO food safety in April 2002, to take effect after July 2002.

China's policy on GM regulation is now under the responsibility of an agency which was established by the State Council, the name of which has been variously reported as either the Joint Monitoring and Management Commission [12] or the Allied Ministerial Meeting [29]. It has a multi-stakeholder membership comprising the highest representatives from ministries like Agriculture, Health, Commerce, Science and Technology, the National Development and Reform Commission, the National Inspection and Quarantine Agency, and the State Environmental Protection

Administration. It is responsible for the coordination of key issues related to the biosafety of agricultural GMOs, the examination and approval of the applications for GMO commercialization, determining the list of GMOs for labeling, and establishing import or export policies for agricultural GMOs and their products. In addition, under the new regulation, foreign investment in biotechnology has been prohibited [5].

The NBC remains the major player in the process of biosafety management. Currently, the NBC is composed of 56 members who come from different administrative departments, academic institutions, etc. They are experts in biological research, production, processing, inspection and quarantine, public health, and environmental protection with respect to agricultural GMOs [31]. The committee meets twice each year to evaluate all biosafety assessment applications related to experimental research, field trials, environmental release, pre-production trials, and commercialization of agricultural GMOs. It makes recommendations to the OGEBA based on the results of its biosafety assessments. OGEBA is responsible for the final approval of decisions, as well as handling routine work and daily operations [29]. In 2005, all 31 provinces in China established biosafety management offices. These biosafety management offices collect local statistics on and monitor the performance of research and commercialization of agricultural biotechnology in their provinces and assess and approve (or disapprove) all applications of GM-related research, field trials, and commercialization in their provinces. Only those cases that are approved by provincial biosafety management offices are submitted to the NBC for further assessment [29].

In May 2007, the National Development and Reform Commission in China announced that it had approved the establishment of a National Biosafety Research Centre. To be completed by 2009, the Centre will manage agricultural and biological-related issues. It is to house several research departments, including laboratories for high-risk plant pathogens, insects, and plants, as well as units for agriculture-related information analysis and quarantine facilities. The Centre will be supervised by the Plant Protection Institute of the Chinese Academy of Agricultural Sciences.

In 2009, a major development in China was the commercialization of transgenic Bt rice, which reached pre-production trials but was still pending final commercialization approval for several years. Influenced in part by opposition to the technology in Europe and to some extent Japan, the final approval of many crops stalled at the level of China's inter-ministerial committee even as research and field trials continued apace. The discovery by Greenpeace that some transgenic seed was planted by farmers without authorization caused international debates about China's biosafety system and may have contributed to regulatory approval delays [33].

Commercialization of GM Crops: Chinese Approval System

If the product is for cultivation, applications must be authorized before the first import of a specific GMO can take place and must be accompanied by a safety assessment carried out in the country of origin of the GM material [5]. For permanent approval of each imported GM product, compulsory field trials are carried out in China in order to re-assess safety within the Chinese context [35]. Generally, the practice in China is to use a comparative risk assessment approach, in which the transgenic crop is compared with the corresponding non-transgenic crop in ecological risk assessment and hazard identification in transgenic foods [36, 37]. Although there are no official guidelines in China for risk assessment on food derived from transgenic crops, the assessments carried out so far on nutrition, toxicity, and allergenicity generally followed the relevant Codex principles and guidelines [31].

In China, agricultural GMOs also need to satisfy the procedures governing the release of new seed varieties. These procedures are governed by the Seed Law in China. Only agricultural GMOs that have previously obtained a biosafety certificate are eligible to be classified as a new seed variety in accordance with the Seed Law and relevant regulations. After the GMO has passed seed variety testing and received the permission for production, it is eligible to enter into the chain of production and marketing [31].

India

In India, a wide variety of crops have been field trialed, but most have not yet been commercialized. The first approval for the commercial production of any GM crop in India occurred in March 2002 when the Indian

competent authority approved three varieties of *Bacillus thuringiensis* (Bt) cotton (MECH 12, MECH 162, and MECH 184 expressing the *cry1Ac* gene for insect resistance) amid widespread protests by anti-GM activists. This was followed by a significant increase in the availability of Bt cotton (currently 809 hybrid varieties) [38] better suited to Indian cultivation. By 2003, more than 34 genes were being tested in a wide variety of crops, including cotton, rice, mustard, maize, potatoes, eggplant, tomatoes, pigeon pea, and cabbage [29]. Most of the varieties initially introduced included insect-resistant cotton varieties as well as some crops modified with herbicide tolerance. Several Bt crop varieties have passed through the field trial stage and have received approval for commercial planting. The primary technology used in India originated in the Monsanto Company, which partnered with Maharashtra Hybrids Company (MAHYCO) to develop transgenic hybrid Bt cotton for sale in India. Once some Bt varieties had been approved for commercial planting, it was discovered that 800,000 ha of unapproved BT cotton had been planted, weakening confidence in the biosafety system [29]. The latest GM crop awaiting regulatory approval for commercial release is Bt brinjal (known also as aubergine or eggplant), which received positive assessments by the regulatory bodies based on years of field trials [3] but was refused authorization by the Environment Minister at the last stage of the commercialization process.

Regulatory Oversight in India

With the signing of the CPB in 2001, India became committed to introducing structures and procedures commensurate with the conditions laid down in the CPB agreement – one of the main guiding principles for India when dealing with products derived from agricultural biotechnology. This commitment provided India with the incentive to strengthen its biosafety capacity and have relevant institutional mechanisms at hand to enable the proficient dealing of GMOs. As the CPB places due importance to national legislations, provided it is developed in accordance with the former, the existing domestic policy on GMOs was required to be fine-tuned and amended wherever necessary. The goal of the Indian regulatory system is therefore to ensure that their GM crops pose no major risk to food safety, environmental safety, or agricultural production and that there are no adverse economic impacts on farmers [29]. As such, the Government of India has adopted a policy of careful assessment of the benefits and risks of GMOs at various stages of their development and field release to ensure biosafety [39].

The existing regulatory framework takes the form of rules and guidelines and is based upon three specific provisions of the Environment Protection Act of 1986 (EPA). These are sections 6, 8, and 25. While Section 6 of the Act empowers the Central Government to make rules on procedures, safeguards, prohibition, and restrictions for handling of hazardous substances, Section 8 of the Act prohibits a person from handling hazardous substances, except in accordance with procedures and after complying with safeguards. Section 25 of the EPA empowers the Central Government to lay down rules regarding procedures and safeguards for handling hazardous substances. Thus, the biosafety rules in India are statutory in nature as they originate from the EPA. These provisions of the EPA led to the adoption of the 1989 Rules for the Manufacture, Use, Import, Export and Storage of Hazardous Micro organisms Genetically Engineered Organisms or Cells ("1989 Rules") [39, 40].

In 1994, the Department of Biotechnology revised its earlier guidelines of 1990, entitled "Revised Guidelines for Safety in Biotechnology." These revised guidelines aimed at regulating large-scale production and the deliberate release of GMOs, plants, animals, and products into the environment and shipment and importation of GMOs for laboratory research [6]. By 2002, an array of legislation likely to impact biosafety regulations had come into existence. This included the National Biodiversity Act 2002 (NBA) and the Protection of Plant Varieties and Farmers' Rights Act 2001 (PPVFR), the latter of which derived from a broad-based consultation with a view to incorporate a form of farmers' rights into the national plant variety rights legislation. The biosafety rules have since been supplemented by the Biotechnology Safety Guidelines issued by the Department of Biotechnology (DBT). These guidelines have been issued in pursuance of Rule 4[2] of the Biosafety Rules, which require manuals of guidelines to be brought out by the Review Committee on Genetic Manipulation [40].

Therefore, the Indian biosafety regulatory framework, comprising the 1989 Rules and the 1990, 1994, and 1998 DBT guidelines, covers the entire spectrum of activities relating to GMOs. This includes "research involving GMOs, as well as genetic transformations of green plants, recombinant DNA (rDNA) technology in vaccine development, and large-scale production and deliberate/accidental release into the environment of organisms, plants, animals and products derived from rDNA technology." Production facilities such as distilleries and tanneries that use GMOs are also covered. In India, the risk assessment and regulatory approval for releases of GMOs and GM products are mandatory. The concept of "biosafety" used in the regulations is a broad one, covering the health safety of humans and livestock, environmental safety (ecology and biodiversity), and economic impact. The first two safety aspects dominate the regulations, while economic impact is given less prominence.

Two nodal agencies, the Ministry of Environment and Forests (MoEF) and the DBT at the Ministry of Science and Technology, are responsible for the implementation of the regulations [39]. The life cycle of a GM product features four domains, pre-research, research, release, and post-release, and is characterized by the presence of six competent authorities [41, 42]. The Recombinant DNA Advisory Committee (RDAC) is in the pre-research domain as it triggers research through its initial approval mechanisms. The Review Committee on Genetic Manipulation (RCGM) resides in the DBT and functions in the research domain, closely monitoring the process of research and experimental releases. It requests food biosafety, environmental impact, and agronomic data from applicants who wish to do research or conduct field trials and will give permits to import GM material for research. Pursuant to Rule 4 [2] of the 1989 "Rules," the RCGM is also required to produce manuals of guidelines. The RCGM is primarily made up of scientists (including agricultural scientists) and can request people with specialized knowledge to review cases. It has a Monitoring cum Evaluation Committee (MEC) that monitors limited and large-scale field trials of GM crops and is primarily made up of agricultural scientists. Commercial production of GM crops, large-scale field trials of GM crops, and the imports of GM commercial products and GM-derived products (e.g., foodstuffs, ingredients in foodstuffs, and additives including processing aids containing or consisting of GMOs) come under the authority of the Genetic Engineering Approval Committee (GEAC) at the MoEF. The committee members are primarily bureaucrats representing different ministries, and they draw on the scientific expertise of each ministry. Additional to these national committees are the State Biotechnology Coordination Committee (SBCC) and the District Level Biotechnology Committee (DLC), who, along with the MEC, basically occupy the post-release domain, although they also contribute to the research domain activities through data provisioning to the RCGM. Completing the regulatory apparatus are the Institutional Biosafety Committees (IBSC) which undertake the monitoring and implementation of safeguards at the R&D sites, under the close supervision of the RCGM, the SBCC, and the DLC. IBSCs must be established in any public or private institute using rDNA in their research and comprise scientists from their respective institutes and a member from the DBT. There are more than 230 IBCs in India, of which 70 deal with agricultural biotechnology. They can approve contained research at institutes unless the research uses a particularly hazardous gene or technique which will require specific approval from the RCGM [29]. In general, these authorities are vested with non-overlapping responsibilities [39, 43].

Under the Constitution of India, it is not the central Government of India but the state governments that exercise formal authority over agriculture. Thus, while the national government may take the initiative in the policy arena and formulate policies concerning agricultural biotechnology and GM crops (in R&D as well as commercialization), as well as being where the decision-making process resides, the agreement and active cooperation of state governments are indispensable for their implementation [44].

The multitude of rules and regulations underline the complexities involved in biosafety as it cuts across ministries and agencies and does not merely govern environmental issues. Most of these regulations deal with GMOs in seclusion without referring to a common agency or secretariat to deal with the risks that are associated with the organism [46], resulting in the biosafety regulations being subjected to criticism both by industry and civil society groups. While

industry associations consider these regulations as affecting their growth, civil society groups consider biosafety regulations as not being strong enough to check the introduction of potentially harmful biotechnology products. Since 2004, there have been serious discussions in India on re-engineering the structure of biosafety regulations. The primary objective of the exercise is to cut down red tape and ensure greater transparency in decision-making. Calls have come for the replacement of the present regulatory system (with its dispersed, unclear, and confusing mandates; responsibilities; and powers) by a new, single, integrated, and professionally led authority, the National Biotechnology Regulatory Authority (NBRA), with a comprehensive mandate and a wide range of responsibilities, with the power to implement the regulatory regime with speed and efficiency [40, 44] and to help the assessment of risks and benefits associated with GM crops in a credible and transparent manner [47]. In May 2007, it was announced that the NBRA will be fully functional in 2 years time and will be administered by the DBT to expedite the application of biotechnology in the agriculture, veterinary, and medicine sectors [48].

The current amendments or changes that have favored the industry relate to changes in the 1998 revised guidelines for research in transgenic plants, whereupon a relaxation was permitted regarding the concept of deliberate release. This amendment, by conferring powers to the RCGM to permit limited conduct of field trials in multi-locations, was at variance with the 1989 Rules that prohibited deliberate or unintentional release for experimental purposes, except where the GEAC approved it as a special case. The distinction between small-scale and large-scale releases brought about by the changed guidelines was unusual and was designed to ensure the control of the DBT and the RCGM over initial field testing of transgenic crops. An amendment was made by the DBT in September 1999 conferring rights to the RCGM to approve small experimental field trials for research, limited to a total area of 20 acres in multi-locations with any one location not exceeding 1 acre. Through this amendment, the DBT removed small experimental trials for research from the deliberate release clause of the 1989 Rules [40].

The changes that have been made to accommodate civil society concerns are basically twofold. The first relates to the formation of the MEC by the DBT in 1998 in order to closely and objectively monitor private sector biosafety data and through the mandatory involvement of state-level agricultural university scientists. The second change, which was induced by the Bt cotton controversy in India, has been the introduction of allergenicity tests of transgenic seeds, leaves, and vegetables on rodents, rabbits, guinea pigs, and goats in the 1998 version of the Biotechnology Safety Guidelines [42]. This precautionary step is viewed by the industry as having contributed to the delay in the regulatory approval for Bt cotton [40]. Additional regulations have recently been added to the PPVFR Act of 2001, requiring applicants to provide relevant GEAC clearances and approvals for registering transgenic varieties, as well as an affidavit stating that the "Terminator" Technology or the Genetic Use Restriction Technology is not involved [52]. Notably, the decision-making circle does not include the participation of industry, civil society, or consumer groups. While the 1989 Rules explicitly say that the RCGM, the GEAC, the SBCCs, and the DLCs may co-opt other members/experts as necessary, they neither include nor exclude representatives of NGOs and the private sector. In practice, however, these non-governmental stakeholders have been excluded [44]. However, following the "Terminator" controversy, the National Bureau of Plant Genetic Resources is now mandated by the government of India to develop probes to detect the presence of terminator genes in imported material, highlighting how the force of public opinion can still shape biosafety rules in India.

To keep up with the rapid pace of developments in plant biotechnology, especially GMOs, the Indian regulatory system revised its existing guidelines in 2008. These were to provide greater clarity on data requirements and include: Standard Operating Procedures (SOPs) for Confined Field Trials of Regulated, Genetically Engineered (GE) Plants [49], Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants [50], and Protocols for Food and Feed Safety Assessment of GE crops [51]. Guidelines for Institutional Biosafety Committees (IBSCs) are also currently under review.

While the overall regulatory system remains unchanged, a notable difference is the classification of all GM field trials into two categories, based on size.

The RCGM, operating in the DBT, is now the regulatory authority for Biosafety Research Level I (BRLI) trials. BRLI trials are limited in size to no more than 1 acre (0.4 ha) per location and a maximum cumulative total of 20 acres (8.1 ha) for all locations for each plant species/construct combination (e.g., one or more events originating from the transformation of a plant species with the same genetic construct), per applicant, per crop season. The GEAC, operating in the MoEF, is now the regulatory authority for Biosafety Research Level II (BRLII) trials. BRLII trials are limited in size to no more than 2.5 acres (1 ha) per location, and the number of locations is decided on a case-by-case basis for each plant species/construct combination, per applicant, per crop season.

Members of the MEC, SBCCs, and DLCs and monitoring teams of SAUs have the authority to inspect and monitor confined field trials at the time of planting, during the growing and harvesting season, and during the period of post-harvest land-use restriction for compliance with the terms and conditions of authorization.

Commercialization of GM Crops: Indian Approval Process

The approval process in India begins with the submission of an application regarding a new LMO event with potential benefits over the conventional variety/hybrid in terms of economic benefit to the farmer and/or the environment. The developer is required to follow a set procedure that involves providing all the necessary information specified by the regulatory body. Such an application is reviewed by the RCGM which, in the first instance, may recommend various limited contained or open field trials to be undertaken in order to generate specific biosafety data which may be lacking in the original submission. Once the full dossier of information from experiments undertaken under confined conditions is submitted, the RCGM will then ascertain as to whether the LMO presents any immediate adverse effects, either to humans, animals, and the environment (including the likely impact of large-scale cultivation on biodiversity). If considered as presenting minimal risk, the RCGM may permit large-scale open field trials to be conducted to generate data concerned with the agronomic performance of the LMO. Once more, possible adverse impacts on the environment, including on non-target organisms, are evaluated. The unconfined, open field trials are conducted either by the applicant or by the ICAR, involving their institutes/State Agriculture Universities, and are monitored by the MEC. The MEC reports their observations directly to the RCGM and the GEAC. Based on the biosafety data and the field performance, the RCGM may recommend the case to the GEAC for further evaluation. The GEAC will consider all the data provided and may ask the company to furnish additional data or repeat the trials in multi-locations during the next season. Based on the overall recorded benefits, the GEAC can approve the commercialization of the GM crop for a limited period and in a specified geographical zone. Data collected during this period will form the basis of the review undertaken by the GEAC for any extension or expansion to the set conditions of commercialization. Any adverse impact on human, animals, and environment derived from such large-scale cultivation is required to be immediately notified by any individual or organization directly to the GEAC.

All commercial authorizations are for a limited period, requiring renewal after the expiry period. Furthermore, approval is conditional upon the observing and collecting of relevant information on the risks, if any, arising from the commercial use of the GMOs and products thereof [42]. A key addition in the 1998 guidelines is the requirement to generate data on comparative economic benefits of a modified plant. Thus, the 1998 guidelines call for a demonstration that a transgenic crop is both "environmentally safe and economically viable." An agronomic evaluation of the transgenic crop to determine economic advantage to farmers is seen as an integral component of the transgenic crop approval process, along with the biosafety evaluation [42]. Thus, when the government granted permission for large-scale field testing of transgenic cotton in India in July 2000 (the first crop to receive such approval), mandatory data to be generated by the applicant included "cost of transgenic seed, projected demand, and the area to be covered under transgenic cotton cultivation" [45].

The Philippines

The Philippines' National Agenda for Sustainable Development for the twenty-first Century (PA 21)

provides the policy framework of the country's strategy for sustainable development. In 2001, the Presidential Policy Statement on Modern Biotechnology [53] reiterated the government policy of promoting the safe and responsible use of modern biotechnology and its products as one of several means to achieve and sustain food security, equitable access to health services, sustainable and safe environment, and industry development. The Philippine government formally funds biotechnology as part of the annual budget for agricultural R&D through legislation [54]. In December 2002, the Philippines became the first country in Asia to commercialize a GM crop for use as food, feed, or for processing [55] when the Department of Agriculture (DA) approved the Bt corn MON810 for import and propagation [56]. By summer 2005, the Philippines had approved 19 different LMOs for direct use as food, feed, or propagation [57], while in 2010, this had increased to 53 (when stacked events are also included).

Regulatory Oversight in the Philippines

The Filipino biotechnology regulatory system was established as a result of the recommendations from the scientists asking the national government to formulate a national policy on biosafety and create a technical body to draft guidelines to ensure that experiments using GMOs do not pose unacceptable risks to human health and the environment [12]. The Philippines has a body of policies aimed at regulating the development, importation, transfer, and use of GMOs. The first guidelines for biosafety were promulgated in October 1990 as Executive Order (EO) 430, which established the National Committee on Biosafety of the Philippines (NCBP). The NCBP was established to "oversee the compliance with policies and guidelines in all institutions, public or private, as well as to coordinate with the appropriate national bodies that have regulatory powers over any violations" [58]. At present, the NCBP is concerned with contained use (confined laboratory and greenhouse experiments on the regulated article), and its primary function is to identify and evaluate potential hazards involved in initiating genetic modification experiments and recommend measures to minimize risks [59]. Additional guidelines were developed and published by the NCBP and the Department of Science and Technology in 1991, 1998, and 2002 before being incorporated into the National Biosafety Framework, which was finalized in 2004 and issued as EO 514 in April 2006 [5, 12]. The rules and regulations for the import and release into the environment of plants and plant products derived from the use of modern biotechnology are set out in Administrative Order no. 8, Series of 2002 of the Philippine Department of Agriculture (AO 8) [35, 55]. The following year, the DA issued Memorandum Circular No. 8, which outlined the import requirements for biotech products. This was quickly followed by the issuance of Memorandum Circulars 11 and 12 in August 2003, which further clarified the import rules for biotech products for direct use as seed, food, feed, or for further processing [57]. Importers of GM plants for contained use, field testing, and propagation (or commercial planting), as well as GMOs for direct use as food, feed, and processing, are required to obtain an approval permit [15, 55] which stipulates that the performance of the GM crop and its effect on the environment as well as human and animal health have been positively assessed [11].

The decision-making process is vested in multiple national competent authorities (NCAs) after consultation with other agencies and/or with a multi-stakeholder advisory body. The Department of Agriculture (DA) is the competent national authority responsible for biosafety decisions concerning plants and plant products derived from modern biotechnology, fisheries and other aquatic resources, domesticated animals and biological products used for animal husbandry or veterinary purposes, and biological agents used for biocontrol. It is the government institution with mandatory responsibility for GM crop field releases and commercialization. Likewise, the Department of Science and Technology is responsible for research and development, the Department of Health for pharmaceuticals which are not addressed by other relevant international agreements or organizations, and the Department of Environment and Natural Resources concerning regulated organisms intended for bioremediation, the improvement of forest genetic resources, and wildlife genetic resources, and applications of modern biotechnology with potential impact on the conservation and sustainable use of biodiversity [12].

Commercialization of GM Crops: Filipino Approval Process

In consultation with the NCBP, the Bureau of Plant and Industry (BPI) of the DA is responsible for the granting of permits issued under AO 8 and are classified according to the intended use of the regulated article: (a) importation for contained use, (b) field testing, (c) release for propagation, and (d) importation for direct use as food or feed or for processing [61]. Applications for import must be accompanied by a certificate from the competent authority in the country of origin stating that the regulated article has been locally approved and a notification in accordance with international obligations. Local applications must be supported with the necessary technical and scientific dossiers, a public information sheet (PIS), and a certificate from the BPI stating that the regulated article has undergone satisfactory field testing in the Philippines. The AO 8 policy for commercial propagation stipulates that no regulated article will be released unless (a) field testing showed that the GM crop will not pose any significant risks to the environment, (b) food and/or feed safety studies showed that the GM crop will not pose any significant risks to human and animal health, and (c) a permit for propagation has been secured from the DA. If the GM crop has transgene-derived pesticidal properties, it must also be duly registered with the Fertilizer and Pesticide Authority (FPA) [61]. Upon receipt of an application, the BPI has 5 days to process and evaluate all of the documentation to ensure that it is sufficient in form and substance. If it is found to be defective, then the applicant is given a 60-day grace period to correct or provide further necessary information. Only a complete application will be accepted for evaluation by a multi-stakeholder advisory body, the results of which must be reported to the BPI within 30 days of acceptance. For the duration of the evaluation process, the reviewers remain anonymous to both the public and the BPI. The Scientific and Technical Review Panel (STRP) and the Bureau of Agriculture and Fisheries Products Standards (BAFPS) evaluate all applications; the STRP comprises of at least three experts from a roster of independent scientists and particularly evaluates the risk assessment and risk management strategies outlined by the applicant, whereas the BAFPS will make a determination of compliance with food-safety standards. Additionally, the Fertilizer and Pesticide Authority (FPA) and the Bureau of Animal Industry (BAI) will also evaluate applications of those regulated articles which are also pest-resistant plants or to be used as feed, respectively. Concurrently, the applicant must carry out a public consultation by publishing the PIS in two newspapers of general circulation and inviting the public to make comments directly to the BPI within 60 days of posting the notice. A decision, together with any agreed permit conditions, is made within 120 days of publication of the notice [62]. Approved products are then included in the registry for direct use maintained by the BPI. Once in the registry, for imported articles, the applicant is no longer required to secure an import permit for succeeding shipments. However, a notification of shipment to BPI is required within 15 days before its arrival at a Philippine port [57].

South Africa

South Africa is a biotechnology leader in Africa and is involved in sophisticated biotechnology activities, including those pertaining to the development and commercialization of GMOs [6, 63]. In 2000, the South African government began to focus on, and substantially increased, its research support for biotechnology. This led to the adoption of the 2001 National Biotechnology Strategy (NBS), a policy framework to create incentives for the biotechnology sector [63], involving several government departments [64]. The NBS commits more than US$300 million per year from government to finance a variety of biotechnology initiatives [6].

South Africa is among only three African countries in which GM crops are commercially grown [2]. In 2006, the commercial release of insect-resistant (Bt) cotton and maize; herbicide-tolerant (RR) soybeans, cotton, and maize; and cotton with the "stacked gene" (Bt and RR) had been approved. At the time, it was estimated that these GM crops accounted for the cultivation of 30.5% of yellow maize, 28.8% of white maize, 59% of soybean, and 90% of cotton in South Africa [65]. The total area of commercialized transgenic crops increased in 2010 to 2.2 million hectares [2], which is mostly due to white and yellow maize, followed by RR soybeans and insect-resistant cotton. Giving an insight

into potential future commercial releases, several varieties of these crops were also in field trials as of 2009, along with additional new crops. Between January and September 2009, the number of field trial permits issued totaled 267 [3]. Maize topped the list with 222 approvals, followed by 24 permits for cotton, 15 for vaccines, 3 for soybeans, and one each for sugar cane, sorghum, and table grapes. The traits associated in these approvals included drought tolerance and herbicide tolerance in maize, herbicide tolerance in cotton, biofortified sorghum, fungus resistance in table grape, alternative sugar production pathways in sugar cane, and cassava with altered starch content.

Regulatory Oversight in South Africa

Concerns regarding the commercial release of GMOs led to South Africa enacting legislation to regulate the development, importation, and application of GMOs. The Genetically Modified Organism Act 1997 (GMO Act) was passed in 1997, and it was subsequently modified in 2006 to bring it in line with the CPB [66]. Regulations for its implementation were initially adopted in 1999, and then, amended regulations took effect in February 2010. The formal structures for the implementation of this act include an Executive Council, which reviews applications for GMO work; a scientific advisory committee (South African Committee for Genetic Experimentation [SAGENE]); a registrar to administer the GMO Act; and an inspectorate to monitor function. According to the legislation, no person may import or export from South Africa, or develop, produce, use, release, or distribute any GMO in South Africa, other than under a permit for undertaking such an activity [67]. Such a permit is to be issued after a technical assessment and risk analysis report has been submitted by the applicant and has been approved by the Executive Council. This Council is responsible for making regulatory decisions and is comprised of ten members: one representative from each of eight government ministries (Agriculture, Science and Technology, Health, Environmental Affairs and Tourism, Trade and Industry, Labour, Water Affairs and Forestry, and the Department of Arts and Culture), the chair of SAGENE who provides scientific and technical analysis of risk assessment data, and the GMO Registrar [68]. The GMO Regulations provide that an applicant shall notify the public of any proposed release of GMOs prior to the application for a permit for such release. Public notifications shall be in the form of a standard notice published in the printed media informing the public of the intended release. It is worth noting, however, that the first field trials were allowed in 1994, and since 1997, several multinational companies have been permitted to grow and import GMOs even before the GMO Act was belatedly implemented in November 1999 [63, 69].

Commercialization of GM Crops: South African Approval Process

Commercial activities concerning GMOs all require a permit, including those for: import, export, contained use (including development, production, distribution, transport, but not those under containment levels 1 & 2, i.e., in the laboratory or growth chamber), deliberate release of GMOs into the environment (trial and general release), and commodity clearance [70]. All applications must be submitted to the GMO Registrar at the DA, along with a copy of the public notice and proof (newspaper clippings) in order for the application to be processed. The public notice allows interested parties to submit comments or objections in connection with the intended release to the Registrar within 30 days after the date of the notification [71]. The Registrar then undertakes an initial review of the application to determine compliance with the provisions of the GMO Act. If the application is not compliant, the application is referred back to the applicant. Once compliant, the application is forwarded to a committee (expertise nominated by SAGENE chairperson) formed under SAGENE to conduct a review of the proposed activity. The review includes an evaluation of the risk assessment data, including food safety (if applicable), submitted in the application. Conclusions of the assessment are detailed in a recommendation report, which is sent to the Registrar on completion of the review. At this stage, the application can be referred back to the applicant to address any concerns raised or to supply additional information, and the response returned to the committee. Once all concerns have been addressed, the committee makes a recommendation on the application. The recommendation document, public input, and a copy of

the application is forwarded to the Executive Council for consideration, who will also take into account the socioeconomical impact that the GMO may have. The Council then submits its decision in writing to the Registrar. Should the Council raise any concerns, the Registrar will once again refer the application back to the applicant for clarification. Based on the information received from the applicant and the assessment done by the Council, the application will be approved or rejected. If the application is approved, the Council authorizes the Registrar to issue a permit to the applicant. This permit will be accompanied by specific containment conditions as prescribed by the Council. If the application was rejected, the Registrar will communicate the decision back to the applicant with reasons for the rejection [70]. Regulations of the Department of Health of 2004 provides for the labeling of foodstuff with genetically modified ingredients that are significantly different to the non-GMO ingredients. The Consumer Protection Act of 2008 also addresses this issue.

United States of America (USA)

In the USA, GM crops have been sold since 1994 and in 2006 were already planted on 54.6 million hectares (soybean, maize, cotton, canola, squash, papaya, and alfalfa), confirming the USA's role as the world leader in agro-biotechnology [10]. The regulatory system in the USA relative to biotechnology products is rather different from the one put in place in the EU, and the discrepancies mainly reflecting the different approaches taken by the governmental authorities, citizens, and firms toward GMOs and GM food, especially in the initial years of the biotechnology revolution.

In the USA, agricultural biotechnology politics has been dominated by a strong and cohesive coalition of pro-biotechnology upstream and downstream producers and farmers. Lower public outrage has made mobilization of NGOs in the United States difficult and, in combination with a less-favorable institutional environment (notably, centralized regulatory policy-making), has resulted largely in their exclusion from agri-biotech policy-making [72].

Regulatory Oversight in the USA

Taking the approach that GM products are essentially an extension of conventional products, the US Government has made use of existing laws to ensure the safety of GM products [15]. US regulatory authorities operate under the assumption that the fact that a plant has been genetically modified is less important than the specific effects of the modification [4]; therefore, the regulation focuses on the characteristics of the products rather than the way in which the product was produced [35]. The current system was delineated by the White House Office of Science and Technology Policy under the 1986 Coordinated Framework for Regulation of Biotechnology [73]. It is still the key document for regulating gene technology in the United States and provides the basis for the regulation of crop varieties produced by rDNA techniques. Under the Framework, the US Department of Agriculture (USDA), the Food and Drug Agency (USFDA), and the Environmental Protection Agency (USEPA) that were responsible for regulatory oversight of certain product categories or for certain product uses are also responsible for evaluating those same kinds of products developed using genetic engineering techniques. Transportation, growing (including field testing), and propagation of GM crops are governed by the USDA's Animal and Plant Health Inspection Service (APHIS) under the Federal Plant Pest Act 1996 (FPPA) and the National Environmental Policy Act 1969. Specifically, the APHIS has two responsibilities: deciding on which GM seeds to oversee, so-called regulated articles, and which GM seeds are safe enough to be free from the agency's oversight, so-called deregulated articles. Deciding on GM seeds to be regulated depends on "familiarity," gained from direct experience through field testing under regulated conditions. Any eventual deregulation, that is, exempting a GMO from the oversight of APHIS, involves a petition process whereby an advertisement is published in the US Federal Register, and a period to comment is provided to the public [74].

If a GM plant is not intended for human consumption and is not modified to contain a pesticide, the USDA is the leading agency. For plants genetically modified to produce their own pest protection, APHIS coordinates its evaluation with the USEPA. Pest-resistant GM crops fall under the authority of the USEPA under the Federal Insecticide, Fungicide, and Rodenticide Act 1996 (FIFRA) and the Toxic Substances Control Act 1976. They are subject to a strict

testing regime, where producers must submit testing data to the USEPA who determines the quantity of pesticidal substances that may be present in food [75]. In fact, industry is required to obtain an Experimental Use Permit (EUP) from the USEPA to test any pest-resistant plant in a field larger than 10 acres. Once the permit is granted, firms are expected to consult the USEPA on the details of the field experiment. While the APHIS analyzes data on the source of the new gene, the nature of the pesticidal substance produced, differences with its natural equivalent, effects on non-target organisms, and environmental fate, the USEPA focuses on the toxicology, the digestive fate, and the potential allergenicity of the toxin. In line with the product-based approach, the USEPA does not assess the GM plant per se but the toxin produced by the plant. As for any pesticides, the USEPA subjects these toxins to a registration process. Pest-resistant plants whose toxin falls under this process are mostly of the Bt variety. Following this registration logic, if the toxin produced by a GM plant has been approved previously as a regular pesticide, a new registration is not required. The USEPA has been requesting for years new regulations to obtain a wider role in the assessment and the management of GM plants, but thus far with only limited success [11, 72, 74]. The US Food and Drug Administration (USFDA) regulates food applications of GM crops and relies on existing laws that hold food manufacturers responsible for food safety. Of the three agencies, the USFDA has had the most influence on biotechnology policy because most biotechnology products on the American market are health care or food products [72]. In 1999, public meetings were held by the agency with the aim of sharing its experience regarding GM foods and soliciting views on whether its policies and procedures should be modified. Public comments indicated considerable public support for a mandatory and more transparent process [4].

Commercialization of GM Crops: US Approval Process

The APHIS oversees the confined and unconfined release of transgenic plants as well as any importation and interstate movement under the FPPA. In addition to the FPPA, the USDA issued rules in 1987 for the "introduction of organisms and products altered or produced through genetic engineering which are plant pests or which there is reason to believe are plant pests" [76]. By these rules, the introduction of a crop produced by rDNA techniques into the environment is only legal with an authorization by the APHIS. The APHIS grants a release permit after preparing an environmental impact assessment and "Finding Of No Significant Impact" ("FONSI"). Exempt from these rules are experiments with plants produced by rDNA technology in a contained environment (e.g., laboratory, green house).

In 1997, the USDA simplified the procedure for the unconfined release of GM crops into the environment by allowing the applicant to petition the APHIS for a "determination of non-regulated status" [77]. When receiving a petition, the APHIS prepares an environmental impact assessment taking into account the following eligibility criteria:

- The crop must not be listed as noxious weed or weed.
- The introduced genetic material must be stable and characterized.
- The introduced genetic materials must not
  - Result in any plant disease
  - Confer an infectious entity or encode toxic substances to non-target organisms
  - Encode products intended for pharmaceutical use
- Any plant virus-derived sequences must not pose a significant risk for new plant virus creation.
- The GM crop must be free of known human and animal pathogens or allergens.

After a complete petition is filed, it is published in the Federal Register to solicit comments from the public. Thereafter, the APHIS reviews the data, taking into account public comments and takes a final decision, which is again announced in the Federal Register. The issuance of a "non-regulated" status for a transgenic crop means that it is deregulated and can be freely commercialized in the US (unconfined release, import, interstate movement), except if it contains a pesticidal substance. In that case, an additional "plant pesticide" approval by the USEPA is required.

The responsibility of the USEPA is to evaluate the risks of GM crops "producing their own pesticide" for

human consumption under the FIFRA. The evaluation process is held to the same standards as those for pesticides applied to plants. To be registered under the FIFRA, a pesticide must not cause "unreasonable adverse effects" on the environment and on human health [78]. Transgenic insect- and virus-resistant plants fall under the jurisdiction of the USEPA; however, viral coat proteins are normally exempted from the requirements as the USEPA considers these proteins as "low-risk applications" based on the principle of familiarity and their ubiquitous presence in the food supply. Today, Bt toxins, one viral coat protein, and the potato leaf roll virus protein are registered as pesticides and supervised by the USEPA. The agency evaluates the risks of these "plant-incorporated protectants" by taking into account the following criteria: toxicological effects, effects on non-target organisms, insect resistance management, and persistence of the substance in the environment. The evaluation process lasts approximately 1 year. If adverse effects of insect- or virus-resistant plants are observed after commercialization, the USEPA has the legal power to amend existing registrations. Moreover, the USEPA may impose new measures such as new pest resistance schemes [79].

Besides the pesticide registration under FIFRA, Section 408 of the FFDCA requires the USEPA to determine tolerance limits for substances used as pesticides on and in food and feed [78, 80]. "Nucleic acids that are part of a plant-incorporated protectant" are exempted from this requirement because the USEPA considers them as "safe" [80]. Once approvals from the APHIS, and from the USEPA when pesticidal substances are used, have been granted, it is legal to commercialize the GM plant or derived product in the USA. However, applicants normally engage in a voluntary consultation process with the USFDA before marketing of the GM plant or derived products. This policy is now under review.

International Obligations Relevant to the Biosafety Frameworks

Countries do not have complete discretion when deciding how to set up their biosafety regulatory system as there are several international treaties and agreements that relate to biosafety. If a country is bound by any or all of those international agreements, then their biosafety regulatory system must be compliant with those obligations [81].

Cartagena Protocol on Biosafety

The conclusion of the CPB was broadly recognized as a step forward in providing an international regulatory framework to reconcile trade with environmental protection by creating an enabling environment for the environmentally sound application of biotechnology [82]. The Convention on Biological Diversity (CBD) [83] in Article 19 [3] states that:

- The Parties shall consider the need for and modalities of a protocol setting out appropriate procedures, including, in particular, advance informed agreement, in the field of the safe transfer, handling and use of and living modified organism resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity.

In 1995, the second Conference of the Parties (COP) to the CBD began the consideration of the need for and modalities of such a protocol. COP Decision II/5 commenced the negotiations for the protocol by launching an open-ended ad hoc Working Group on Biosafety [1]. Meeting six times between 1996 and 1999, the Working Group concluded its work with the submission of a draft protocol for consideration by the first extraordinary meeting of the COP, convened with the purpose of adopting a protocol on biosafety to the CBD. The result of this first extraordinary meeting, which took place in two separate meetings in 1999 and 2000, was the adoption of the Protocol [84]. In accordance with its Article 36, the Protocol was opened for signature by States and regional economic integration organizations from 15 to 26 May 2000 and remained open for signature from 5 June 2000 to 4 June 2001. By that date, the Protocol had received 103 signatures. The Protocol entered into force on 11 September 2003, 90 days after receipt of the 50th instrument of ratification [85]. There are presently 160 parties to the Protocol [86], with the COP to the CBD serving as the meeting of the Parties to the Protocol (COP-MOP), the Protocol's governing body. Since the coming into force of the Protocol, the COP-MOP has met five times. The fifth meeting of the

COP-MOP took place from 11 to 15 October 2010 in Nagoya, Japan, and it approved a Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety [87].

The Protocol's scope is the "transboundary movement, transit, handling, and use of all living modified organisms that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health" [82]. To ensure the safe transfer, handling, and use of GMOs, the Protocol sets up two separate procedures. The first time that a GMO is to be intentionally introduced into the environment, the Protocol sets up an Advance Informed Agreement (AIA) procedure. That procedure requires that the exporter of the GMO provides a notice with detailed information about the GMO to the importing country. The importing country then reviews the information, conducts a risk assessment, and decides, based on the risk assessment results, whether to approve or reject the GMO. The second procedure set up by the Protocol is for GMOs to be used for food or feed or for processing (such as corn, soybeans, wheat, or other grains that will be fed directly to humans or animals or used for processing). For those GMOs, the AIA procedure is not required. Instead, the Protocol establishes a simpler system that reflects the decreased likelihood that those GMOs will affect the importing country's biodiversity. Before the GMO can be exported to another country, the only requirement is that the safety decision in the exporting country is communicated to other countries through the Biosafety Clearing-House. For LMOs used for other purposes, such as LMOs used in the laboratory, the Protocol leaves any regulation to the discretion of the individual country. The Protocol also does not cover products derived from LMOs, such as processed foods that have ingredients that came from LMOs. Although the Protocol comprehensively covers many issues, there are a few remaining to be addressed by the individual Party when establishing their biosafety regulatory regime [81].

In addition to the CPB, other relevant international agreements exist. Under international law, countries shall comply with all treaties to which they are parties, provided that the provisions of these treaties are not contradictory (principle of accumulation of international obligations). According to the principle of integration contained in the Rio Declaration on Environment and Development (Principle 4) and the 2002 Plan of Implementation of the World Summit on Sustainable Development (Paragraph 92), environmental treaties and trade goals shall mutually support each other. In fact, the various agreements and instruments on the topic of the development and use of agrobiotechnology are all closely interrelated and need to be carefully considered in the decision-making process, both individually and collectively. This regulatory and public policy background consists of relevant international instruments and processes, such as: the entry into force of the CPB, the Codex Alimentarius Guidelines on Food Safety and Labelling of GMOs, and the World Trade Organization (WTO) Agreements on Sanitary and Phytosanitary Measures and Technical Barriers to Trade. Table 1 presents a summary of the main international instruments and processes of relevance for biosafety.

A Comparative Analysis of Regulatory Processes in All Study Countries

Attributes of a Modern Regulatory Biosafety System

Although the development of regulatory regimes is essentially based on national specific practices, legal systems, public management strategies, and local socioeconomic considerations, national regulatory biosafety frameworks will generally always consider the following elements [110–112]:

- Biosafety policy, occasionally as part of a broader biotechnology or biodiversity policy
- Regulatory policy
- A mechanism to handle requests and permits related to LMO use, in particular, notifications required by the CPB (i.e., an administrative system)
- A mechanism for monitoring and inspections
- A system that allows public participation and information

According to Jaffe [113]:

> The purposes of a national biosafety regulatory system are to scientifically assess the safety of genetically engineered (GE) organisms to humans and the environment, manage any potential risks, and authorize the development and marketing of safe GE organisms and

Commercialisation of GM Crops: Comparison of Regulatory Frameworks. Table 1 Relevant international instruments and processes (Taken from [88])

Agreement, declaration, or process	Content
Rio Declaration on Environment and Development	The precautionary principle is a fundamental instrument for the safe use of biotechnology. This principle is contained in Principle 15, which sets forth that: "*In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation*".
United Nations Environment Program – International Technical Guidelines for Safety in Biotechnology	Enacted in Cairo in 1995, these guidelines may assist governments, intergovernmental organizations, private organizations, and others to strengthen capacities and exchange biosafety information.
	The guidelines are based on the following principles: (a) identification of hazards; (b) risk assessment, taking into account the probability of any hazards arising and the potential consequences of such hazards; and (c) risk management, applying adequate management strategies which include developing procedures and methods to minimize risks and their consequences, or making decisions not to proceed. Such management strategies shall be proportional to the risk assessment results.
World Trade Organization (WTO) Agreement	The WTO Agreement recognizes the goal of sustainable development, considering that free trade shall protect and preserve the environment [89]. There are three WTO Agreements which can be associated with GMOs: The Agreement on the Application of Sanitary and Phytosanitary Measures (SPS; [90]), the Agreement on Technical Barriers to Trade (TBT; [91]), and the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS; [92]). It is also important to understand the regulations and procedures that govern Dispute Settlement Understanding (DSU) in the WTO context [93].
Agreement on Technical Barriers to Trade (TBT)	The TBT Agreement is relevant to biotechnological products as it applies to technical regulations and rules, including requirements for packaging and labeling [91]. The TBT Agreement recognizes that no country shall be prevented from taking necessary measures to ensure the quality of its exports, the prevention of deceptive practices, and the protection of the environment and human, animal, and plant life or health. However, such measures shall not be taken as means of arbitrary or unjustifiable discrimination between countries, where the same conditions prevail, or as disguised restrictions on international trade. Furthermore, such measures must otherwise comply with the provisions of the Agreement [91].
The Agreement on the Application of Sanitary and Phytosanitary Measures (SPS)	SPS rules are summarized in the following manner [90]: 1. Members have the right to take necessary sanitary and phytosanitary measures to protect human, animal, and plant life or health, provided that such measures are not inconsistent with the provisions of the Agreement. 2. Any sanitary or phytosanitary measures are applied only to the extent that is necessary to protect human, animal, and plant life or health, based on scientific principles. Furthermore, such measures cannot be maintained in the absence of sufficient scientific evidence of their necessity. 3. Members shall ensure that their sanitary and phytosanitary measures do not arbitrarily or unjustifiably discriminate between members among whom identical or similar conditions prevail. Moreover, sanitary and phytosanitary measures shall not be applied in a manner that would constitute a disguised restriction on international trade.

Commercialisation of GM Crops: Comparison of Regulatory Frameworks. Table 1 (Continued)

Agreement, declaration, or process	Content
	4. Sanitary or phytosanitary measures, which are taken in accordance with the relevant provisions of the Agreement, are presumed to comply with the members' obligations under GATT 1994, which relate to the use of sanitary or phytosanitary measures, in particular, the provisions of Article XX(b) [94]. 5. In principle, members shall base their sanitary or phytosanitary measures on international standards, guidelines, or recommendations, where these exist. 6. Sanitary or phytosanitary measures which comply with international standards, guidelines, or recommendations are deemed necessary to protect human, animal, and plant life or health and are presumed to be consistent with the relevant provisions of GATT 1994 [94] and the SPS Agreement [90]. 7. Members may introduce or maintain sanitary or phytosanitary measures which result in a higher level of sanitary or phytosanitary protection than the level that is required by the relevant international standards, guidelines, or recommendations, provided that there is a scientific justification to this heightened level of protection or that this level of protection is determined by a member country to be appropriate according to Article 5 of the SPS Agreement regarding risk assessment and adequate protection levels [90]. However, measures which result in a heightened level of sanitary or phytosanitary protection need not go against any other provisions in the Agreement. The above-mentioned standards, guidelines, and recommendations are defined as those established by international organizations, such as the Codex Alimentarius Commission, the Office International des Epizooties, and the Secretariat of the International Plant Protection Convention. As for other subjects that are not within the scope of the aforementioned organizations, they shall be regulated by "other international organizations" identified by the WTO Committee on Sanitary and Phytosanitary Measures as being open to the Parties' membership.
The Codex Alimentarius	Created by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations, the Codex Alimentarius (http://www.codexalimentarius.net) is the organization which is in charge of establishing regulations related to food safety. Codex Alimentarius standards need to ensure fair trade practices in food trade, according to WTO rules. The Codex Alimentarius Commission established an Ad Hoc Intergovernmental Task Force on Food Derived from Biotechnology [95] to handle issues associated with food obtained through biotechnological processes, particularly food used for health and nutrition purposes. In July 2003, the Codex Alimentarius Commission held a meeting during which three risk assessment standards for food resulting from biotechnology were approved [96]. These standards establish risk assessment principles for food derived from modern biotechnology. The principles refer to the concept of "tracing," which is a risk assessment tool whose meaning is the subject of an important debate. In fact, the United States of America consider this concept to be different from traceability and limit themselves to following only the previous and subsequent link in the LMO movement chain. The approved standards are the following: – Principles for the Risk Analysis of Foods Derived from Modern Biotechnology [97] – Guideline for the Conduct of Food Safety Assessment of Foods Derived from – Recombinant-DNA Plants [98] – Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Micro-organisms [99]

Commercialisation of GM Crops: Comparison of Regulatory Frameworks. Table 1 (Continued)

Agreement, declaration, or process	Content
	In 2008, the Ad Hoc Intergovernmental Task Force on Food Derived from Biotechnology completed a Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Animals [100]. At its 31st session, the Commission noted that the Task Force had completed its work, one year ahead of schedule, and agreed to its dissolution [101]. Likewise, the Codex Committee on Food Labelling is working on draft guidelines for the Labelling of Food and Food Ingredients Obtained through Certain Techniques of Genetic Modification/Genetic Engineering, as well as on a Draft Amendment to the General Standard for the Labelling of Pre-packaged Foods (in order to address the issue of genetically modified foods) [102]. Finally, in 2007, the Codex Alimentarius Commission adopted the "Working Principles for Risk Analysis for Food Safety for Application by Governments" that were proposed by the Codex Committee on General Principles [103]. These principles allude to the precautionary approach. For more information on Codex activities, see www.codexalimentarius.net.
International Plant Protection Convention (IPPC)	The IPPC (https://www.ippc.int/) aims to prevent the international spread and introduction of pests of plants and plant products and to promote appropriate measures for their control. The Convention was reviewed in 1997 and entered into force in 2005 [104]. A Commission Working Group studied plant pest risks associated to LMO issues and an international standard (ISPM No.11 pest risk analysis for quarantine pests, including analysis of environmental risk and LMOs) [105]. This text intends to protect plants and ecosystems from LMO-related risks. According to the text, such protection measures should be cost effective, nondiscriminatory, and feasible and should not limit basic trade needs.
World Organization for Animal Health (OIE)	The World Organization for Animal Health, also known as the Office International des Epizooties (OIE; http://www.oie.int/), is an intergovernmental organization created by the International Agreement signed on 25 January 1924. OIE's mission is to ensure the transparency of animal health conditions worldwide. Member countries promise to declare animal diseases detected on their territory. In addition, the OIE is in charge of safeguarding international trade through the elaboration of health regulations destined to be applied to international transboundary movements of animals and animal products. The WTO recognizes such regulations as international reference rules.
Regional International Organization for Plant Protection and Animal Health (OIRSA)	In 2000, the Regional International Organization for Plant Protection and Animal Health (OIRSA; http://www.oirsa.org) produced a Regional Guideline on the Safety of Plant Biotechnology [106]. In fact, the Guideline is based on the CPB and essentially regulates the same issues as the Protocol. However, in view of its nature, the guideline also aims to harmonize the various regional laws and practices pertaining to the issue of biosafety.
Central American Commission for Environment and Development (CCAD)	The Central American Commission for Environment and Development (CCAD), which is the environmental authority of the Central American Integration System (SICA), through its biodiversity program and territorial legal system, prepared a Central American Protocol on the Safety of Modern Biotechnology. A series of technical consultations was undertaken and led to the adoption of a draft Protocol, which was then approved by the Central American Ministries of Environment in 2002. Although the Central American Protocol is based on the CPB, some of its provisions go beyond the scope of the CPB. In fact, provisions regarding labeling, documentation, liability, contained use, and transit, as well as various biosafety principles, have been included in the Central American Protocol in an attempt to avoid the inconsistencies and ambiguities which resulted from the multilateral negotiations that lead up to the adoption of the CPB.

Commercialisation of GM Crops: Comparison of Regulatory Frameworks. Table 1 (Continued)

Agreement, declaration, or process	Content
The Inter-American Institute for Cooperation in Agriculture (IICA)	As requested by the Ministries of Agriculture in 2002, IICA, in collaboration with the OIRSA and the Tropical Agricultural Research and Higher Education Centre (CATIE), was put in charge of preparing a regional biosafety regulatory framework, in response to the lack of regulations in certain countries that are receiving, or may potentially receive, requests for field trials or trade in LMOs. A group comprising of various agencies was formed, along with a consultative process, which led to the preparation of a Regulatory Framework Draft for Living Modified Organisms for Agricultural and Livestock Use in Central American Countries. This initiative intends to promote a model regulation on agricultural biotechnology that is to be adapted by each country, according to its particular needs and situation. There is hope that this regulation will be enacted in a similar fashion by the various nations, thereby triggering a process of harmonization of regulatory frameworks in the region.
Other relevant agreements and declarations	– CGRFA draft Code of Conduct on Biotechnology [107] – United Nations Model Regulations on the Transport of Dangerous Goods (UN Recommendations) [108] – The Aarhus Convention on Access to Information, Public Participation in Decision-making and Access to Justice in Environmental Matters, which contains specific guidelines on public participation in LMO-related decision-making processes [109]. The Convention entered into force in 2001. Although it was enacted by the United Nations Economic Commission for Europe, the convention is open to other nations. The Convention was amended in 2005 in order to set out more precise provisions on the deliberate release of genetically modified organisms, but the amendment has not yet entered into force. The amendment will enter into force once it has been ratified by at least three-quarters of the Parties. In September 2007, the amendment had only been ratified by four countries. The third meeting of the Parties was held in Riga, Latvia, on 11–13 June 2008. The meeting adopted the Riga Declaration and a strategic plan for the Convention, resolved the issue of how to calculate ratification of amendments, and renewed the mandates of task forces dealing with access to justice, electronic information tools, and public participation in international forums.

their products. To develop such a regulatory system, a government can use existing laws or develop new laws. Any national biosafety regulatory system that is proposed, however, must be functional, protective, and also comply with international trade standards that are evolving in recognition of the growing importance of GE organisms in world affairs.

A national biosafety regulatory system is a regulatory regime responsible for assessing and managing the full range of potential risks that might be posed by a GMO and its products. It addresses potential risks to the environment and biological diversity as well as any food/feed risks or other safety-related issues involving GMOs (e.g., worker health, drug safety, etc.). A protective biosafety regulatory system ensures that any risks from GMOs are managed and allows safe GMOs to be developed, marketed, and utilized for their intended purpose. Such a system, however, must also be functional, which means that it should be understandable, workable, equitable, fair, adaptive, and enforceable [114].

Existing biosafety regulatory systems from around the world reflect, among other things, the type of government in the country, the politics of the country, the country's view on the relative safety of GMOs, and the country's regulation of food, agriculture, and environmental issues. Establishing those systems required balancing numerous goals and trading off different interests. Through an analysis and comparison of

different existing biosafety regulatory systems, however, one can identify key characteristics and components that are generally important to a functional and protective biosafety regulatory system [113]. Incorporating each of those characteristics and components in a functional and protective biosafety regulatory system involves problem solving because there can be tensions between the different characteristics.

Jaffe [113] indicates the following attributes of a system:

- Comprehensive.
- Adequate legal authority to subject each GMO to a food-safety and environmental risk assessment approval.
- A clear safety standard.
- Proportionate risk-based reviews.
- Transparent and understandable.
- Participatory.
- Post-approval oversight. A biosafety regulatory system does not stop its oversight once a GMO has been approved for a confined field trial or for a commercial release.
- Flexible and adaptable.
- Efficient, workable, and fair.

Decision-makers are facing an important challenge because biotechnology and biosafety fields are ever evolving at a dramatic pace. In this context, overly precise and detailed regulatory frameworks can easily become obsolete in a short period of time. In order to avoid forever having to enact new legislation and policies, it is preferable to develop a regulatory framework that takes on the form of a general guide, thus regulating more specific biosafety aspects by way of by-laws and other regulations. In fact, this alternative allows more efficient regulation of future situations but has the effect of conferring the power of regulating specific aspects of biotechnology to the executive branch of government and other administrative institutions, instead of to the Parliament [115].

General Comments on Evolution of Biosafety Regulatory Systems

The nature of GM crop regulations around the world has as much to do with social and political values as with concerns about health and safety. Consumers' growing awareness of their rights and farmers' increasing fear of dependence on multinational companies are symptoms of a deeper concern about values and priorities, the type of environment that people want, the role of biodiversity, their tolerance to risk, and the price that people are prepared to pay for regulation. Therefore, the regulations in the study countries were formed, or amended, during this period of heightened public awareness and are a reflection of how the various governments have attempted, or not, to address these concerns. A comprehensive discussion of regulatory requirements for GM crops at the national and international levels is a broader topic than can be covered here, and previous studies have addressed them in detail [5, 116].

In general terms of regulatory systems, several approaches have been considered worldwide with respect to the safe use of modern biotechnology: (a) certain countries decided to apply preexistent plant or animal health regulations to GMO-related issues or to incorporate biosafety provisions into plant and animal health protection laws; (b) other countries decided to adopt specific biosafety regulations that need to be enacted by the Parliament, the executive power (through agreements or resolutions), or public sector institutions; (c) certain countries decided to mention biosafety in their environmental regulations; (d) some countries decided to apply seeds, pest control, and plant or animal regulations to modern biotechnology, enacting but a few coordination rules and specific provisions associated to biosafety.

The original trend of agricultural biosafety regulations focusing on transgenic plants continues to exist in some countries but is in the process of being replaced by a broader focus that comprises biosafety regulation of animals, microorganisms, fish, and forest species. This progressive expansion of biosafety toward new areas results from the need to regulate research and eventual trade of other types of organisms created through genetic engineering. The CPB, whose scope extends beyond the issue of plants, and the subsequently developed regulatory frameworks implementing its provisions, brought on a gradual increase in the number and variety of organisms and activities being regulated. In addition, health authorities have increased participation in biosafety advisory commissions and committees.

In view of the complexity of biosafety issues, particularly issues of risk assessment and risk management, and several other issues associated to the introduction of GMOs into the environment, almost all of the countries have established a committee or a national commission to assist decision-makers in the development of biosafety regulations. The composition of these committees and commissions varies considerably in the region, mainly with respect to the inclusion of the productive sector, consumers, and non-governmental organizations. However, the trend points toward the inclusion of these organizations, even though such an inclusion can potentially lead to conflicts of interest. Currently, these committees or commissions are for the most part directly connected to the regulatory authorities in place. Nevertheless, independent national commissions (commissions that are not connected to the regulatory authorities) were created in some countries and were assigned political and coordinative functions, leaving the issue of technical recommendations to be dealt with by other sector-based authorities established by each country's competent Ministry or Secretariat.

The entry into force of the CPB had the effect of introducing the Ministries of Environment to the biosafety debate. For the reasons mentioned in the previous paragraph, the Ministries of Agriculture have traditionally been the authorities involved in the decision-making process regarding GMOs. Gradually, environmental authorities started demanding and assuming an active role in the regulation of GMOs in view of their mandate to protect the environment. This trend has been strengthened by the incorporation of biosafety provisions into environmental laws and regulations. In some cases, GMOs are even subject to environmental impact assessments (at least based on a literal interpretation of the pertinent legal provisions).

Comparison of Approval Systems

In Asia, the only major GM crops approved for commercial release are Bt cotton, which is grown commercially in China, India, and Indonesia, and GM corn recently approved in the Philippines. To date, no Asian government has given official permission to plant GM soybeans or rice.

In the context of GM crops, the concept of "biosafety" is, in principle, a broad one, covering three areas: the health safety of humans and livestock, the safety of the environment (i.e., ecology and biodiversity), and socioeconomic safety (i.e., the economic and social impact on farmers, consumers, and different social classes, as well as on trade and economy in general) [44]. While the biosafety regulations in force in industrialized countries (e.g., Canada, the European Union, and the USA) address only the health and environmental risks and exclude socioeconomic considerations, the regulations in developing countries (e.g., India, Argentina, SA, and the Philippines) tend to include all three areas.

Countries have responded differently to the opportunities presented by GM crops and the potential risks associated with them (Table 2). The composition of the "trade off" between potential benefits and risks in each case depends upon whether a government adopts a permissive, precautionary, or prohibitive policy approach to GM crops. Three basic conditions may thus trigger application of protective measures: uncertainty, risk, and lack of proof of direct causal link [5]. As major agricultural exporters, Argentina, Canada, and the USA have each adopted a permissive attitude very early on, widely authorizing most GM products for production and consumption, thereby benefiting from lower production costs and greater export profits. Regulators in India, Europe, and the Philippines, on the other hand, have taken a more cautious approach based on guaranteeing a very low level of risk to human health and the environment. They have therefore imposed strict control measures on approval and marketing of GMOs and GM products [5]. While China had initially moved quickly on the approval of GM crops for environmental and commercial releases, the approval process has slowed considerably since 2000, and strict regulations have been implemented for GMO imports [15].

Further differences are obvious. Process-based regulation is the rule in almost all countries that have developed national biosafety regulatory systems. Even in countries employing a product-focused RA process, the scope of regulatory oversight is defined by the process of genetic modification. Canada is the only country in which regulatory oversight is triggered solely by the novelty of the trait(s) expressed by plants, irrespective of the means by which the novel traits were introduced – an approach that is most consistent with

Commercialisation of GM Crops: Comparison of Regulatory Frameworks. Table 2 Main characteristics of commercialization approval systems in study countries

Element of approval system	Country/region							
	Argentina	Canada	China	Europe	India	Philippines	S. Africa	USA
Biosafety/GMO-specific law/act	X	X	X	✓	X	X	✓	X
Regulatory trigger: process (A) or product (B)	B	B[a]	B	A	A	A	A	B
Responsible ministry or government department: agriculture (A), environment (E), or health (H)	A	A,H	A	A or E[b]	E	A		A, E[c]
ERA committee composition: academia (A), commercial (C), government (G), or public (P) representatives	A,C, G,P	G	A,G	A	G	A,G	A,G	G
Obligatory domestic field testing	✓	X[d]	✓	X[d]	✓	✓[e]		
Obligatory prior approval in export country		X	✓	X		✓	✓	
Compulsory compliance with food-safety requirements	✓	✓	✓	✓	✓	✓	✓	X[f]
Socioeconomic impacts considered	✓	X	X[g]	X	✓		✓	X
Compulsory variety registration	✓	✓	✓		✓			
Mandatory post-market monitoring[h]		X		✓	✓			X
Time- or spatially restricted authorization		✓		✓	✓			X
Public consultation (days)				30	30[i]	60	30	✓

[a]In contrast to all other countries, Canada relies on the concept of novelty to trigger regulatory oversight and has declared that all plants derived through genetic modification are considered novel

[b]The European Commission sends its draft approval to the Council of Ministers (agricultural or environmental ministers), which has three months to reject or adopt it. If they do not act within this time, the Commission may adopt its own decision and authorize the new GM product

[c]For those GM plants producing their own pesticide, the evaluation is coordinated between APHIS and the USEPA

[d]Where data from field studies on other continents are supplied, the applicant should submit a reasoned argument that the data is applicable to domestic conditions

[e]For local applications only, not for applications for import

[f]Developers of GM crops engage in a voluntary, but recommended, consultation process with the USFDA (voluntary pre-market review). This process is currently under review

[g]Taking socioeconomic considerations into account during the risk assessment process is not legally required in China [30]

[h]✓ – Mandatory requirement for approved post-marketing monitoring plans and reporting. X – No specific approval requirement, but the developer is expected to monitor for existing and emerging risks that may be associated with its product and notify the regulatory authorities whenever new information is uncovered

[i]Thirty-day time period is provided for public consultation after the formal approval

the scientific principle that the risks associated with GM crops are not inherently different than those posed by more conventional crops [117, 118]. Indeed, the US National Research Council has explicitly recommended using objective compositional changes, not breeding method, as the basis for regulatory scrutiny and even then, only "when warranted" [119]. India, Argentina, Canada, most EU countries, and South Africa have all used non-statutory guidelines to manage the environmental impact of GM crops before promulgating new acts or regulations. There is no evidence that this approach has ever compromised environmental safety. India is the exception among the study countries in locating its biosafety decision-making authority solely within the ministry responsible for the environment, while the Ministry of Agriculture predominates among the remaining study countries. As a rule, environment ministries have a more precautious or preventative approach to introducing new technologies, as compared to those with the responsibility for agriculture. Different structural approaches are used to secure the necessary scientific advice for the decision-making process. The EU has implemented a system of expert advisory committees, while others, such as India, Canada, and the USA, rely primarily on scientists and professionals working within government departments and agencies. Other countries, e.g., Argentina, China, and South Africa, have a combination of both. Only India and the EU mandate post-market monitoring in attempts to gauge the impacts of the introduction of GM crops over the long-term as well as larger spatial scales. Other countries may address this indirectly by authorizing time-limited or geographically limited introductions (e.g., Canada), whereas the USA does neither.

Conclusion and Future Directions

Governments have an important role in ensuring that novel foods are safe for human consumption and that novel agricultural inputs do not cause major negative impacts on the environment and long-term agricultural production. The adoption of biosafety regulatory frameworks is a challenging task since decision-makers are faced with numerous difficulties, such as ever-evolving technology, which can quickly render specific regulations obsolete. Countries and policy-makers are responding to these various problems with different legislative and policy-based strategies. As seen above, most countries, with the notable exception of the USA, consider GM crops to be novel foods, regardless of the characteristics of their final product. Hence, new laws and institutions to regulate potential biosafety and food-safety issues have and continue to be established, requiring that GM products be approved before they may be grown in, consumed in, or imported into a country. Concurrently, public opinion in many parts of the world still regards the use of GM crops as controversial. Concerns about new risks have led biosafety, food-safety, and labeling regulations to become complex and costly, with no tiered mechanisms in place to regulate the various and different GMOs based upon the level of risk presented and the amount of regulatory experience gained with similar products. As a result, regulation has evolved which implicitly assumes that all GMOs have the ability to present the same (high) risk unless proven otherwise, requiring the over-production of data of questionable value to decision-making. This regulatory position has become a real threat to the future development of GM crops in the non-corporate and public sectors, especially for those subsistence crops involving tolerance to abiotic stress and higher nutrient contents being specifically developed for the benefit of farmers and consumers in the developing world. Should regulatory safety standards be set to an impossibly high threshold (e.g., at zero risk), these GM crops are unlikely to be approved in those countries who stand to gain most from their potential benefits.

The requirements of setting up effective and efficient regulations and legislative systems pertaining to products of rDNA technology inevitably involve additional costs, e.g., the development and maintenance of institutions, procedures, and management tools, costs which many developing countries cannot afford. Even should developing countries decide to form their regulatory frameworks by adapting regulatory guidance already implemented elsewhere, cost sharing still carries a financial burden. Once a system is in place, other relevant costs include the cost of compliance with biosafety regulations and risk management conditions, as well as the economic, environmental, and health costs related to delayed access to new technologies and products and their associated benefits. The variety

and disparity of potential frameworks call for a normalization of information requirements and, wherever synergies and cooperation mechanisms are promoted, a harmonization of the scientific and technical aspects of regulatory oversight at the national and subregional level. As such, an expanded use of internationally accepted consensus can promote the acceptance of regional approaches to regulation. Examples include OECD documents on scientific aspects of risk assessment, as well as guidelines issued by international standard-setting bodies such as the International Plant Protection Convention and the Codex Alimentarius. Programs that create networks on regional and subregional levels may also facilitate the acquisition and dissemination of biosafety expertise at reduced costs. Providing useful, relevant information is a worthwhile task, but it can also prove expensive to provide training on how best to utilize such information in decision-making. It is therefore imperative that external funding streams continue to be directed toward supporting the establishment of effective regulatory systems in developing countries that respond to national needs and policies, until such time that they become self-sustainable.

Disclaimer

Opinions and views expressed in this chapter are strictly those of the authors and may not necessarily represent those of the organizations where the authors are currently employed.

Bibliography

1. Cordonier Segger M-C, Perron-Welch F, Frison (eds) (2012) Introduction. In: Legal aspects of implementing the Cartagena protocol: Biosafety becomes binding. Cambridge University Press, UK
2. James C (2010) Global status of commercialized biotech/GM crops: 2010. ISAAA Brief No. 42. International service for the acquisition of agri-biotech applications (ISAAA), New York, USA. http://www.isaaa.org/resources/publications/briefs/42/executivesummary/pdf/Brief42-ExecutiveSummary-English.pdf
3. James C (2009) Global status of commercialized biotech/GM crops: 2009. The first fourteen years, 1996 to 2009. ISAAA Brief No. 41. International service for the acquisition of agri-biotech applications (ISAAA), New York, USA. http://www.isaaa.org/resources/publications/briefs/41/executivesummary/pdf/Brief41-ExecutiveSummary-English.pdf
4. Falck-Zepeda J, Cavalieri A, Zambrano P (2008) Delivering genetically engineered crops to poor farmers. Recommendations for improved biosafety regulations in developing countries. IFPRI Policy Brief 14. Washington DC, USA. http://www.ifpri.org/sites/default/files/publications/bp014.pdf
5. Zarrilli S (2005) International trade in GMOs and GM products: National and multilateral legal frameworks. Policy issues in international trade and commodities study series, 29. United Nations conference on trade and development, New York, USA and Geneva, Switzerland. http://www.eldis.org/static/DOC18565.htm
6. Mugabe J (2003) International trends in modern biotechnology: Entry by and implications for African countries. ATPS special paper series No. 15, African technology policy studies network (ATPS), Nairobi, Kenya. http://www.atpsnet.org/content/files/documents/SpecialPaperSeries15.pdf
7. Anderson K, Valenzuela E, Jackson LA (2006) Recent and prospective adoption of genetically modified cotton: a global CGE analysis of economic impacts. World Bank policy research working paper 3917. http://www-wds.worldbank.org/external/default/WDSContentServer/IW3P/IB/2006/05/10/000016406_20060510092736/Rendered/PDF/wps3917.pdf
8. US Department of Agriculture (2006) Argentina. Biotechnology Annual Report 2006. Foreign agricultural service GAIN Report AR6027, Washington DC, USA. http://www.fas.usda.gov/gainfiles/200610/146249372.pdf
9. Trigo EJ, Cap EJ (2003) The impact of the introduction of transgenic crops in Argentinean agriculture. J Agrobiotechnol Manage Econ (AgBioForum) 6(3):87–94, http://www.agbioforum.org/v6n3/v6n3a01-trigo.pdf
10. James C (2006) Global Status of Commercialized Biotech/GM Crops: 2006. ISAAA Brief No. 35. ISAAA, New York, USA. http://www.isaaa.org/resources/publications/briefs/35/executivesummary/pdf/Brief35-ExecutiveSummary-English.pdf
11. Nap J, Metz P, Escaler M, Conner A (2003) The release of genetically modified crops into the environment. Part 1. Overview of current status and regulations. Plant J 33(1):1–18
12. UNEP-GEF Biosafety Unit (2006) A comparative analysis of experiences and lessons from the UNEP-GEF biosafety projects. United Nations Environment Programme (UNEP)-Global Environment Facility (GEF), Geneva, Switzerland. http://www.unep.ch/biosafety/development/devdocuments/UNEPGEFBiosafety_comp_analysisDec2006.pdf
13. Burachik M, Traynor P (2002) Analysis of a national biosafety system: regulatory policies and procedures in Argentina. ISNAR Country Report no. 63, International Service for National Agricultural Research, The Hague, The Netherlands. ftp://ftp.cgiar.org/isnar/Publicat/cr63.pdf
14. BATS (2003) Genetically modified (GM) crops: molecular and regulatory details. Version 2. http://www.bats.ch/gmo-watch/GVO-report140703.pdf
15. Baumüller H (2004) Domestic import regulations for genetically modified organisms and their compatibility with WTO rules. Asian Biotechnol Dev Review 6(3):33–42. http://www.ris.org.in/images/RIS_images/pdf/abdr_July044.pdf
16. SAGPyA (2003) Resolution No. 39/03. Annex. Application for permit to release genetically modified plant organisms into

the environment. Secretariat of agriculture, livestock, fisheries and food (SAGPyA), Argentina. http://www.sagpya.mecon.gov.ar/new/0-0/programas/biotecnologia/resolutionn39annex.pdf
17. SAGPyA (2002) Resolution No. 412/02. Requisitos para la evaluación de la aptitud alimentaria de los de organismos genéticamente modificados. Secretariat of agriculture, livestock, fisheries and food (SAGPyA), Argentina. http://www.sagpya.mecon.gov.ar/new/0-0/programas/biotecnologia/marco_regulatorio/028_412.php
18. Macdonald P, Yarrow S (2003) Regulation of Bt crops in Canada. J Invertebr Pathol 83:93–99
19. Finstad K, Bonfils A-C, Shearer W, Macdonald P (2007) Trees with novel traits in Canada: regulations and related scientific issues. Tree Genet Genomes 3:135–139
20. Agriculture and Agri-Food Canada (1994) Regulatory directive Dir94-08: Assessment criteria for determining environmental safety of plants with novel traits. Ottawa, Canada. http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml#ch1
21. Industry Canada (1998) The federal regulatory framework for biotechnology (1993), Annex C of the 1998 Canadian biotechnology strategy: an ongoing renewal process. http://www.biostrategy.gc.ca/CMFiles/1998strategyE49RAI-8312004-5365.pdf
22. CFIA (2005) Long term testing/substantial equivalence. http://www.inspection.gc.ca/english/sci/biotech/reg/equive.shtml
23. Health Canada (2006) Guidelines for the safety assessment of novel foods. Ottawa, Canada. http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/gmf-agm/guidelines-lignes directrices_e.pdf
24. CFIA (2006) Directive 96–13: import permit requirements for plants with novel traits (including transgenic plants), and their products. http://www.inspection.gc.ca/english/plaveg/protect/dir/d-96-13e.pdf
25. Huang J, Wang Q (2002) Agricultural biotechnology development and policy in China. J Agro-biotechnol Manag Econ (AgBioForum) 5(4):122–135, http://www.agbioforum.org/v5n4/v5n4a01-huang.htm
26. Karplus V, Deng XW (2008) Agricultural biotechnology in China: origins and prospects. Springer, New York
27. Chen ZL (2006) Chinese agricultural biotechnology in the field. In: Agricultural biotechnology: economic growth through new products, partnerships and workforce development, NABC Report 18. National agricultural biotechnology council (NABC), Ithaca, USA. http://nabc.cals.cornell.edu/pubs/nabc_18/NABC18_Chen.pdf
28. Pray CE, Naseem A (2007) Supplying crop biotechnology to the poor: opportunities and constraints. J Dev Stud 43(1): 192–217
29. Pray C, Ramaswami B, Huang J, Hu R, Bengali P, Zhang H (2006) Costs and enforcement of biosafety regulations in India and China. Int J Technol Globalisation 2(1/2):137–157
30. Wang Y, Johnston S (2007) The status of GM rice R&D in China. Nat Biotechnol 25(7):717–718
31. Wang Y, Johnston S (2007) Review on GM rice risk assessment in China. UNU-IAS Working Paper No. 152. United Nations University-Institute of Advanced Studies, Yokohama, Japan. http://www.ias.unu.edu/resource_centre/152YanqingWang.pdf
32. US Department of Agriculture (2004) China, Peoples republic of: food and agricultural import regulations and standards (FAIRS) Country Report 2004. Foreign Agricultural Service GAIN Report CH4028, Washington DC, USA. http://www.fas.usda.gov/gainfiles/200408/146107026.doc
33. OECD (2011) Adapting agriculture to climate change: what role for biotechnology? ENV/EPOC/WPCID(2011)2, Working party on climate, Investment and Development, Environment Policy Committee, Environment Directorate, Organisation for Economic Co-operation and Development (OECD), Paris, France
34. US Department of Agriculture (2005) China, Peoples Republic of: Biotechnology Annual Report 2005. Foreign Agricultural Service GAIN Report CH5069, Washington DC, USA. http://www.fas.usda.gov/gainfiles/200601/146176661.pdf
35. Foster M, Berry P, Hogan J (2003) Market access issues for GM products. Australian bureau of agricultural and resource economics (ABARE) eReport 03.13, Canberra, Australia. http://abareonlineshop.com/PdfFiles/PC12559.pdf
36. Conner A, Glare T, Nap J (2003) The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. Plant J 33:19–46
37. König A, Cockburn A, Crevel RWR, Debruyne E, Grafstroem R, Hammerling U, Kimber I, Knudsen I, Kuiper HA, Peijnenburg AACM, Penninks AH, Poulsen M, Schauzu M, Wal JM (2004) Assessment of the safety of foods derived from genetically modified (GM) crops. Food Chem Toxicol 42:1047–1088
38. GEAC (2010) Yearwise list of commercially released varieties of Bt cotton hybrids by GEAC. Genetic engineering approval committee (GEAC), Ministry of Environment and Forests, Government of India. http://igmoris.nic.in/files/Final_commercially_approved.pdf
39. APCoAB (2006) Bt cotton in India – a status report. Asia-Pacific consortium on agricultural biotechnology (APCoAB), New Delhi, India. p 34. http://www.apcoab.org/documents/bt_cotton.pdf
40. Damodaran A (2005) Re-engineering biosafety regulations in India: towards a critique of policy, law and prescriptions. Law Environ Develop J 1(1):1–16, http://www.lead-journal.org/content/05001.pdf
41. Government of India (1989) Rules for the manufacture, use, import, export and storage of hazardous micro organisms genetically engineered organisms or cells. Ministry of Environment and Forests, Government of India. http://www.envfor.nic.in/legis/hsm/hsm3.html
42. Government of India (1998) Revised guidelines for research in transgenic plants & guidelines for toxicity and allergenicity evaluation of transgenic seeds, plants and plant parts. Department of Biotechnology, Ministry of Science and Technology, Government of India. August 1998. http://dbtindia.nic.in/policy/guidelines_98.pdf
43. US Department of Agriculture (2005) India biotechnology annual 2005. Foreign Agricultural Service GAIN Report

IN5078, Washington DC, USA. http://www.fas.usda.gov/gainfiles/200507/146130314.pdf
44. Indira A, Bhagavan MR, Virgin I (2005) Agricultural biotechnology and biosafety in India: expectations, outcomes and lessons. Stockholm Environment Institute, Sweden. http://www.agbios.com/docroot/articles/05-268-001.pdf
45. Gupta A (2002) Ensuring "Safe use" of biotechnology: key challenges. Economic and Political Weekly, 6 July 2002, pp 2762–2769. http://www.epw.org.in/epw/uploads/articles/9705.pdf
46. Chaturvedi S, Chawii L (2005) RIS-DP # 99. Research and information system for developing countries, New Delhi, India
47. National Commission on Farmers (2006) Fifth and final report. Ministry of Agriculture, Government of India. http://krishakayog.gov.in/revdraft.pdf
48. Bhan MK (2007) BIO 2007, Boston, 9 May 2007. http://www.ciionline.org/news_new/newsMain09-05-2007_2.asp
49. Department of Biotechnology and the Ministry of Environment and Forests (2008) Guidelines & standard operating procedures (SOP) for confined field trials of genetically engineered (GE) plants. Government of India. http://igmoris.nic.in/Guideline_index.htm
50. Indian Council of Medical Research (2008) Guidelines for the safety assessment of foods derived from genetically engineered plants. New Dehli, India. http://igmoris.nic.in/files%5CCoverpage.pdf
51. Department of Biotechnology (2008) Protocols for food and feed safety assessment of GE crops. Ministry of Science and Technology, Government of India. http://igmoris.nic.in/files%5CCoverpage1.pdf
52. Indian Ministry of Agriculture (2006) Protection of plant varieties and farmers' rights regulations, 2006. The Gazette of India: Extraordinary (Part II Sec. 3(i)), 7th December 2006, New Delhi, India. http://www.plantauthority.in/PDFile/Indgazette.pdf
53. Secretary of Agriculture, the Republic of the Philippines (2001) Policy statement on modern biotechnology. http://www.ncbp.dost.gov.ph/downloads/PSMB.pdf
54. Republic of the Philippines (1998) Republic Act No. 8435, Agriculture and Fisheries Modernization Act, 10 June 1998. http://www.da.gov.ph/agrilaws/ra/afma.pdf
55. APCoAB (2005) Commercialization of Bt corn in the Philippines. A status report. Asia-Pacific consortium on agricultural biotechnology (APCoAB), New Dehli, India. p 41. http://www.apcoab.org/documents/bt_corn.pdf
56. Yorobe JM Jr, Quicoy CB (2006) Economic impact of Bt corn in the Philippines. The Philippine Agric Sci 89(3):258–267
57. US Department of Agriculture (2005) Philippines Biotechnology Annual Report 2005. Foreign Agricultural Service GAIN Report RP5027, Washington DC, USA. http://www.fas.usda.gov/gainfiles/200507/146130361.pdf
58. Republic of the Philippines (1990) Executive Order 430 Constituting the NCBP, 1990. Government of Republic of the Philippines. http://www.ncbp.dost.gov.ph/index.php?option=$com_remository&Itemid=55&func=download&filecatid=25
59. Manalo AJ, Ramon GP (2007) The cost of product development of Bt corn event MON810 in the Philippines. J Agrobiotechnol Manag Econ (AgBioForum) 10(1):19–32, http://www.agbioforum.org/v10n1/v10n1a03-manalo.pdf
60. de Leon A, Manalo A, Cielo Guilatco F (2004) The cost implications of GM food labeling in the Philippines. http://www.bcp.org.ph/downloads/CostImplicationsofGMFoodLabelinginthePhilippines.pdf
61. Department of Agriculture's Administrative Order No. 8, s. 2002. Government of Republic of the Philippines. http://www.ncbp.dost.gov.ph/downloads/commercialization.pdf.
62. Andanda AP (2006) Developing legal regulatory frameworks for modern biotechnology: the possibilities and limits in the case of GMOs. Afr J Biotechnol 5(15):1360–1369, http://academicjournals.org/ajb/PDF/pdf2006/3Aug/Andanda.pdf
63. Department of Science and Technology (2001) National biotechnology strategy for South Africa. Government of the Republic of South Africa. http://www.dst.gov.za/publications/reports/dst_biotechnology_strategy.PDF
64. Cloete TE, Nel LH, Theron J (2006) Biotechnology in South Africa. Trends Biotechnol 24(12):557–552
65. van der Walt WJ (2006) South African GM crop adoption in 2006. African Crops News Service. Newsletter May 2006. http://www.africancrops.net/news/may06/gm-southafrica.htm
66. US Department of Agriculture (2005) Republic of South Africa. Biotechnology Annual Report 2005. Foreign Agricultural Service GAIN Report SF5024, Washington DC, USA. http://www.fas.usda.gov/gainfiles/200512/146131662.pdf
67. Government of the Republic of South Africa (1997) Genetically Modified Organisms Act (No. 15 of 1997), Government Gazette, vol 383, no. 18029. 23 May 1997. http://www.info.gov.za/acts/1997/act15.htm
68. Government of the Republic of South Africa (2005) The GMO Amendment Bill. Government Gazette 27913, 26 August 2005. http://www.info.gov.za/gazette/bills/2005/b34-05.pdf
69. Gouse M, Pray CE, Kirsten J, Schimmelpfennig D (2005) A GM subsistence crop in Africa: the case of Bt white maize in South Africa. Int J Biotechnol 7(1/2/3):84–94, http://inderscience.metapress.com/media/7hxdqlmumk0y0qgunm13/contributions/j/d/2/p/jd2pphettplpyg50.pdf
70. RSA Department of Agriculture (2004) Guideline document for use by the advisory committee when considering proposals/applications for activities with genetically modified organisms. Government of the Republic of South Africa (RSA). http://www.nda.agric.za/docs/geneticresources/GuidelinesforAC-May2004.pdf
71. RSA Guidelines. Public notification in terms of the Genetically Modified Organisms Act, 1997 (Regulation 6). Republic of South Africa (RSA). http://www.nda.agric.za/docs/geneticresources/publicnotice.pdf
72. Bernauer T, Meins E (2003) Technological revolution meets policy and the market: explaining cross-national differences in agricultural biotechnology regulation. Eur J Polit Res 42:643–683

73. US Office of Science and Technology Policy (1986) Coordinated framework for regulation of biotechnology, United States Federal Register, 51:23302, June 26, 1986. http://usbiotechreg.nbii.gov/Coordinated_Framework_1986_Federal_Register.html
74. MacKenzie DJ (2000) International comparison of regulatory frameworks for food products of biotechnology. Prepared for the Canadian Biotechnology Advisory Committee Project Steering Committee on the Regulation of Genetically Modified Foods. http://www.cbac-cccb.ca/epic/internet/incbac-cccb.nsf/vwapj/InternatComparisons_MacKenzie.pdf/$FILE/InternatComparisons_MacKenzie.pdf
75. US Department of Agriculture (2001) Economic issues in biotechnology. economic research service, Agriculture Information Bulletin no. 762, Washington DC, USA. http://www.ers.usda.gov/Publications/aib762/
76. US Department of Agriculture (1987) Introduction of organisms and products altered or produced through genetic engineering which are plant pests or which there is reason to believe are plant pests, 7 CFR Parts 330 and 340. United States Federal Register 52(115), June 16 1987. http://www.aphis.usda.gov/brs/687rule.txt
77. US Department of Agriculture (1997) Simplification of requirements and procedures for genetically engineered organisms and products, 7 CFR Part 340. United States Federal Register 62(85), May 2 1997. http://www.aphis.usda.gov/brs/pdf/r597rule.pdf
78. US National Research Council (2000) Genetically modified pest-protected plants, science and regulations. National Research Council, National Academy, Washington DC, USA, pp 104–207. http://newton.nap.edu/catalog/9795.html#toc
79. US Environmental Protection Agency (2001) Plant incorporated protectants. US Environmental Protection Agency, Washington DC, USA. http://www.epa.gov/pesticides/biopesticides/pips/index.htm
80. US Environmental Protection Agency (2001) Plant-incorporated protectants, final rules and proposed rule, 40 CFR Parts 152 and 174. United States federal register 66(139), 19 July 2001. http://www.epa.gov/pesticides/biopesticides/pips/pip_rule.pdf
81. Jaffe G (2012) Crafting national biosafety regulatory systems. In: Cordonier Segger M-C, Perron-Welch F, Frison (eds) Legal aspects of implementing the Cartagena protocol: Biosafety becomes binding. Cambridge University Press, UK
82. Secretariat of the Convention on Biological Diversity (2000) Cartagena Protocol on Biosafety to the Convention on Biological Diversity. Montreal, Canada. http://www.cbd.int/biosafety/protocol.shtml.
83. Secretariat of the Convention on Biological Diversity (1992) Convention on biological diversity. Montreal, Canada. http://www.cbd.int/doc/legal/cbd-en.pdf
84. Secretariat of the Convention on Biological Diversity (2000) Decision EMI/3. Adoption of the Cartagena protocol and interim arrangements. First extraordinary meeting (ExCOP 1), 22–23 Feb 1999, Cartagena, Colombia 1999, and Montreal, 24–29 Jan 2000. http://www.cbd.int/doc/meetings/cop/excop-01/official/excop-01-03-en.pdf
85. Secretariat of the Convention on Biological Diversity (2003) The Cartagena protocol on biosafety: a record of the negotiations. Montreal, Canada. http://bch.cbd.int/database/attachment/?id=10886
86. Secretariat of the Convention on Biological Diversity (2011) Cartagena protocol on biosafety ratification list. Montreal, Canada. http://www.cbd.int/doc/lists/cpb-ratifications.pdf
87. Secretariat of the Convention on Biological Diversity (2011) Nagoya – Kuala lumpur supplementary protocol on liability and redress to the cartagena protocol on biosafety. Montreal, Canada. http://bch.cbd.int/database/attachment/?id=10898
88. Cabrera Medaglia J (in press) Biosafety regulatory frameworks in the Americas. In: Cordonier Segger M-C, Perron-Welch F, Frison (eds) Legal aspects of implementing the Cartagena protocol: Biosafety becomes binding. Cambridge University Press, UK
89. WTO (1994) Marrakesh agreement establishing the world trade organization. Final Act of the 1986–1994 uruguay round of trade negotiations. World Trade Organization (WTO), Geneva, Switzerland. http://www.wto.org/english/docs_e/legal_e/04-wto_e.htm
90. WTO (1994) Agreement on the application of sanitary and phytosanitary measures (SPS Agreement) (Article 1 – 14). Final Act of the 1986 –1994 uruguay round of trade negotiations. Annex 1a, Multilateral agreements on trade in goods. World Trade Organization (WTO), Geneva, Switzerland. http://www.wto.org/english/docs_e/legal_e/15sps_01_e.htm
91. WTO (1994) Agreement on technical barriers to trade (TBT Agreement) (Article 1 – 15). Final Act of the 1986 – 1994 Uruguay round of trade negotiations. Annex 1a, multilateral agreements on trade in goods. World Trade Organization (WTO), Geneva, Switzerland. http://www.wto.org/english/docs_e/legal_e/17-tbt_e.htm
92. WTO (1994) Agreement on trade-related aspects of intellectual property rights (TRIPS Agreement). Final Act of the 1986 –1994 uruguay round of trade negotiations. Annex 1, Multilateral agreements on trade in goods. World Trade Organization (WTO), Geneva, Switzerland. http://www.wto.org/english/docs_e/legal_e/27-trips_01_e.htm
93. WTO (1994) Understanding on rules and procedures governing the settlement of disputes. Final Act of the 1986 – 1994 uruguay round of trade negotiations. Annex 2, multilateral agreements on trade in goods. World Trade Organization (WTO), Geneva, Switzerland. http://www.wto.org/english/docs_e/legal_e/28-dsu_e.htm
94. WTO (1994) General agreement on tariffs and trade. Final act of the 1986 –1994 uruguay round of trade negotiations. Annex 1A, multilateral agreements on trade in goods. World Trade Organization (WTO), Geneva, Switzerland. http://www.wto.org/english/docs_e/legal_e/06-gatt_e.htm

95. CAC (1999) Summary and conclusions. Report of the twenty-third session, FAO headquarters, Rome, Italy, 28 June–3 July 1999. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland, pp 124. www.codexalimentarius.net/download/report/518/Al99_37e.pdf
96. CAC (2003) Alinorm 03/41. Report of the twenty-sixth session, FAO headquarters, Rome, Italy, 30 June–7 July 2003. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. ftp://ftp.fao.org/codex/alinorm03/al03_41e.pdf
97. CAC (2003) Principles for the risk analysis of foods derived from modern biotechnology. CAC/GL 44–2003. Amended in 2008. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/standards/10007/CXG_044e.pdf
98. CAC (2003) Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants. CAC/GL 45–2003. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/standards/10021/CXG_045e.pdf
99. CAC (2003) Guideline for the conduct of food safety assessment of foods produced using recombinant DNA microorganisms. CAC/GL 46–2003. Amended in 2008. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/standards/10025/CXG_046e.pdf
100. CAC (2008) Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA animals. CAC/GL 68–2008. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/standards/11023/CXG_068e.pdf
101. CAC (2008) Alinorm 08/31/REP. Report of the thirty-first session, FAO headquarters, Rome, Italy, 30 June–4 July 2008. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/report/698/al31REPe.pdf
102. CAC (2007) Alinorm 07/30/22. Report of the thirty-fifth session of the Codex committee on food labelling, Ottawa, Canada, 30 April–4 May 2007. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/report/682/al30_22e.pdf
103. CAC (2007) Working principles for risk analysis for food safety for application by governments. CAC/GL 62–2007. Codex Alimentarius Commission (CAC) of the World Health Organisation (WHO), Geneva, Switzerland. http://www.codexalimentarius.net/download/standards/10751/CXG_062e.pdf
104. FAO (1997) International plant protection convention. New revised text approved by the FAO conference at its 29th session, November 1997. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. https://www.ippc.int/file_uploaded//publications/13742.New_Revised_Text_of_the_International_Plant_Protectio.pdf
105. Secretariat of the IPPC (2004) Pest risk analysis for quarantine pests including analysis of environmental risks and living modified organisms. International plant protection convention (IPPC), Food and Agriculture Organization of the United Nations, Rome, Italy. https://www.ippc.int/file_uploaded/1146658377367_ISPM11.pdf
106. OIRSA (2000) Eighth meeting of the executive committee, 31 March 2000. Organismo Internacional Regional de Sanidad Agropecuaria (OIRSA), San Salvador, El Salvador. http://www.oirsa.org/documentos/actasyresoluciones/resoluciones/xiii-31-mar.-2000-recomendaciones.pdf
107. CGRFA (2007) Code of conduct on biotechnology as it relates to genetic resources for food and agriculture. Commission on Genetic Resources for Food and Agriculture (CGRFA), Food and Agriculture Organization of the United Nations, Rome, Italy. http://www.fao.org/ag//CGRFA/biocode.htm
108. UNECE (2001) UN recommendations on the transport of dangerous Goods – model regulations. Twelfth revised edition. United Nations Economic Commission for Europe (UNECE), Geneva, Switzerland. http://www.unece.org/trans/danger/publi/unrec/12_e.html
109. UNECE (1998) Convention on access to information, public participation in decision-making and access to justice in environmental matters (Aarhus Convention), United Nations Economic Commission for Europe (UNECE), Geneva, Switzerland. http://www.unece.org/env/pp/welcome.html
110. UNEP-GEF Biosafety Unit (2003) Guide for the implementation of national biosafety frameworks. United Nations Environment Programme (UNEP)-Global Environment Facility (GEF) Projects on Implementation of national biosafety frameworks, Geneva, Switzerland. http://www.unep.org/biosafety/Documents/Impl.Guide-RegReg.pdf
111. McLean MA, Frederick RJ, Traynor P, Cohen JI, Komen J (2002) A conceptual framework for implementing biosafety: linking policy, capacity and regulation. Briefing paper No. 47, Program for biosafety systems, International Food Policy Research Institute, Washington DC, USA. http://programs.ifpri.org/isnararchive/publications/pdf/bp-47.pdf
112. Burachik M, Traynor P (2002) An analysis of a national biosafety system: regulatory policies and procedures in Argentina. Country Report No. 63, Program for biosafety systems, International Food Policy Research Institute, Washington DC, USA. http://programs.ifpri.org/isnararchive/publications/pdf/ cr63.pdf
113. Jaffe G (2004) Regulating transgenic crops: a comparative analysis of different regulatory processes. Transgenic Res 13(1):5–19

114. UNEP-GEF Biosafety Unit (2005) Toolkit for the development project, phase 3 – Drafting the NBF. Part I: Formulation of the regulatory regime, project on development of national biosafety frameworks. United Nations Environment Programme (UNEP) – Global Environment Facility (GEF), Geneva, Switzerland. http://www.unep.org/biosafety/Documents/Drafting_the_NBF_Formulation_of_the_regulatory_regime.pdf
115. Garforth K (2005) When biosafety becomes binding. In: Cordonier Segger M-C, Weeramantry CG (eds) Sustainable justice: reconciling economic, social and environmental law. Martinus Nijhoff, Boston
116. Pew Initiative on Food and Biotechnology (2004) Issues in the regulation of genetically engineered plants and animals. Washington, DC, USA. http://www.pewtrusts.org/uploadedFiles/wwwpewtrustsorg/Reports/Food_and_Biotechnology/food_biotech_regulation_0404.pdf
117. OECD (1986) Recombinant DNA safety considerations: safety considerations for industrial, agricultural and environmental applications of organisms derived by recombinant DNA techniques. Organisation for Economic Co-operation and Development (OECD), Paris, France. http://www.oecd.org/dataoecd/45/54/1943773.pdf
118. US National Research Council (1987) Introduction of recombinant DNA-engineered organisms into the environment, Key Issues. White Paper. National Academies Press, Washington DC, USA
119. US National Research Council (2004) Safety of genetically engineered foods. National Academies Press, Washington DC, http://www.nap.edu/books/0309092094/html/

Crop Breeding for Sustainable Agriculture, Genomics Interventions in

Pawan L. Kulwal[1], Mahendar Thudi[2]
Rajeev K. Varshney[2,3,4]
[1]State Level Biotechnology Centre, Mahatma Phule Agricultural University, Rahuri, Maharashtra, India
[2]Centre of Excellence in Genomics (CEG), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, AP, India
[3]Genomics Towards Gene Discovery Sub programme, Generation Challenge Programme (GCP), Generation Challenge Programme (GCP) c/o CIMMYT, Mexico, DF, Mexico
[4]School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley, WA, Australia

Article Outline

Glossary

Association mapping Association mapping is a high-resolution method for mapping quantitative trait loci (QTLs) or gene(s) for traits of interest based on linkage disequilibrium (LD) and holds great promise for the dissection of complex genetic traits.

Back cross (BC) Back cross is a cross of the F_1 with either of the parental genotype and the resultant progeny is called BC_1. The progeny of the cross between BC_1 and the recurrent parent is called as BC_2.

Gene pyramiding Gene pyramiding is a process of accumulating the favorable genes/alleles from different genotypes into an elite/commercial cultivar. Gene pyramiding is often performed through marker-assisted selection (MAS).

Genome-wide selection or genomic selection (GS) Genome-wide selection or genomic selection is a concept for accelerating genetic gain especially for complex traits in elite genotypes by utilizing genomic information and estimating their breeding values in breeding strategies. GS is becoming very popular over marker-assisted selection that was focused on few individual genes or few QTLs to improve genotypes, especially when recent advances in genomic technologies have drastically reduced the cost on marker genotyping.

Genomics-assisted breeding (GAB) Genomics-assisted breeding is a holistic approach, where genomics technologies including molecular markers, trasncriptomics, metabolomics, proteomics, bioinformatics, and phenomics are integrated with conventional breeding strategies for breeding crop plants resistant/tolerant to biotic and abiotic stresses or improved for quality and yield.

Haplotype Haplotype is a set of alleles of closely linked loci on a chromosome that tend to be inherited together.

Linkage disequilibrium (LD) Linkage disequilibrium is a nonrandom association of alleles at different loci, describing the condition with non-equal (increased or reduced) frequency of the haplotypes in a population at random combination of alleles at different loci. LD is not the same as linkage, although tight linkage may generate high levels of LD between alleles.

Marker-assisted selection (MAS) Marker-assisted selection is a process of indirect selection for improving the traits of interest by employing morphological, biochemical, or DNA-based markers. DNA-based markers/molecular markers, in the recent past, were proven to be the markers of choice for MAS.

Narrow genetic base Narrow genetic base does frequently exists in modern crop cultivars or breeding lines due to the continuous use of small number of elite genotypes in breeding programs. In fact, it is

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

a serious obstacle to sustain and improve crop productivity due to rapid vulnerability of genetically uniform cultivars to emerging biotic and abiotic stresses.

Next-generation sequencing (NGS) technologies Next-generation sequencing (NGS) technologies include various novel sequencing technologies for example 454/FLX (Roche Inc.), ABI SOLiD (Applied Biosystems), Solexa (Illumina Inc.), etc., that have surpassed traditional Sanger sequencing in through-put and in cost-effectiveness for generating large-scale sequence data.

Polygenes Polygenes are a group of non-allelic genes, each having a small quantitative effect, that together produce a wide range of phenotypic variation.

Quantitative trait loci (QTLs) Quantitative trait loci are the loci or regions in the genome that contribute towards conferring tolerance to abiotic stresses (e.g., drought, salinity) or resistance to biotic stresses (e.g., fungal, bacterial, viral diseases) or improving agronomic traits (e.g., yield, quality) which are generally controlled by polygenes and greatly depend on gene × environmental (G × E) interactions.

Sustainable agriculture Sustainable agriculture refers to efficient agricultural production while maintaining the environment, farm profitability, and prosperity of farming communities.

Sustainable development Sustainable development is defined as balancing the fulfillment of human needs with the protection of the environment so that these needs can be met not only at the present time, but also in the future.

Definition of the Subject

There has been significant improvement in production and productivity of important cereal crops globally as a consequence of the "Green Revolution" and other initiatives [1]. However, today the stage has reached that the available traditional methods of crop improvement are not sufficient to provide enough and staple food grains to the constantly growing world population [2]. This situation is projected to be worse by the year 2050, especially in context of climate change [3]. In other words, the conventional plant breeding practices may not be able to achieve the sustainability in today's agriculture.

It is under such circumstances that advances in plant genomics research are opening up a new era in plant breeding, where the linkage of genes to specific traits will lead to more efficient and predictable breeding programs in future. Several initiatives have been started towards use of genomics technologies in number of crop plants to ensure the sustainable production of healthy and safe crops and the results are encouraging. It is therefore expected that the genomics will be the integral part of the agricultural/plant breeding practices in future for improving crop productivity leading to achieve food security and sustainable production.

Introduction and Importance of Sustainable Agriculture

The goal of agricultural science is to increase crop productivity coupled with the quality of the products, and maintain the environment [1]. Food security is a growing concern worldwide, and more than 1 billion people are estimated to lack sufficient dietary energy availability [2]. The issue of "food security" has become so important that prominent scientific journals, including Science, have also published a special issue on this subject recently (February 12, 2010 issue). With the current rate of growth, the global population is likely to plateau at some 9 billion people by roughly the middle of this century [3]. With this ever-increasing human population and amidst the fear of shrinking resources in terms of cultivable area, irrigation resources, newly emerging insect pests, stagnated yields, etc., it has become difficult to maintain agricultural sustainability. In order to make today's agriculture sustainable, it is necessary that plant breeders adopt innovative technologies that can increase the efficiency of selection with more precision [4]. Under such circumstances, molecular approaches including modern genomics and genetic engineering technologies have emerged as powerful tools to assure rapid and precise selection for the trait(s) of interest. Maintaining effective and environmentally friendly agricultural practices is a necessary prerequisite for maintaining sustainability.

Plant genomics is a rapidly developing field, which is radically improving our understanding of plant biology by making available novel tools for the improvement of plant properties relevant to sustainable agricultural production. Recent advances in high-throughput genomics technologies, including that of next-generation sequencing and high-throughput genotyping, have helped immensely in understanding the functions and regulation of genes in crop plants [5]. The ever-increasing availability of genome sequences in crop plants have facilitated greatly the development of genomic resources that will allow us to address biological functions and a number of basic processes relevant to crop production leading to sustainable agriculture.

One of the myths linked to sustainable agriculture means going back to past techniques/farm practices, which were followed by our ancestors. In fact, sustainable agriculture can be achieved by combining some of the wisdom of past practices with careful use of current technology, including the vast array of information technologies now available. Sustainable agriculture is a key element of sustainable development and is essential to the future well-being of the human race and the planet. A compelling need exists for restorative and sustainable agriculture to help address the pressing trends of population, climate, energy, water, soil, and food. Sustainable agriculture needs to be economically viable, environmentally sound, and socially acceptable. In other words, it is a system of agricultural production that, over the long term, will: (1) satisfy human food, feed, and fiber needs; (2) enhance the environmental quality and the natural resource base upon which the agricultural economy depends; (3) make the most efficient use of available technologies, nonrenewable resources, and on-farm resources, and integrate, where appropriate, natural biological cycles and controls; (4) sustain the economic viability of farm operations; and (5) enhance the quality of life for farmers and society as a whole.

There are various components of sustainable agriculture, which include technological interventions, and environmental and socio-economic factors. As the factors related to socio-economics and environments have been discussed in a number of reviews earlier, in this article, we focus on the interventions of plant genomics technologies in crop breeding.

Contribution of Plant Genomics Technologies to Crop Breeding

Plant genomics technologies have contributed immensely in today's agriculture which has led to better understanding of how plants function, and how they respond to the environment. This has also helped in achieving targeted objectives in breeding programs to improve the performance and productivity of crops. The DNA-based molecular markers have facilitated smarter and knowledge-based breeding, by enabling early-generation selection for key traits, thus reducing the need for extensive field selection. Besides this, the molecular tools can effectively be used for the characterization, conservation, and use of genetic resources.

Recent advances made in the area of molecular biology and bioinformatics offer substantial opportunities for enhancing the effectiveness of classical plant breeding programs. These tools can be integrated into breeding work in order to analyze efficiently high numbers of crosses at the early seedling stage. This approach is known as "genomics-assisted breeding" [6]. Through this approach, both the phenotype and the genotype of new varieties can be analyzed and the performance of new specific introgressed traits can be predicted. The goals of the integration of these technologies in classical breeding are to create genotype-to-phenotype trait knowledge for breeding objectives and to use this knowledge in product development and deployment for the resource poor farmer.

For successful utilization of genomics-assisted breeding approach in a crop, availability of basic molecular tools, such as molecular markers, genetic maps, etc., is a prerequisite. Among molecular markers, though a variety of molecular markers such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), microsatellite or simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), and single nucleotide polymorphism (SNP) markers have been developed in a range of crops, SSR and SNP markers have emerged as the markers of choice [7, 8]. Because of advent of NGS technologies [5] and high-throughput genotyping platforms, SNP marker system and array-based genotyping platforms are becoming more

popular [9, 10]. An overview on availability of genomic resources in some selected important crop species is shown in Table 1. It is evident that cereal crops, especially rice, maize, wheat, barley, etc., are on top in terms of availability of genomic resources (see [11]). Genome sequences have already become available for several crop species, including rice (http://rgp.dna.affrc.go.jp/IRGSP/), sorghum [12], and maize (http://gbrowse.maizegdb.org/cgi-bin/gbrowse/maize/). Recent investments coupled with advances in genomics technologies have contributed towards developing a good resource of genomics tools in legumes as well [13, 14].

Some Modern Breeding Approaches

The availability of genomic resources in almost all important crops combined with information on pedigrees as well as optimized methods of precise phenotyping make it possible to undertake genomics-assisted breeding approaches for crop improvement. In fact, some molecular breeding approaches like advanced backcross QTL (AB-QTL) analysis and marker-assisted selection (MAS) have been successfully employed in several crops, leading to improved cultivars, some other approaches such as marker-assisted recurrent selection (MARS) or genomics selection (GS) are being used in several crops [15, 16].

Marker-Assisted Selection (MAS)

There are three major steps involved in MAS: (1) identification of molecular marker(s) associated with trait(s) of interest to breeders, (2) validation of identified marker(s) in the genetic background of the targeted genotypes to be improved, and (3) marker-assisted backcrossing (MABC) to transfer the QTL/gene from the donor genotype into the targeted genotype. In context of marker-trait association, linkage mapping has been extensively used for identifying the markers associated with a trait of interest in a range of crops including cereals, legumes, horticultural crops, etc. These studies have been reviewed in detail in several reviews [14, 17] and books [18]. Although hundreds of studies have been undertaken, only a few studies were taken further to marker validation and MABC. This may be attributed to (1) identification of few markers associated with small-effect QTLs, (2) non-validation of markers in elite genotypes, and (3) slow adoption of markers by breeders in their breeding programs. Recent advances in association genetics, however, offer opportunities to overcome the first two constraints.

Association mapping (AM) is considered an alternative strategy to linkage mapping for identifying marker-trait associations and has been used extensively in human and animal systems. AM has a number of advantages over linkage mapping, including the potential for increased QTL resolution and an increased sampling of molecular variation (for reviews see [19, 20]). AM involves studying a natural population rather than the offspring of crosses, and associations in natural populations are typically on a much finer scale because they reflect historical recombination events. Several examples on marker-trait association using AM are available [21]; however, there is a need for optimization of more advanced analytical tools in the area of association genetics [22]. It is anticipated that because of reduction in costs on marker genotyping [10], AM will be extensively used for trait mapping in the future.

Once the markers associated with a trait of interest are identified through linkage mapping or AM, the next step is to use these markers in the breeding programs. In this context, the selection of one or a few genes (QTLs) through molecular markers using backcrossing is a very efficient technique [23, 24]. Important advantages of MAS are that it can be effectively utilized for traits with low heritability; for gene pyramiding, selection can be made at seedling stage; and, above all, there are no issues involving GE crops [25]. Although use of markers in breeding programs through MABC is a common practice in the private sector [26], MAS is in routine use in wheat and barley breeding programs in Australia [8, 27–29] and USA (www.maswheat.ucdavis.edu; http://barleycap.cfans.umn.edu/). Nevertheless, there are several success stories in many crops including wheat, rice, barley, maize, soybean, etc., where MAS has successfully been utilized to develop superior lines/varieties/hybrids for improving quality, resistance to diseases or tolerance to abiotic stresses. For example, Gupta et al. [17] has recently summarized success stories of molecular breeding in wheat.

A widely discussed success story of molecular breeding is the introgression of the FR13A *Sub1* locus conferring resistance against submergence in an Asian rice cultivar, Swarna [30, 31] that can confer tolerance

Crop Breeding for Sustainable Agriculture, Genomics Interventions in. Table 1 Genomic resources among selected cereals and legumes

Crop plant	Molecular markers (SSRs and SNPs)	Molecular maps (Genetic/QTL map/ comparative/physical maps)	Transcript data and expression profiling	Genome sequence data
Rice	++++[a]	++++[b,c]	++++[d]	++++[e]
Maize	++++[f]	++++[b,g,h]	++++[d]	++++[i]
Wheat	+++[j]	+++[b,k,l]	+++[d]	++[m]
Sorghum	+++[n]	++++[b,o]	+++[d]	++++[p]
Barley	+++[q]	+++[r]	+++[d,s]	+++[t]
Soybean	++++[u,v]	+++[w,x,y]	+++[z]	++++[aa]
Groundnut	++[bb]	+[cc]	+[z]	
Cowpea	+++[bb]	++[cc]	+[z]	
Common bean	++[bb]	++[cc]	+[z]	
Chickpea	+++[bb]	++[cc]	+[z]	
Pigeonpea	+++[bb]			

+, Very few; ++, Few; +++, Moderate; ++++, Abundant

[a] http://www.gramene.org/markers/index.html

[b] http://www.gramene.org/cmap/

[c] http://www.gramene.org/db/qtl/qtl_display?query=&search_field=&species=Oryza+sativa&submit=Submit

[d] http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html; http://compbio.dfci.harvard.edu/tgi/plant.html

[e] http://www.gramene.org/Oryza_sativa/Info/Index

[f] http://www.maizegdb.org/probe.php

[g] http://www.maizegdb.org/map.php

[h] http://www.gramene.org/db/qtl/qtl_display?query=*&search_field=trait_name&species=Zea+mays+subsp.+mays&submit=Submit

[i] http://www.maizesequence.org/Zea_mays/Info/Index

[j] http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker

[k] http://wheat.pw.usda.gov/GG2/maps.shtml#wheat

[l] http://wheat.pw.usda.gov/cgi-bin/graingenes/quickquery.cgi?query=qtls&arg1=*

[m] http://wheat.pw.usda.gov/cgi-bin/graingenes/search.cgi?class=sequence

[n] http://www.gramene.org/db/markers/marker_view?
marker_name=*&marker_type_id=&taxonomy=sorghum&action=marker_search&x=0&y=0

[o] http://www.gramene.org/db/cmap/map_set_info?species_acc=sorghum&map_type_acc=-1

[p] Paterson et al. [12]

[q] http://wheat.pw.usda.gov/GG2/Barley/

[r] http://wheat.pw.usda.gov/GG2/maps.shtml#barley

[s] http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=sequence&query=barley1_*

[t] http://www.public.iastate.edu/~imagefpc/IBSC%20Webpage/IBSC%20Template-home.html

[u] http://soybeanbreederstoolbox.org/

[v] http://soybase.org/BARCSOYSSR/index.php

[w] http://lis.comparative-legumes.org/cgi-bin/cmap/viewer?changeMenu=1

[x] http://soybeanbreederstoolbox.org/search/search_results.php?category=QTLName&search_term=

[y] http://soybeanphysicalmap.org/

[z] http://lis.comparative-legumes.org/lis/lis_summary.html?page_type=transcript

[aa] http://www.phytozome.net/cgi-bin/gbrowse/soybean/?name=Gm09

[bb] See Varshney et al. [13]

[cc] See Varshney et al. [14] marker assisted recurrent selection (MARS) or genomic selection (GS) are being used in several crops [15, 16].

up to 2 weeks of complete submergence. This has offered great relief to the large number of Asian farmers whose rice land is located in deltas and low-lying areas that are at risk from flooding during the monsoon season every year. Some selected examples of molecular breeding in rice and wheat (adopted from [17]) are summarized in Table 2.

Advanced Backcross QTL (AB-QTL) Analysis

Although MAS has been quite successful, it has always been a difficult task to tackle linkage drag, especially when a QTL or a gene is to be introgressed from wild/exotic species. Furthermore, in MAS, QTL/gene discovery and variety development are two separate processes. To deal with this problem and to harness the potential of the wild/unadapted germplasm in breeding programs, a new approach referred as advanced backcross QTL (AB-QTL) analysis was proposed by Tanksley and Nelson [32]. AB-QTL aims at simultaneous detection and transfer of useful QTLs from the wild/unadapted relatives to a popular cultivar for improvement of a trait. In this context, a superior cultivar/variety is crossed with a wild species leading to the production of a backcross population (BC_2, BC_3), and molecular markers are used to monitor the transfer of QTLs by conventional backcrossing. The advanced backcross approach has already been successfully utilized in different crops, including tomato [59], rice [60, 61], barley [62], and wheat [63]. It is anticipated that the use of AB-QTL will be accelerated in a range of crops for improving important traits such as disease resistance as well as yield traits.

Marker-Assisted Recurrent Selection (MARS)

In the majority of traits of interest, quantitative variation is controlled by many QTLs, each with minor effect. Moreover, minor QTLs show an inconsistent QTL effect in different environments and over different seasons. Even when the effect of these minor QTLs is consistent, their introgression into the desired genotype through MABC becomes extremely difficult as a larger number of progenies are required to select appropriate lines. In such cases, MARS has been proposed for pyramiding of superior alleles at different loci/QTLs in a single genotype [64, 65].

It was demonstrated in recent studies that the response of MARS is larger when prior knowledge of the QTLs exists and the response decreases as the knowledge of the number of minor QTL associated with the trait decreases [66]. In sweet corn, MARS was employed to fix six marker loci in two different F_2 populations which showed an increase in the frequency of marker allele from 0.50 to 0.80 [64]. Similarly, in a separate study, enrichment of rust resistance gene (*Lr34/Yr18*) with an increase in frequency from 0.25 to 0.60 was reported in wheat BC_1 through MARS [28]. MARS can be utilized effectively for selection of traits associated with multiple QTLs by increasing the frequency of favorable QTLs or marker alleles. Several companies are using MARS in their maize, soybean, etc., breeding programs [66, 67]. Recently, some institutes like International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), the French Centre for International Agricultural Research (CIRAD), and the University of California-Riverside, USA, have also initiated MARS programs in chickpea (PM Gaur, personal communication 2010), sorghum (J-F Rami, personal communication 2010), cowpea (J Ehlers, personal communication 2010), etc., for pyramiding favorable drought-tolerant alleles.

Genome-Wide or Genomic Selection (GS)

Although MAS has been practiced for the improvement of quantitative traits, it has its own limitations. Therefore, in addition to MARS, Genomic Selection can be used to pyramid favorable alleles for minor effect QTLs at the whole genome level [68, 69]. Genomic selection predicts the breeding values of lines in a population by analyzing their phenotypes and high-density marker scores. A key to the success of GS is that, unlike MABC or MARS, it calculates the marker effects across the entire genome that explains the entire phenotypic variation. In simple terms, genome-wide selection refers to marker-based selection without significance testing and without identifying of a subset of markers associated with the trait [68]. The genome-wide marker data (marker loci or haplotypes) available or generated on the progeny lines, therefore, are used to calculate genomic estimated breeding values (GEBV) as the sum of the effects of all QTLs across the genome, thereby potentially exploiting all the genetic variance for a trait [68, 69]. The GEBVs are calculated for every individual

Crop Breeding for Sustainable Agriculture, Genomics Interventions in. Table 2 Some examples of improved cultivars or varieties developed through marker-assisted introgression of important genes/QTLs in rice and wheat

Crop	Genes/QTL introgressed	Function	Variety developed/ released	Reference
Rice	*GBSS*	Unique cooking and processing quality traits including amylose content	Cadet and Jacinto	[33]
	Xa33t	Bacterial blight resistance	BC_3F_2	[34]
	Xa21	Bacterial blight resistance	Zhongyou 6 and Zhongyou 1176	[35]
	Sub1	Submergence tolerance	BC_3F_2	[30]
	Sub1	Submergence tolerance	Samba Mashuri-*Sub1*	[36]
			IR64-*Sub1*	
			TDK1-*Sub1*	
			CR1009-*Sub1*	
			BR11-*Sub1*	
	SUB1QTL	Submergence tolerance	*Sub1* introgression lines	[37]
	Piz-5+Xa21	Blast and bacterial blight resistance	BC_4F_2	[38]
	Xa4+xa5 and *Xa4+Xa7*	Bacterial blight resistance	Angke and Conde	[39]
	xa7 and *Xa21*	Bacterial blight resistance	Zhenshan97 × Minghui 63	[40]
	xa13 and *xa21*	Bacterial blight resistance, strong aroma	Pusa1460, IET18990	[41]
	xa13 and *Xa21*	Bacterial blight resistance	Improved Pusa RH1	[42]
	xa5, *xa13* and *Xa21*	Bacterial blight resistance	Improved PR106	[43]
	Xa5, xa13 and xa21	Bacterial blight resistance	BC_3F_2	[44]
	Xa5, Xa13 and Xa21	Bacterial blight resistance	IET19046	http://www.drricar.org/four_varieites.htm
	Xa4, xa8, xa13 and *Xa21*	Bacterial blight resistance	BC_1F_3	[45]
	Xa4, Xa5, Xa13 and *Xa21*	Improved bacterial blight resistance	Pusa1526–04–25	http://www.iari.res.in/?q = node/233

Crop Breeding for Sustainable Agriculture, Genomics Interventions in. Table 2 (Continued)

Crop	Genes/QTL introgressed	Function	Variety developed/ released	Reference
	–	Bacterial blight resistance	Xieyou218	[46]
	QTL	Drought-tolerant aerobic rice	MAS946–1	www.hindu.com/2007/11/17/stories/2007111752560500.htm
	q*SALTOL* and q*SUB1*	Enhanced salt and submergence tolerance	F_6	http://open.irri.org/sabrao/images/stories/conference/site/papers/apb09final00098.pdf
	QTL	Improved performance under drought	Birsa VikasDhan111 (PY 84)	http://claria13.securesites.net/News/releases/2009/may/26018.htm
Wheat	*QPhs.ccsu-3A.1* and *Lr24 +Lr28*	Preharvest sprouting tolerance and leaf rust resistance	BC_3F_3	[47]
	Lr47	Resistance to leaf rust	BIOINTA2004	[48]
	*Gpc-B*1	High grain protein content	Lillian	[49]
	Qfhs.ndsu-3AS	Resistance to fusarium head blight	Bena	[50]
	Sm1	Resistance to the insect orange blossom wheat midge	Goodeve	[51]
	Stb4	Resistance to *Septoria*	Kern	Cited from [17]
	Wsm-1	Resistance to wheat streak mosaic virus (WSMV)	Mace	[52]
	Yr15	Seedling stripe rust	$BC_3F_{2:3}$	[53]
	Qss.msub-3BL	Resistance to wheat stem sawfly	McNeal, Reeder, Hank	http://www.wheatworld.org/pdf/dubcovsky.pdf
	Bdv2	Resistance to yellow dwarf virus	Above, Avalanche, Ankor	http://www.wheatworld.org/pdf/dubcovsky.pdf
	Yr17 and Lr37	Stripe rust and leaf rust resistance	Patwin	[54]
	CreX and *CreY*	Cyst nematode resistance	F_3 progenies	[55]
	Yr36 and *Gpc-B*1	Resistant to stripe rust and high grain protein content	Westmore	[56]
	Yr17 and *Yr36*	Resistance to stripe rust	Lassik	Cited from [17]
	Lr19 and *Sr25*	Resistant to stem rust race UG99	UC1113 (PI638741)	[57]
	Yr36 and Gpc-B1	Resistance to stripe rust, high grain protein content	Farnum (WA7975)	http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1671746
	Yr15 and *Gpc-B*1	Resistance to stripe rust and high grain protein content	Scarlet (WA7994)	http://css.wsu.edu/Proceedings/2005/2005_Proceedings.pdf
	Lr1, Lr9, Lr24, Lr47	Leaf rust resistance	BC_1F_2	[58]

of the progeny based on genotyping data using a model that was "trained" from the individuals of another training populations having both phenotyping and genotyping data. These GEBVs are then used to select the progeny lines for advancement in the breeding cycle. Thus GS provides a strategy for selection of an individual without phenotypic data by using a model to predict the individual's breeding value [69].

Recently, Wong and Bernardo [70] simulated the comparative responses of phenotypic selection (PS), MARS, and GS with small population sizes in oil palm, and assessed the efficiency of each method in terms of years and cost per unit gain (i.e., the time and cost saved by these different methods over each other for making selection). They used markers significantly associated with the trait to calculate the marker scores in MARS, whereas all markers (without significance tests) to calculate the marker scores in GS. Responses to PS and GS were consistently greater than the response to MARS. Furthermore, with population sizes of $N = 50$ or 70, responses to GS were 4–25% larger than the corresponding responses to PS, depending on the heritability and number of QTLs. In terms of economics, cost per unit gain was 26–57% lower with GS than with PS when markers cost US $1.50 per data point, and 35–65% lower when markers cost $0.15 per data point. Reduction in costs in sequencing and high-throughput marker genotyping may enhance uptake of GS for crop improvement in the future.

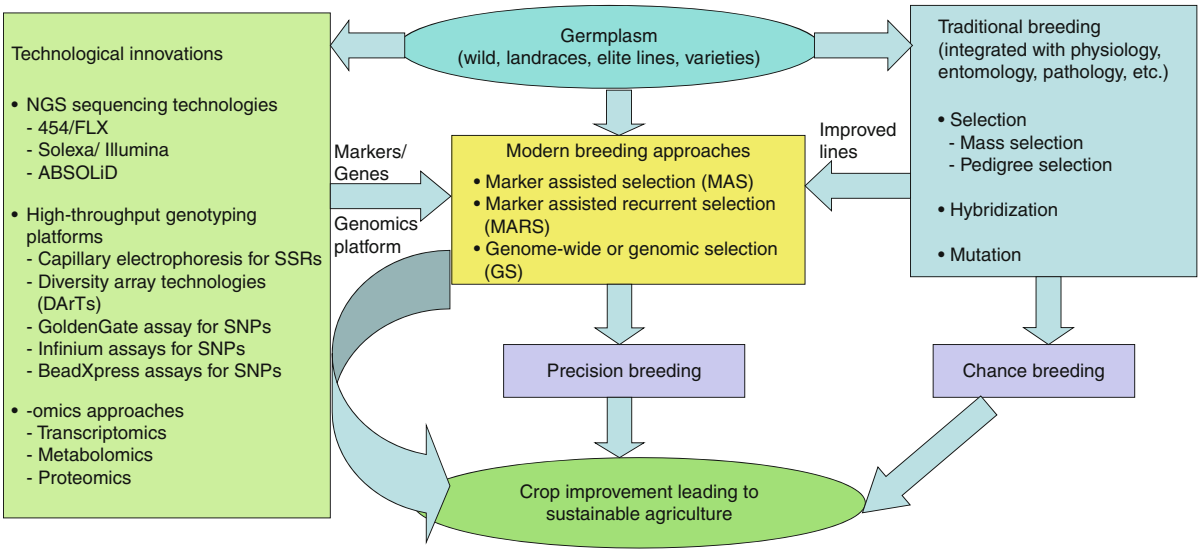
Challenges in Adoption of Genomics Technologies

Developing sustainable approaches to agriculture is one of the most difficult challenges facing growers and scientists today. Agricultural sustainability involves successful management of resources for agriculture to satisfy changing human needs, while maintaining or enhancing the quality of the environment and conserving natural resources [71]. However, sustainable production is hampered by the decline in land and soil productivity as a result of inappropriate soil and water management and other agricultural practices, as well as misguided policies and frequent opposition to technological advances that have the potential to improve the quality of life of billions of people worldwide. This is in addition to the postulated challenges of climate change, the number of hazardous chemicals (pesticides and fungicides) that are constantly being released into the environment and are becoming increasingly toxic to human and animal life [71]. In recent years, the use of promising biotechnology tools like genetic engineering (GE) has offered potential solutions to the above problems. However, the adoption of any new technique, particularly related to genetic engineering, remains a policy matter and as mentioned above faces stiff opposition many a times. In a recent review, Farre et al. [25] addressed several of these issues and advocated to overcome on the major barriers to adoption, which are political rather than technical, for realization of the potential of GE crops in developing countries.

It is thus obvious that the challenges facing agriculture are massive, particularly with the controversies over GE crops world over. It is clear that current methods of food production, in both the developing as well as the developed countries, are neither sufficient nor sustainable [72]. Under these circumstances, genomics interventions have great role to contribute to sustainable agriculture. As mentioned in this article, genomics approaches are very powerful to predict the phenotype, with higher precision and efficiency, based on the genotype. A variety of approaches ranging from MAS to GS are available to become integral part of plant breeding. While in the past, plant breeders were hesitant to use genetic variation existing in wild relatives of crop species in commercial breeding programs due to the long time it takes to recover desired phenotypes because of linkage drag, approaches like AB-QTL, in addition to MAS, can be successfully utilized. Availability of NGS technologies, associated with low costs and high-throughput, offers the opportunity to sequence either entire or major proportion of the germplasm collection for a species present in the gene banks around the word to understand genome variation. In case, the genome variation can be associated with the phenotype, which is not trivial, it will be possible to develop the ideotype, based on haplotype, of the variety to be developed.

Future Directions

While success stories of genomics-assisted breeding are available in several crops, it must also be recognized that much of the genome information generated is not being routinely used by plant breeders, especially in public breeding programs [26]. This may be due to



Crop Breeding for Sustainable Agriculture, Genomics Interventions in. Figure 1
Schematic representation of genomics technologies for crop improvement and sustainable agriculture
In general, traditional crop improvement programs (shown in the box on the right hand side in Fig. 1) employ different breeding strategies integrated with physiology, pathology, entomology, etc., and generate superior lines or improved crop varieties. These approaches, however, take more time, and sometimes such breeding is referred as "chance breeding" due to uncertainty in successes predicted in these approaches. On the other hand, genomics technologies (shown in the box on the left hand side) such as a number of next-generation sequencing (NGS) technologies, availability of high-throughput genotyping such as capillary electrophoresis for large-scale SSR genotyping, microarray-based DArTs, GoldenGate/Infinium/BeadXpress assays for large-scale SNP genotyping, and a range of -omics technologies provide candidate markers, gene(s), and QTLs to be integrated into the breeding programs by using high-throughput genomics platforms. Integrated breeding approaches (shown in the box in the middle) such as marker-assisted selection (MAS), marker-assisted recurrent selection (MARS), and genome-wide selection (GS) offer "precision breeding" with a great potential, versus "chance breeding" to contribute to sustainable crop improvement

shortage of trained personnel, inadequate access to genotyping, inappropriate phenotyping infrastructure, unaffordable bioinformatics systems, and a lack of experience of integrating these new technologies with traditional breeding [4, 26]. However, recently, several international initiatives such as the Integrated Breeding Platform (formerly Molecular Breeding Platform, www.mbp.genertaioncp.org), a joint initiative of The Bill and Melinda Gates Foundation and The Generation Challenge Program, have been started so that plant breeders especially from developing countries can have access to many genotyping, phenotyping, as well as information technologies to integrate their breeding programs with modern genomics approaches.

We believe that integration of modern genomics in combination with other cutting edge technologies in breeding programs is invaluable for crop improvement (Fig. 1) and will lead to sustainable agriculture for food security, especially in developing countries.

A vital task facing the plant breeding community today is to enhance food security in an environmentally friendly and sustainable manner. Though genomics interventions will not solve all the problems associated with agricultural production leading to sustainability, they have the potential, especially when they are used in an integrated manner as described in Fig. 1, to improve the breeding efficiency to address specific problems. These include increasing crop productivity, diversification of crops, enhancing nutritional value of food (biofortification), and reducing environmental impacts of agricultural production. However, only through judicious, rational, and science- and need-based exploitation

of genetic resources through genomic technologies coupled with conventional plant breeding and genetic engineering will lead to sustainable agriculture.

Acknowledgments

We thank the Generation Challenge Program (www.generationcp.org), the Indian Council of Agriculture Research (ICAR) and the Department of Biotechnology (DBT) of Government of India for funding various research projects (RKV) on genomics applications in breeding.

Bibliography

Primary literature

1. Briggs SP (1998) Plant genomics: more than food for thought. Proc Natl Acad Sci USA 95:1986–1988
2. Barrett CB (2010) Measuring food insecurity. Science 327: 825–828
3. Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010) Food security: the challenge of feeding 9 billion people. Science 327:812–818
4. Tester M, Langridge P (2010) Breeding technologies to increase crop production in a changing world. Science 327:818–822
5. Varshney RK, Nayak SN, May GD, Jackson SA (2009) Next generation sequencing technologies and their application for crop genetics and breeding. Trends Biotechnol 27:522–530
6. Varshney RK, Graner A, Sorrells ME (2005) Genomics-assisted breeding for crop improvement. Trends Plant Sci 10:621–630
7. Gupta PK, Varshney RK (2000) The development and use of microsatellite markers for genetics and plant breeding with emphasis on bread wheat. Euphytica 113:163–185
8. Varshney RK, Langridge P, Graner A (2007) Application of genomics to molecular breeding of wheat and barley. Adv Genet 58:121–155
9. Gupta PK, Rustgi S, Mir RR (2008) Array-based high-throughput DNA markers for crop improvement. Heredity 101:5–18
10. Varshney RK (2009) Gene-based marker systems in plants: high throughput approaches for discovery and genotyping. In: Jain SM, Brar DS (eds) Molecular techniques in crop improvement, vol II. Springer, The Netherlands, pp 119–140
11. Varshney RK, Hoisington DA, Tyagi AK (2006) Advances in cereal genomics and applications in crop breeding. Trends Biotechnol 24:490–499
12. Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, Haberer G, Hellsten U, Mitros T, Al P et al (2009) The *Sorghum bicolor* genome and the diversification of grasses. Nature 457:551–556
13. Varshney RK, Close TJ, Singh NK, Hoisington DA, Cook DR (2009) Orphan legume crops enter the genomics era! Curr Opin Plant Biol 12:202–210
14. Varshney RK, Thudi M, May GD, Jackson SA (2010) Legume genomics and breeding. Plant Breed Rev 33:257–304
15. Varshney RK, Dubey A (2009) Novel genomic tools and modern genetic and breeding approaches for crop improvement. J Plant Biochem Biotechnol 18:127–138
16. Phillips RL (2010) Mobilizing science to break yield barriers. Crop Sci 50:S99–S108
17. Gupta PK, Kumar J, Mir RR, Kumar A (2009) Marker-assisted selection as a component of conventional plant breeding. Plant Breed Rev 33:145–217
18. Kole C (2007) Genome mapping and molecular breeding in plants, a series of seven volumes: cereals and millets; oilseeds; pulses, sugar and tuber crops; fruits and nuts; vegetables; technical crops; and forest trees. Springer, Berlin/Heidelberg
19. Flint-Garcia SA, Thornsberry JM, Buckler ES IV (2003) Structure of linkage disequilibrium in plants. Ann Rev Plant Biol 54:357–374
20. Gupta PK, Rustgi S, Kulwal PL (2005) Linkage disequilibrium and association studies in higher plants: present status and future prospects. Plant Mol Biol 57:461–485
21. Buckler ES IV, Ersoz E, Yu J (2007) Applications of linkage disequilibrium and association mapping in crop plants. In: Varshney RK, Tuberosa R (eds) Genomics assisted crop improvement, vol I, Genomics approaches and platforms. Springer, Dordrecht, pp 97–119
22. Rafalski JA (2010) Association genetics in crop improvement. Curr Opin Plant Biol 13:174–180
23. Collard BCY, Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Phil Trans R Soc B 363:557–572
24. Varshney RK, Hoisington DA, Nayak SN, Graner A (2009) Molecular plant breeding: methodology and achievements. In: Somers D, Langridge P, Gustafson PJ (eds) Methods in molecular biology: plant genomics. Humana, Totowa, pp 283–304
25. Farre G, Ramessar K, Twyman RM, Capell T, Christou P (2010) The humanitarian impact of plant biotechnology: recent breakthroughs *vs* bottlenecks for adoption. Curr Opin Plant Biol 13:219–225
26. Ribaut J-M, de Vicente MC, Delannay X (2010) Molecular breeding in developing countries. Curr Opin Plant Biol 13:213–218
27. Langridge P (2003) Molecular breeding of wheat and barley In: Tuberosa R, Phillips RL, Gale M (eds) Proceedings of the international congress "In the wake of the double helix: from the green revolution to the gene revolution," Bologna, Italy, 27–31 May 2003, pp 279–286
28. Kuchel H, Fox R, Reinheimer J, Mosionek L, Willey N, Bariana H, Jefferies S (2007) The successful application of a marker-assisted wheat breeding strategy. Mol Breed 20:295–308
29. Cakir M, McLean R, Wilson R, Barclay I, Moore C, Loughman R, Haley S, Kidwell K, Anderson J, Sorrells M, Manes Y, Hayden M, Feuille C, William M (2007) Genome level targeted breeding in wheat. In: Plant and Animal Genomes conference XV,

San Diego, 13–17 Jan 2007 (http://www.intl-pag.org/15/abstracts/PAG15_W40_265.html)
30. Neeraja CN, Maghirang-Rodriguez R, Pamplona A, Heuer S, Collard BCY, Septiningsih EM, Vergara G, Sanchez D, Xu K, Ismail AM, Mackill DJ (2007) A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. Theor Appl Genet 115:767–776
31. Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM et al (2006) *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 442:705–708
32. Tanksley SD, Nelson JC (1996) Advanced backcross QTL analysis: a method for simultaneous discovery and transfer of valuable QTL from unadapted germplasm into elite breeding lines. Theor Appl Genet 92:191–203
33. Hardin B (2000) Rice breeding gets marker assists. Agricultural Research Magazine 48:12, USDA-ARS, USA (www.ars.usda.gov/is/AR/archive/dec00/rice1200.pdf)
34. Natarajkumar P, Sujatha K, Laha GS, Viraktamath BC, Reddy CS, Mishra B, Balachandran SM, Ram T, Srinivasarao K, Hari Y, Sundaram RM (2010) Identification of a dominant bacterial blight resistance gene from *Oryza nivara* and its molecular mapping. Rice Genetics Newsletters 25:54–56
35. Cao L, Zhuang J, Zhan X, Zeng K, Cheng S, Cao LY, Zhuang JY, Zhan D, Zheng KL, Cheng SH (2003) Hybrid rice resistance to bacterial blight developed by marker assisted selection. Chin J Rice Sci 17:184–186
36. Septiningsih EM, Pamplona AM, Sanchez DL, Neeraja CN, Vergara GV, Heuer S, Ismail AM, Mackill DJ (2009) Development of submergence-tolerant rice cultivars: the *Sub1* locus and beyond. Ann Bot 103:151–160
37. Singh S, Mackill DJ, Ismail AM (2009) Responses of *SUB1* rice introgression lines to submergence in the field: Yield and grain quality. Field Crops Res 113:12–23
38. Narayanan NN, Baisakh N, Vera Cruz CM, Gnanamanickam SS, Datta K, Datta SK (2002) Molecular breeding for the development of blast and bacterial blight resistance in rice cv. IR50. Crop Sci 42:2072–2079
39. Toennissen GH, Toole JCO, DeVries J (2003) Advances in plant biotechnology and its adoption in developing countries. Curr Opin Plant Biol 6:191–198
40. He Y, Li X, Zhang J, Jiang G, LiuS, Chen S, TuJ, Xu C, Zhang Q (2004) Gene pyramiding to improve hybrid rice by molecular-marker techniques. In: New directions for a diverse planet: proceedings 4th international crop science congress, Brisbane, Australia (http://www.cropscience.org.au/icsc2004/)
41. Gopalakrishnan S, Sharma RK, Anand Rajkumar K, Joseph M, Singh VP, Singh AK, Bhat KV, Singh NK, Mohapatra T (2008) Integrating marker assisted background analysis with foreground selection for identification of superior bacterial blight resistant recombinants in Basmati rice. Plant Breed 127:131–139
42. Basavaraj SH, Singh VK, Singh A, Singh A, Singh A, Anand D, Yadav S, Ellur RK, Singh D, Gopalakrishnan S, Nagarajan M, Mohapatra T, Prabhu KV, Singh AK (2010) Marker-assisted improvement of bacterial blight resistance in parental lines of Pusa RH10, a superfine grain aromatic rice hybrid. Mol Breed 26:293–305
43. Singh S, Sidhu S, Huang N, Vikal Y, Li Z, Brar DS, Dhaliwal HS, Khush GS (2001) Pyramiding three bacterial blight resistance genes (*xa5, xa13 and Xa21*) using marker assisted selection into *indica* rice cultivar PR106. Theor Appl Genet 102:1011–1015
44. Sundaram RM, Vishnupriya MR, Laha GS, Rani NS, Rao PS, Balachandran SM, Reddy GA, Sarma NP, Sonti RV (2009) Introduction of bacterial blight resistance into Triguna a high yielding, mid-early duration rice variety. Biotechnol J 4:400–407
45. Joseph M, Gopalakrishnan S, Sharma RK, Singh VP, Singh AK, Singh NK, Mohapatra T (2004) Combining bacterial blight resistance and Basmati quality characteristics by phenotypic and molecular-marker assisted selection in rice. Mol Breed 13:377–387
46. Cheng S-H, Zhuang J-Y, Cao L-Y, Chen S-G, Peng Y-C, Fan Y-Y, Zhan XD, Zheng KL (2004) Molecular breeding for super rice. Chinese J Rice Sci 18:377–383
47. Kumar J, Mir RR, Kumar N, Kumar A, Mohan A, Prabhu KV, Balyan HS, Gupta PK (2009) Marker assisted selection for pre-harvest sprouting tolerance and leaf rust resistance in bread wheat. Plant Breed doi:10.1111/j.1439-0523.2009.01758.x
48. Bainotti C, Fraschina J, Salines JH, Nisi JE, Dubcovsky J, Lewis SM, Bullrich L, Vanzetti L, Cuniberti M, Campos P, Formica MB, Masiero B, Alberione E, Helguera M (2009) Registration of 'BIOINTA2004' wheat. J Plant Registr 3:165–169
49. DePauw RM, Townley-Smith TF, Humphreys G, Knox RE, Clarke FR, Clarke JM (2005) Lillian hard red spring wheat. Can J Plant Sci 85:397–401
50. Elias EM (2005) Fusarium resistant tetraploid wheat. United States Patent Application 20050273875
51. DePauw RM, Knox RE, Thomas JB, Smith M, Clarke JM, Clarke FR, McCaig TN, Fernandez MR (2009) Goodeve hard red spring wheat. Can J Plant Sci 89:937–944
52. Graybosch RA, Peterson CJ, Baenziger PS, Baltensperger DD, Nelson LA, Jin Y, Kolmer J, Seabourn B, French R, Hein G, Martin TJ, Beecher B, Schwarzacher T, Heslop-Harrison P (2009) Registration of 'Mace' hard red winter wheat. J Plant Registr 3:51–56
53. Randhawa HS, Mutti JS, Kidwell K, Morris CF, Chen X, Gill KS (2009) Rapid and targeted introgression of genes into popular wheat cultivars using marker-assisted background selection. PLoS ONE 4:e5752
54. Helguera M, Khan IA, Kolmer J, Lijavetzky D, Zhong-Qi L, Dubcovsky J (2003) PCR assays for the *Lr37-Yr17-Sr38* cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. Crop Sci 43:1839–1847
55. Barloy D, Lemoine J, Abelard P, Tanguy AM, Rivoal R, Jahier J (2007) Marker-assisted pyramiding of two cereal cyst nematode resistance genes from *Aegilops variabilis* in wheat. Mol Breed 20:31–40
56. Brevis JC, Dubcovsky J (2008) Effect of the Gpc-B1 region from *Triticum turgidum* ssp. *dicoccoides* on grain yield, thousand grain weight and protein yield. In: Appels R, Eastwood R, Lagudah E, Langridge P, Mackay M, McIntyre L, Sharp P (eds) Proceedings of 11th international wheat genet symposium,

Brisbane, Australia, 24–29 Aug 2008. Sydney University Press, pp 1–3 (http://hdl.handle.net/2123/3179)
57. Yu L-X, Abate Z, Anderson JA, Bansal UK, Bariana HS, Bhavani S, Dubcovsky J, Lagudah ES, Liu S, Sambasivam PK, Singh RP, Sorrells ME (2009) Developing and optimizing markers for stem rust resistance in wheat. The Borlaug Global Rust Initiative 2009 Technical Workshop, 17–20 March 2009, Ciudad Obregon, Mexico
58. Nocente F, Gazza L, Pasquini M (2007) Evaluation of leaf rust resistance genes *Lr1, Lr9, Lr24, Lr47* and their introgression into common wheat cultivars by marker-assisted selection. Euphytica 155:329–336
59. Tanksley SD, Grandillo S, Fulton TM, Zamir D, Eshed Y, Petiard V, Lopez J, Beck-Bunn T (1996) Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium*. Theor Appl Genet 92:213–224
60. Xiao J, Li J, Grandillo S, Ahn S, Yuan L, Tanksley SD, McCouch SR (1998) Identification of trait-improving quantitative trait loci alleles from a wild rice relative, *Oryza rufipogon*. Genetics 150:899–909
61. Moncada P, Martínez CP, Borrero J, Chatel M, Gauch H Jr, Guimaraes E, Tohme J, McCouch SR (2001) Quantitative trait loci for yield and yield components in an *Oryza sativa* × *O. rufipogon* BC_2F_2 population evaluated in an upland environment. Theor Appl Genet 102:41–52
62. Pillen K, Zacharias A, Léon J (2003) Advanced backcross QTL analysis in barley (*Hordeum vulgare* L.). Theor Appl Genet 107:340–352
63. Huang XQ, Cöster H, Ganal MW, Röder MS (2003) Advanced backcross QTL analysis for the identification of quantitative trait loci alleles from wild relatives of wheat (*Triticum aestivum* L.). Theor Appl Genet 106:1379–1389
64. Edwards M, Johnson L (1994) RFLPs for rapid recurrent selection. In: Analysis of molecular marker data. Joint plant breeding symposia series, ASA, Madison, pp 33–40
65. Bernardo R, Charcosset A (2006) Usefulness of gene information in marker-assisted recurrent selection: a simulation appraisal. Crop Sci 46:614–621
66. Ragot M, Gay G, Muller J-P, Durovray J (2000) Efficient selection for the adaptation to the environment through QTL mapping and manipulation in maize. In: Ribaut J-M, Poland D (eds) Molecular approaches for the genetic improvement of cereals for stable production in water-limited environments. CIMMYT, México DF, pp 128–130
67. Ribaut J-M, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations, and alternative. J Exp Bot 58:351–360
68. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using dense marker maps. Genetics 157:1819–1829
69. Heffner EL, Sorrells ME, Jannink J-L (2009) Genomic selection for crop improvement. Crop Sci 49:1–12
70. Wong CK, Bernardo R (2008) Genome-wide selection in oil palm: increasing selection gain per unit time and cost with small populations. Theor Appl Genet 116:815–824
71. Gregory P (1989) Sustainability and CIP's research. CIP Circular 17,2: 8–11, CIP, Lima, Peru
72. Charlafti I (2003) Science and sustainability. EMBO Rep 4: 454–456

Books and Reviews

Bradford KJ, Van Deynze A, Gutterson N, Parrott W, Strauss SH (2005) Regulating transgenic crops sensibly: lessons from plant breeding, biotechnology and genomics. Nat Biotech 23:439–444

Gupta PK, Varshney RK (2004) Cereal genomics. Kluwer, The Netherlands

Hall D, Tegström C, Ingvarsson PK (2010) Using association mapping to dissect the genetic basis of complex traits in plants. Brief Funct Genom Pro 9:157–165

Innes NL (2008) A plant breeding contribution to sustainable agriculture. Ann Appl Biol 126:1–18

Moose SP, Mumm RH (2008) Molecular plant breeding as the foundation for 21st century crop improvement. Plant Physiol 147:969–977

Persley GJ, Peacock J, van Montagu M (2002) Biotechnology and sustainable agriculture. ICSU series on science for sustainable development No. 6, ICSU

Reganold JP, Papendick RI, Parr JF (1990) Sustainable agriculture. Sci Am 262:112–120

Ribaut J-M (2007) Drought adaptation in cereals. Food Products Press, Binghamton

Salmeron J, Herrera-Estrella LR (2006) Plant biotechnology: fast-forward genomics for improved crop production. Curr Opin Plant Biol 9:177–179

Serageldin I (1999) Biotechnology and food security in the 21st century. Science 285:387–389

Varshney RK, Tuberosa R (2007) Genomics-assisted crop improvement, vol I, Genomics approaches and platforms. Springer, The Netherlands

Varshney RK, Tuberosa R (2007) Genomics-assisted crop improvement, vol II, Genomics Applications in Crops. Springer, The Netherlands

Crop Development Related to Temperature and Photoperiod

Marc Moragues[1], Gregory S. McMaster[2]
[1]Department of Soil and Crop Sciences, Colorado State University, Fort Collins, CO, USA
[2]USDA-ARS, Agricultural Systems Research Unit, Fort Collins, CO, USA

Article Outline

Glossary

Base temperature Lower temperature threshold below which development ceases.

Epigenetics Genetic information other than DNA sequence information.

Phenology Study of the sequence of developmental stages of a plant and how it relates to climate.

Photoperiod sensitivity Requirement for a minimum (or maximum) day length for reproductive phase induction.

Phytomer Fundamental building block of plant canopies. A vegetative phytomer is comprised of leaf, node, internode, and axillary bud.

Phyllochron Rate of appearance of leaves on a shoot.

Shoot apex The tip of the shoot where usually there is meristematic tissue producing new organs.

Thermal time Temperature response curve used to estimate development rate.

Vernalization sensitivity Requirement for a period of low temperatures for reproductive induction.

Definition of the Subject

Plant development, or the progression of plants through their life cycle, has been of great interest in human history because of the need to know and predict when the harvested part of the plant was at the optimum stage. This knowledge was especially important (even vital) in medicinal plants, where the timing of harvesting defines the medicinal value of the product. This interest increased as groups moved from hunting and gathering to agrarian societies.

Crop development can be defined with the number and rate of appearance, growth, and senescence of phytomers. However, that definition lacks information about when the switch of vegetative to reproductive phytomers occurs, which is defined by the phenology of the crop. Crop development is of great importance in agriculture because it is the main mechanism for plants to escape both biotic and abiotic stresses, and adapt to the environment. At a more practical level, it affects the management of the crop because cultural practices are more effective at specific stages of crop development.

Introduction

Major food, feed, and industrial crops were domesticated in a few centers of origin, including the "Fertile Crescent," the Americas, and China. The wild relatives of modern crops were adapted to survival in the environment prevalent in the center of origin. Those original crops were locally grown in the region of origin, but some species showed a significant ability to adapt to new environments and were spread globally with human migrations and trade. For instance, wheat was domesticated in the Middle East (the "Fertile Crescent") [1, 2], around 32°N and is currently cultivated from between 30°N and 60°N and from 27°S to 40°S. However, it can grow beyond those limits, in lower latitudes, in high altitudes, or even in the arctic circle. This range of environments where wheat can grow makes wheat one of the most plastic crops currently grown. It requires a large range of developmental mechanisms to adapt to such different environments, where photoperiods vary from 13–14 h to nearly 20 h. One of the major accomplishments of the "Green Revolution" was the discovery of photoperiod insensitive mutants in wheat that could be grown at lower latitudes.

Plasticity in development is key for the adoption of crops in a wide range of environments. Although growth and development are related, they are different processes. Development is the initiation and

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

differentiation of organs and the progression of stages through which cells, organs, and plants go during their life cycle, whereas growth is the change in size or weight of the initiated organs. Biotic (e.g., genetics, weeds, and diseases) and abiotic (e.g., temperature, light, water, and nutrients) factors influence the initiation, growth, and senescence of plant organs.

Since the "The Metamorphosis of Plants" by Johann Wolfgang von Goethe, originally published in 1790 [3], there has been extensive research on how plants proceed from germination to maturity in an orderly and predictable manner. This research has led to an extensive conceptual framework of plant development resulting in many tools to predict plant development. Plant shoots develop by forming a series of nearly identical building blocks, called phytomers [4, 5]. The vegetative phytomer is associated to a leaf, and phytomers are produced in an orderly manner on a shoot; for example, the phytomer of leaf 2 is formed after the phytomer of leaf 1.

Phenology is the study of the plant (or animal) life cycle and how it is influenced by seasonal and interannual variations in climate. The phenology of a crop is defined by the sequence of stages, which, in turn, define phases. Identification of certain stages may require examination of the shoot apex. For example, after germination, the apical meristem produces vegetative structures such as leaf primordia. When temperature and photoperiod requirements are met, the shoot apex will start initiating reproductive structures (e.g., spikelet and floret primordia).

The rate of appearance of phytomers and changes at the shoot apex are regulated by the genetics of the plant, the environment, and often an interaction of both. The main environmental drivers of plant development are temperature and photoperiod; their effects on phenology interact with the genetics of the plant responses by photoperiod sensitivity genes, vernalization genes, and earliness *per se*.

This entry covers how plant development is regulated by temperature and photoperiod. Temperate cereals are emphasized because they are adapted to a wide array of environments and show a diverse set of adaptive mechanisms regulating their development. However, defining plant parts and how canopies are built as well as the sequence of events throughout the crop cycle is required before their regulation can be explained. This entry starts by defining the canopy structure and the sequence of events that define the developmental processes of a crop, followed by the current knowledge on how temperature (including vernalization) and photoperiod regulate crop development. This entry finalizes with two important parts: modeling crop development and future directions. Modeling crop development is twofold important; first to understand the physiology and genetic basis of crop development, and second to predict when key developmental events are likely to happen as accurately as possible.

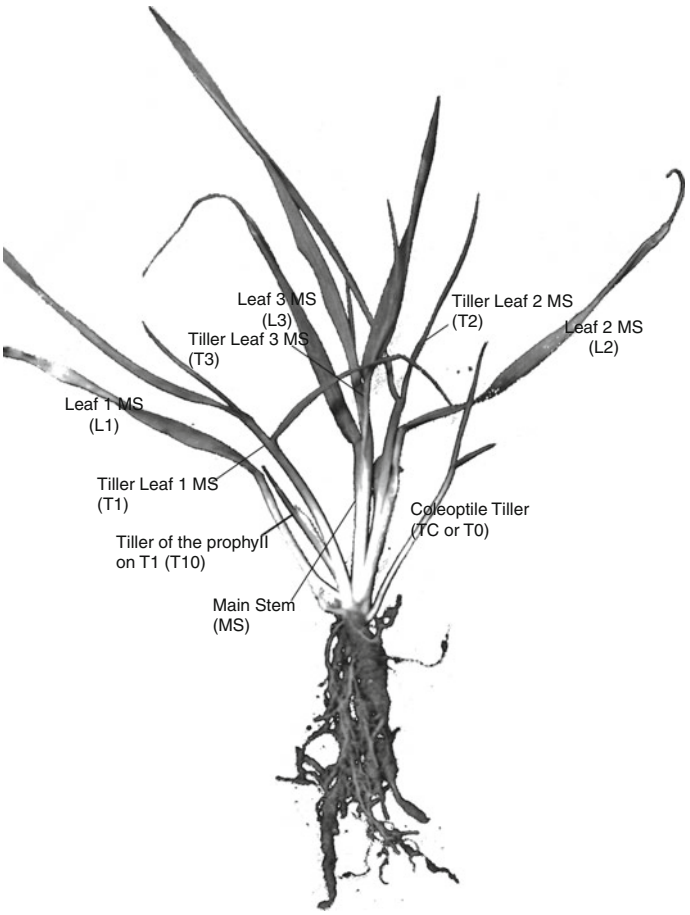
Canopy Structure: Phytomers, Phyllochron, and Plastochron

The phytomer is considered the basic building block of plant canopies and is most commonly defined as the leaf, node, internode above the node, and the axillary bud [6]. Therefore, canopy architecture and development is determined by the dynamic appearance, growth, and abortion/senescence of phytomers (and components of the phytomer). Phytomers originate at the shoot apex with the initiation of a leaf, and the potential for a new shoot is formed with the presence of the axillary bud. The growth and differentiation of each component of a phytomer will lead to their visual appearance. For example, the internode can continue differentiating and growing, resulting in its appearance from the leaf sheath. Tillers may appear when the axillary bud differentiates and grows.

Naming Plant Parts

Because plant development is an orderly process, accurately identifying plant parts aids in describing the process and quantifying the developmental rate.

Several naming systems of plant parts have been proposed, but most are quite similar. For example, true leaves can be numbered acropetally for each shoot with an *L* [7–9] (Fig. 1) beginning with the first foliar leaf, L1 [9]. Similarly, Jewiss [7] proposed a system for naming tillers that has been modified and extended by many, but the modified system proposed by Klepper et al. [8, 9] is increasingly being adopted. This system uses the leaf axil number of the parent shoot to name the tiller. The first shoot to appear from the seed is the main stem (MS).



Crop Development Related to Temperature and Photoperiod. Figure 1
Naming leaves and tillers of a winter wheat plant

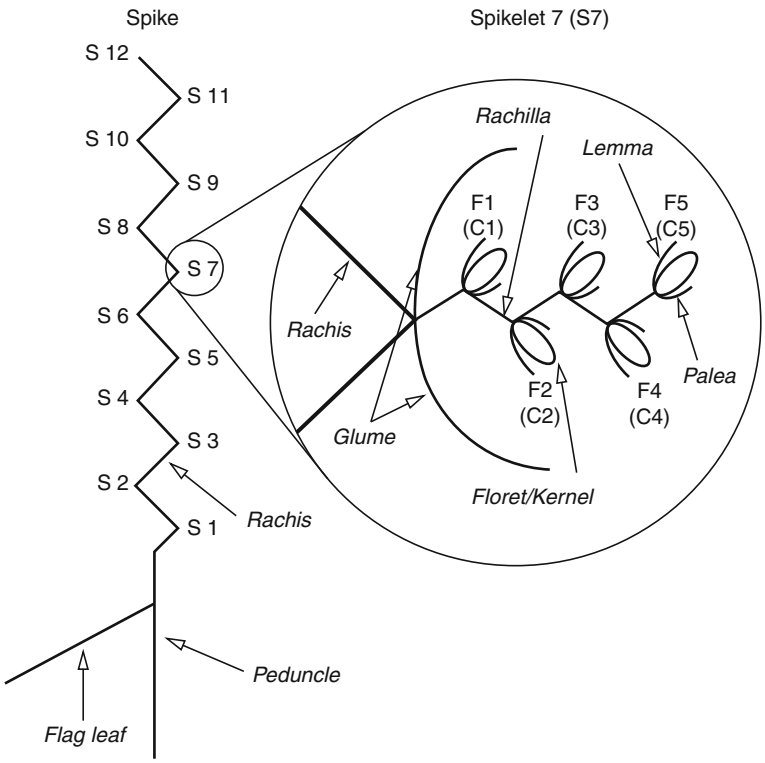
Tillers appearing from the axils of leaves on the main stem are primary tillers and are named with a T and a digit corresponding to the leaf number. For example, the tiller appearing from the first leaf (L1) on the main stem is called T1. This system is however limited by the potential production of tillers from the axil of leaf 10 and above, which rarely happens. Primary tillers can produce tillers that are called secondary tillers. Secondary tillers can arise from the axil of the prophyll of primary tillers and their second digit is a zero (Fig. 1).

Haun [10] proposed a numerical leaf staging system to quantify the number of leaves appearing on the main stem (which can be extended to any shoot):

$$\text{Haunstage} = (n-1) + \frac{L_n}{L_{n-1}}, \left(0 < \frac{L_n}{L_{n-1}} \leq 1\right) \quad (1)$$

where n is the number of leaves that have appeared on a shoot, L_{n-1} is the blade length of the penultimate leaf, and L_n is the blade length of the youngest visible leaf extending from the sheath of the penultimate leaf. Therefore, when a shoot is identified, it can be further characterized using the Haun system. For example, the Haun stage for shoots on the plant shown in Fig. 1 is: MS (5.3), T0 (1.5), T1 (2.4), T2 (1.4), and T3 (0.7). The Haun stage of a tiller with one leaf not fully unfolded is arbitrarily assigned as 0.1 or 0.9 when only the tip of the leaf is visible or most of the leaf blade has appeared, respectively.

Similarly, the leaf and tiller naming scheme has been extended to the wheat inflorescence. Klepper et al. [11] defined a numerical index for the development of the inflorescence and Wilhelm and



Crop Development Related to Temperature and Photoperiod. Figure 2
Naming scheme for reproductive organs of spike inflorescence. Spikelet positions are denoted by the letter S and numbered acropetally along the rachis. Florets/caryopsis positions are denoted by the letters F/C and numbered acropetally along the rachilla (From Wilhelm and McMaster [12])

McMaster [12] extended it to uniquely identify each plant part. Spikelets are named with an S followed by the position from the base of the spike. Then, S1 is the basal spikelet and S2 is the second spikelet from the peduncle (Fig. 2). Florets are designated with an F and numbered acropetally from the base of the rachilla. After fertilization, the letter F designating a floret is changed to a C for caryopsis. This system allows naming reproductive structures in grasses with one spikelet per rachis node such as wheat.

These naming systems of leaves, tillers, spikelets, and florets or caryopsis allow the accurate identification of each plant part. For example, the second caryopsis on the third spikelet on the primary tiller from the axil of the second leaf of a wheat plant would be T2S3C2.

The systems for naming individual plant organs can be easily extended to name vegetative and reproductive wheat phytomers [13]. A phytomer would be denoted with a "P" followed by an "L," if it is a phytomer associated with a leaf or an "S," if it is a reproductive phytomer and the leaf or spikelet number. The name of the shoot can be added to identify the tiller being described. For example, MS PL2 is the second phytomer on the main stem and T1 PS1 is the basal spikelet phytomer in the spike of the first tiller.

Dynamic Appearance of Plant Organs: The Plastochron and Phyllochron

The naming systems of plant parts described earlier are the landmarks to describe the development and structure of a wheat plant. However, plant development is a dynamic process that follows the formation, growth, and senescence of phytomers and their components resulting in a continually changing architecture.

The creation of the vegetative phytomer is dependent on the initiation of the leaf primordium. Therefore, the rate of leaf primordia initiation controls the timing of phytomer formation. The plastochron was first defined as the interval between the formation of two successive internode cells of the green alga *Nitella flexilis* [14], as cited in [15]. Milthorpe [16] and Esau [17] defined the plastochron as the interval between the formations of successive leaf primordia at the shoot apex, which is now commonly accepted. Similarly, the phyllochron was defined as the interval between appearances of consecutive leaves on a shoot [18] as cited in [6]. Wilhelm and McMaster [6] further refine the definition of phyllochron by defining appearance as "visible without magnification, dissection or changing leaf display." The inverse of the phyllochron is termed development rate (DR), which can be generalized as the inverse of the time interval between two developmental events. The relationship between the plastochron and the phyllochron depends on the species. In wheat, leaf primordia are produced more rapidly than they appear, suggesting that different mechanisms are involved in regulating each process. Leaf primordia are initiated at the meristem on the shoot apex, where new cells are produced very quickly. After a leaf primordium is formed, it continues to grow in cell number and cell size, but this growth does not happen at the shoot apex meristem, rather at the intercalary meristem at the base of the leaf. The amount a leaf grows until it appears through the curl of leaves is much larger than the growth involved in forming a leaf primordia, therefore it is more dependent on the available resources (e.g., water, carbohydrates, and nutrients).

Developmental Stages and Phases: Phenology

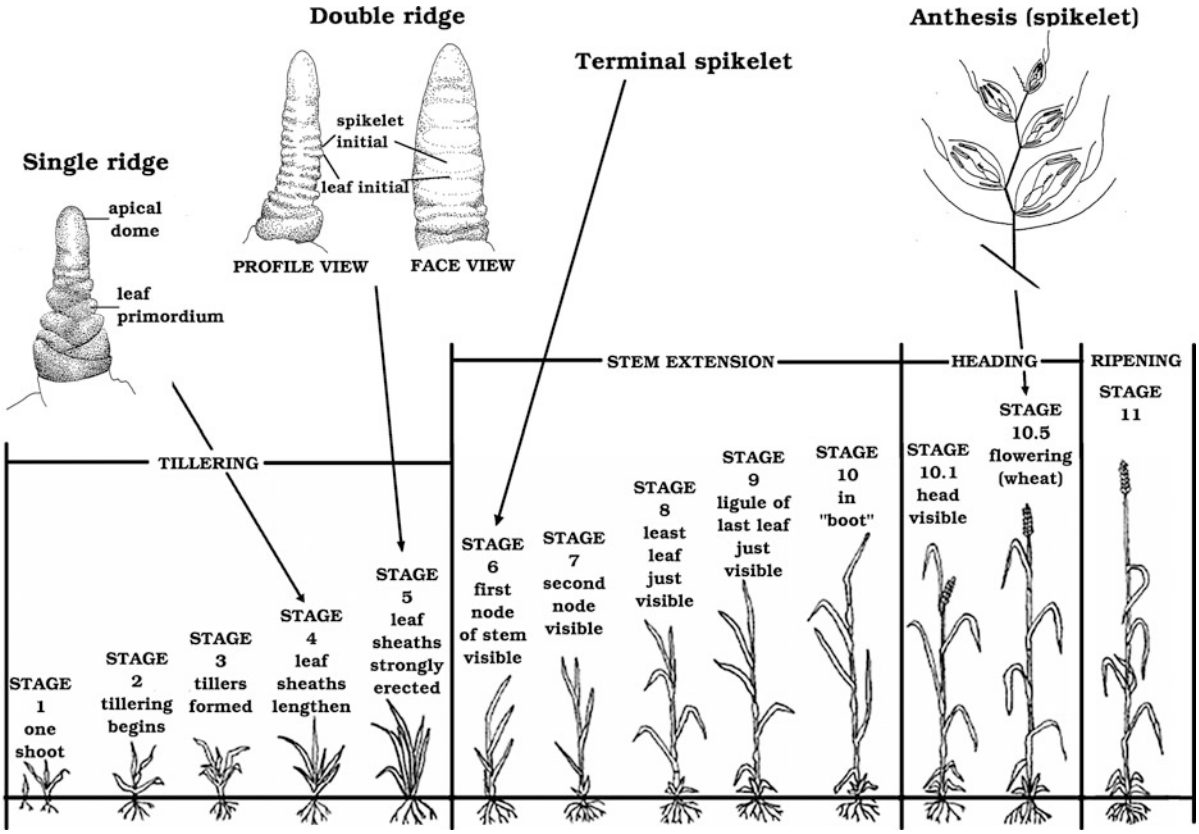
Plants develop by the repetition of elementary building blocks (i.e., phytomers), whose morphological, dimensional, functional, and anatomical features change during ontogeny and according to several processes called heteroblasty, phase change, life stages, maturation, aging, age states, or morphogenetic progression [19]. In this entry, those changes will be referred as *developmental stages* (or simply *stages*) and the time between stages will be referred as *developmental phases* (or *phases*). It is common in the literature referring to these stages as "growth stages." However, there is little growth in some developmental events such as anthesis. The phenology of a crop is the ordered succession of stages and phases that can have different lengths determined by internal factors (e.g., the genetics of a variety or species) or biotic and abiotic external factors (e.g., diseases, temperature, light, and nutrients).

Plant development can broadly be divided into vegetative and reproductive phases that often overlap. The switch from vegetative to reproductive phase happens at the meristem level, which stops producing vegetative phytomers (i.e., leaves, nodes, and internodes) to start producing reproductive phytomers (rachis, spikelets, and florets).

Phenological Scales

Developmental stages occur in a consistent pattern in a crop each year and numerous approaches exist that characterize crop phenology. The most widely used phenological scales for temperate cereals are Feekes [20], Zadoks [21], Haun [10], and BBCH [22]. The Feekes scale is shown in Fig. 3 and described, jointly with the Zadoks' decimal code, in Table 1. The BBCH scale for cereals is mainly based on the Zadoks scale, and the Haun scale primarily describes the development of shoots until the last leaf is fully expanded.

Phenological scales consider basic developmental stages like germination, emergence, tillering, stem elongation, heading, flowering, grain filling, and physiological maturity, with differences among scales primarily in how much detail each stage is characterized. Some developmental stages are not well defined, leading to confusion in measuring and reporting these stages. For example, the beginning of stem elongation is usually recorded as the jointing date or when the first node is visible above ground; however, the first node is formed when the apex is underground and is only visible after the stem has elongated sufficiently to elevate the apex and the node above ground. Likewise, physiological maturity is defined as when the maximum dry weight is reached. In wheat, determining physiological maturity is somewhat difficult because there is not a clear morphological change in the plant morphology as it happens in maize (and sunflower). In maize, a black layer near the base of the kernel appears when the maximum dry weight is reached. The Feekes scale defines harvest maturity as when the grain is



Crop Development Related to Temperature and Photoperiod. Figure 3
Feekes developmental scale with the approximate timing of some shoot apex developmental events (From [13])

difficult to divide along the crease and Zadoks [21] defines the 90% of ripeness of rice (*Oryza sativa* L.) when the kernel cannot be dented with the fingernail. These definitions likely are not precisely correlated with maximum seed biomass. Therefore, it is now commonly accepted practice to assume physiological maturity for temperate cereals occurs when all green color has disappeared from the spike. This definition seems reasonable as leaves and internodes have long since lost all green color so that no photosynthesis occurs, and there is no report showing retranslocation from carbohydrate reserves to the grain at this time.

Regulation of Crop Development

Plant development is highly dependent on temperature, which controls the rate of development and the switch from vegetative to reproductive states. Both high and low temperatures may have a major effect on plant development, especially controlling the switch to the reproductive state. For example, winter wheat requires a period of low temperatures to start producing reproductive structures (vernalization). The length of the cold period varies with genotype.

Besides vernalization, day length (or photoperiod) modifies the temperature-controlled rate of development. Photoperiod refers to the number of daylight hours, which changes through the seasons and with latitude. Photoperiod increases after the winter solstice (December 21 in the northern hemisphere or June 21 in the southern hemisphere) and decreases after the summer solstice. Crops and genotypes vary in their sensitivity to photoperiod and the minimum amount of daylight required to switch from vegetative to reproductive phases.

Developmental Response to Temperature

The relationship between temperature and phenology has been long recognized. Temperature is a better

Crop Development Related to Temperature and Photoperiod. Table 1 Description of the main developmental stages according to Feekes [20] and Zadoks [21], and suggested measurements characteristics (Modified from [23])

Stage or phase	Description		Measurement characteristics
	Feekes	Zadoks	
Germination	No stage	Stages: 00 – dry seed, 01 – beginning of imbibition, 03 – imbibition complete, 05 – radicle emerged from caryopsis, 07 – coleoptile emerged from caryopsis	Beginning of imbibition: Seed begins to swell
Emergence	Stage 1 – main shoot only	Stage 09 – leaf at the tip of the coleoptile	Beginning of emergence: First true leaf emerges through the coleoptile and the tip is visible above the soil surface
Tillering	Stage 2 – beginning of tillering	Stages 21–29 – main stem plus 1 to 9 tillers	Beginning of tillering: The first tiller is visible
Single ridge	No stage	No stage	Shoot apex shape changes from dome to more elongated and leaf primordia begin to form a ridge around the apex
Double ridge	No stage	No stage	Formation of double ridges around the apex. Bottom ridge is leaf primordia and top ridge is spikelet primordia
Terminal spikelet	No stage	No stage	Apical spikelet primordium appears and noted by a 90° rotation from the plane of previous spikelets
Jointing	Stage 6 – first node visible	Implicit in stage 31 – first node detectable and change of plant habit from prostrate to erect	First node visible above the soil surface
Stem elongation	Stages: 4 – change of plant habit from prostrate to erect, 5 – pseudo-stem clearly erect, 6 – first node visible, 7 – second node visible, 8 – last leaf appearance, 9 – last leaf ligule visible, 10 – last leaf sheath swelling	Stages: 30 – pseudo-stem erect, 31 to 36 first to sixth node detectable, 39 – flag leaf ligule visible, 43 to 49 – flag leaf sheath swelling	Beginning when the first node is formed, usually below soil surface, and pushed above the soil surface
Flag leaf	Stage 9 – last leaf ligule visible	Stage 39 – Flag leaf ligule visible	Ligule of last leaf is visible and no new leaf is emerging

Crop Development Related to Temperature and Photoperiod. Table 1 (Continued)

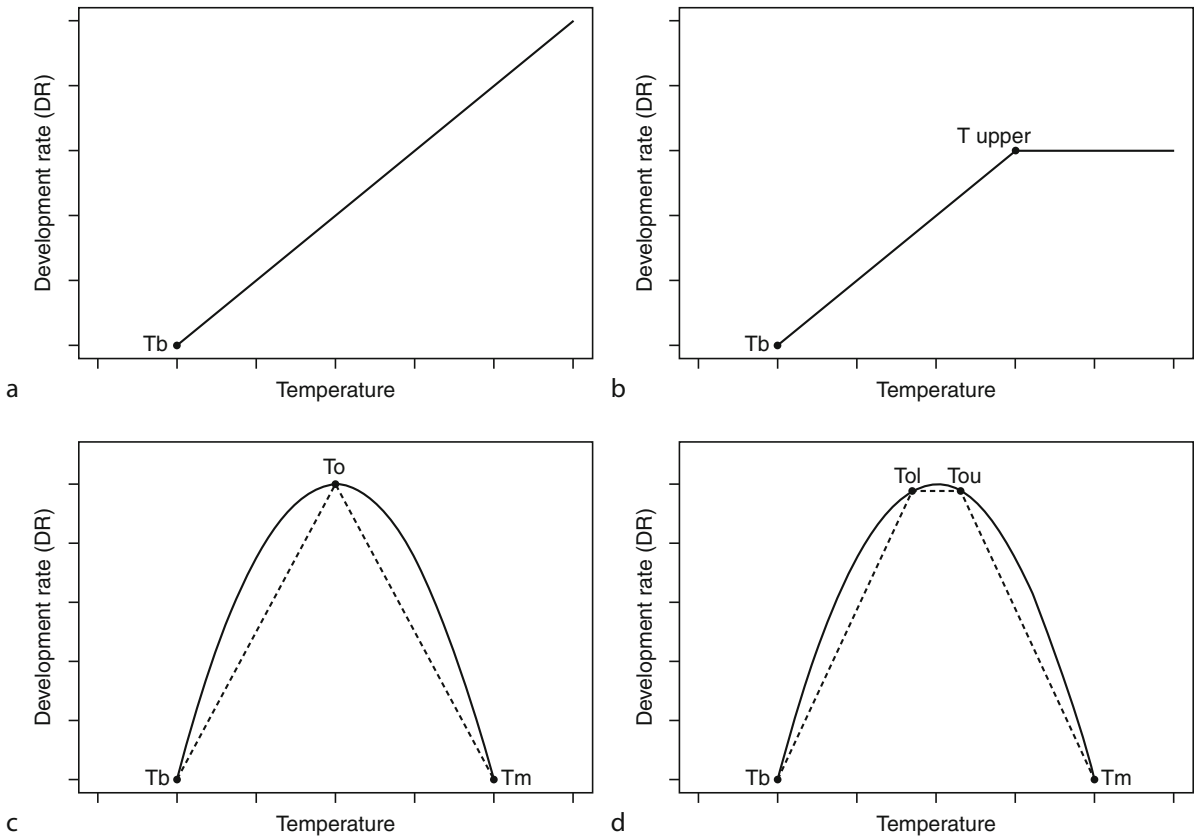
Stage or phase	Description		Measurement characteristics
	Feekes	Zadoks	
Booting	Stage 10 – last leaf sheath visible	Stages 43–49 – flag leaf sheath swelling	Begins at flag leaf and ends at heading
Heading	Stages: 10.1 – first ears just visible, 10.2 – ¼ of heading completed, 10.3 – ½ of heading completed, 10.4 – ¾ of heading completed, 10.5 – all ears out of the sheath	Stages: 51 – first spikelet just visible, 53 – ¼ of the inflorescence visible, 55 – ½ of the inflorescence visible, 57 – ¾ of the inflorescence visible, and 59 – inflorescence completely emerged	Begins when first spikelet is visible and ends when the inflorescence is fully emerged
Anthesis	Stages: 10.5.1 – anthesis starts, 10.5.2 – flowering completed to the top of the ear, 10.5.3 – flowering completed to the bottom of the ear, 10.5.4 – flowering over/kernel watery ripe	Stages: 61 – beginning of anthesis, 65 – mid-anthesis, 69 – anthesis completed	Starts when the first anther is visible on an inflorescence and ends when no more anthers appear on the inflorescence
Grain filling	Stages: 10.5.4, 11.1 – milky ripe, 11.2 – mealy ripe	Stages: 71 to 77 – milk grain, 83–87 – dough grain	Begins at fertilization, usually considered anthesis and ends at physiological maturity
Physiological maturity		No Stage	When all spike components, internodes, and leaves lose green color
Ripening	Stages: 11.3 – kernel hard, 11.4 – ripe for cutting	Stages 91–99	Ripening and dormancy

predictor of many developmental processes than calendar time. Reamur [24] formalized this relationship by creating the concept of heat units, now referred to as thermal time. The relationship between temperature and developmental rate is a curve with a maximum development rate at the optimum temperature (T_o) and developmental rate reaching zero at temperatures below the base temperature (T_b) or above the maximum temperature (T_m; Fig. 4). This nonlinear relationship is shown in several studies [25, 26].

Thermal time has two components: (1) the average temperature (T_a) over some time interval (e.g., hourly, daily), and (2) a temperature response curve describing the effectiveness of T_a on the development rate for the process (e.g., phyllochron, phenology). T_a is the integral of temperature over the time period of interest; however, in practice the average of the maximum and minimum temperatures in the time interval is often used:

$$T_a = \frac{T_{max} + T_{min}}{2} \quad (2)$$

This approximation is fairly accurate, but its error increases with deviations of from 12 h photoperiods and if sudden changes in temperature happen within the time interval. The time interval mostly depends on data availability, but common intervals range from daily to hourly time intervals, with daily the most commonly used. There are many temperature–response curves, which greatly diversifies the



Crop Development Related to Temperature and Photoperiod. Figure 4

Development rate as a function of temperature. Temperature response curves: (**a**) linear response, (**b**) extended linear response with an upper temperature threshold, (**c**) bilinear model with two zero development temperatures (base temperature, T_b, and maximum temperature, T_m) and an optimal temperature (T_o), and (**d**) trilinear model with two zero development temperatures (T_b and T_m) and a range of optimal temperatures defined by optimal lower temperature (T_{ol}) and optimal upper temperature (T_{ou})

calculation of thermal time (Tt). The most simple form is a linear relationship with the temperature at which there is some plant development (Fig. 4a), i.e., the difference between T_a and the temperature at which development is zero or base temperature (T_b):

$$Tt = T_a - T_b, (Tt \geq 0) \quad (3)$$

where thermal time is expressed as growing degree-days (GGD, °C·days). Modifying Eq. 2 to include a maximum development rate (Fig. 4b) is useful if plants are grown at higher temperatures. Given that the relationship between temperature and development rate is not linear [25–27], further refinements to the accumulation of thermal time can include changing the development rate based on certain cardinal temperatures (Fig. 4c, d). These approaches assume development rate increases with temperatures above T_b until an optimum temperature (T_o, or a range of optimal temperatures, T_{ol} to T_{ou}) is reached, and then decreases until development stops at a maximum temperature (T_m). This can be approximated by two- or three-segmented linear models, or curvilinear models such as a quadratic curve [25] or beta distribution [27, 28].

Vernalization

The term vernalization was first used by Lysenko in 1928, but research on the need of a cold period for

winter cereals to flower started as early as 1857 [29]. Vernalization can be viewed as an adaptive mechanism to avoid unfavorable periods for development (e.g., winter) and ensure flower development and subsequent seed growth occurs under favorable conditions (e.g., spring and summer). Hence, vernalization synchronizes plant development with seasonal climate changes.

Commonly, genotypes requiring vernalization are referred to as "winter" wheat (or "winter" barley) and are normally planted late summer or early fall with vernalization occurring during the late fall or early winter. Conversely, "spring" genotypes are commonly viewed as not requiring vernalization, and normally are planted in the spring or in regions where temperatures are often above effective vernalizing temperatures. This well-entrenched distinction between "winter" and "spring" genotypes does not reflect that "spring" genotypes (1) often have at least some vernalization requirements, (2) reach flowering faster if experiencing vernalizing temperatures, and (3) mask the continuum of vernalization requirements present among all wheat genotypes.

Effective vernalizing temperatures range from 0° C to 10° C [30], and a few weeks of cold are usually sufficient to promote the switch from vegetative to reproductive phases and longer periods of cold temperatures can shorten the time to flowering until the vernalization response is saturated [30]. Genotypes vary in the length of the cold period required to saturate the vernalization response. For instance, time to flowering in wheat was reduced in response to longer cold periods [31, 32], and interestingly, genotypes thought to require vernalization to flower eventually flowered without undergoing vernalization treatment [32]. Quantifying responses to varying periods of vernalizing temperatures in calendar time or thermal time does not reflect the biology of the response. A better method to quantify the effects of vernalization would be by counting the number of leaves produced at flowering time [33]. Using this quantification method, vernalization reduces the time to flowering by reducing the number of leaves being produced rather than the phyllochron [31, 33]. That is, the number of leaves produced at flowering increases with shorter vernalization periods, while the phyllochron is not affected by the duration of the vernalization period.

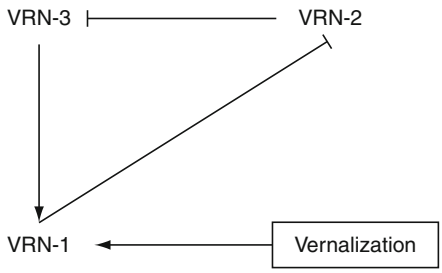
Genetic Regulation of Vernalization Although the mechanisms by which plants sense cold and initiate the cellular signaling to induce flowering are not known, the genetic regulation of vernalization is fairly well known in cereals like wheat and barley, which benefited of the research done on the model plant *Arabidopsis thaliana*. Four major genes are involved in the expression of vernalization sensitivity in wheat and barley, *VRN-1*, *VRN-2*, *VRN-3*, and *VRN-4*. The first three genes have been cloned and identified [34–36]. However, these three genes do not explain the spring habit of all varieties [35], suggesting that other genetic mechanisms may be involved.

The *VRN-1* gene encodes the MADS box transcription factor similar to *APETALA-1*, which is responsible for meristem identity in several plants [34]. This gene is up-regulated by vernalizing temperatures and the degree at which it is up-regulated depends on the length of the vernalization period. However, spring varieties also show an up-regulation of *VRN-1* during the initiation of the reproductive phase and remain high throughout the reproductive phase, suggesting an involvement of *VRN-1* in meristem identity but not limited to vernalization response.

The *VRN-2* gene encodes the *ZCCT1* protein, which shows high similarity to the CCT domain of the *Arabidopsis CONSTANS* and *CONSTANS*-like genes. *VRN-2* represses flowering and is down-regulated by vernalization [35]. The spring allele *vrn-2* in wheat has a point mutation at the CCT domain that replaces an arginine with a tryptophan. It has been suggested that the CCT domain may be involved in protein–protein interactions [37], so a mutation in this domain can alter these interactions.

The *VRN-3* gene is a RAF kinase inhibitor like protein with high homology to the *Arabidopsis FLOWERING LOCUS T* (*FT*) gene [36], which induces flowering when expressed. *FT* is a flowering signal that moves from leaves to stem apices.

VRN-1, *VRN-2*, and *VRN-3* interact with each other regulating flowering under the vernalization pathway (Fig. 5). Before vernalization occurs, *VRN-2* is expressed and represses the expression of *VRN-3*. When plants are vernalized, *VRN-1* is induced and it represses the expression of *VRN-2*, allowing *VRN-3* to express and promote flowering. At the same time, there is a feedback mechanism by which *VRN-3* up-regulates



Crop Development Related to Temperature and Photoperiod. Figure 5
Model of the vernalization pathway in temperate cereals. Arrows mean induction (or up-regulation) of gene expression; for example, vernalization induces the expression of VRN-1. Bar-headed lines mean repression (or down-regulation) of gene expression; for example, VRN-2 represses the expression of VRN-3

VRN-1. It seems that *VRN-1* is the primary target of vernalization and is essential for flowering.

VRN-4 has been recently fine mapped and its cloning is underway [38]. Identifying and including this gene in the vernalization pathway may increase the understanding of vernalization responses in wheat.

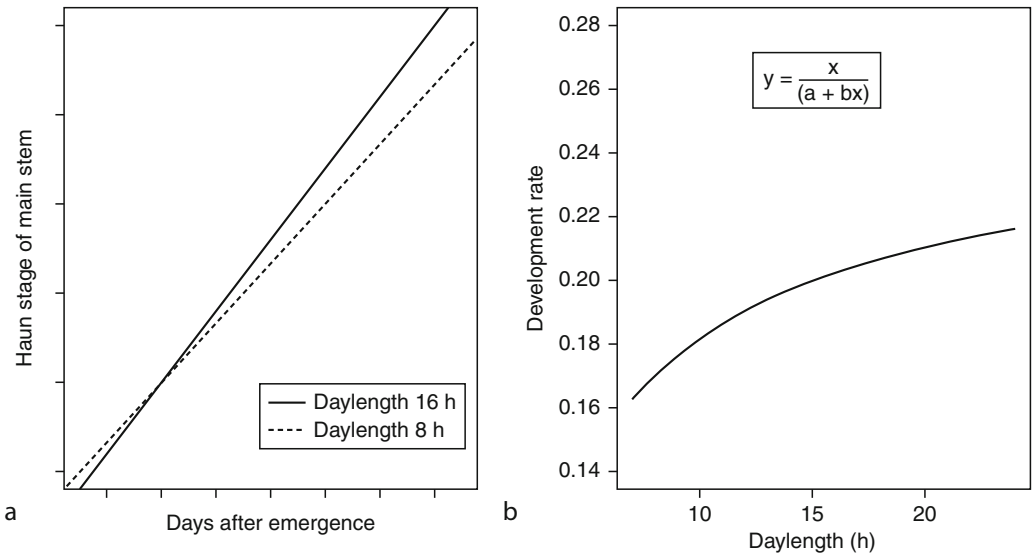
Photoperiod

Photoperiod can be defined as the number of hours of light in a 24-h period, which changes throughout the season depending on the latitude. It has been long recognized that plants, including crops, normally flower only when the length of the day was favorable [39]. Crops sense the amount of light they receive daily and respond to it by accelerating or slowing their development. There is, however, genetic variation of a quantitative nature in the response to photoperiod within a crop, meaning that different varieties respond differently to changes in the photoperiod, and some show no response to photoperiod (or are photoperiod insensitive). Photoperiod sensitivity is thought to be the wild type phenotype because it is wide spread in wild barley (*Hordeum spontaneum* L.) and confers strong adaptive features in the center of origin of barley, where there might be late spring frosts. Photoperiod insensitivity, however, brings wide geographical adaptability because plants do not require long days to flower, and, hence, they are suitable for environments with short seasons or latitudes where long days do not occur. For example, in wheat, day length neutrality, jointly with semi-dwarfism and rust resistance traits, were used to develop the high yielding varieties of the "green revolution" [40]. Photoperiod insensitivity promotes earliness thought to be a desirable adaptive trait for environments closer to the equator where high temperatures and drought can be expected at the end of the season. Therefore, earliness allows wheat and barley varieties to escape this terminal stress.

Although the rate of development mainly responds to temperature, when temperature is fixed, longer photoperiod alters the development rate by shortening the phyllochron [41, 42]. In Fig. 6, the appearance of leaves over time follows a linear model when plants are grown at a constant temperature, and the rate of appearance (slope of the curve) increases with the photoperiod; however, as day length increases, fewer leaves are formed per hour of light, hence there is a reduction in photoperiod efficiency for leaf emergence.

Different crops respond differently to photoperiod; crops can be classified as long day (LD) and short day (SD) depending on which photoperiod accelerates development (promotes flowering). Temperate cereals are long-day crops because flowering is promoted by photoperiod longer than 12–14 h, while maize and rice are short-day crops, meaning that short days induce the switch to reproductive phase. In wheat and barley, increased photoperiod shortens the time to flowering by modifying the length from emergence to terminal spikelet initiation. The effects of photoperiod on the duration from terminal spikelet initiation to flowering depend on what environments they are measured (field versus controlled environments). This reduction of time to flowering is not only due to an accelerated phyllochron, but also by the production of fewer leaves.

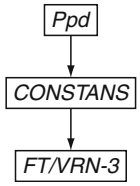
Genetic Regulation of Photoperiod Sensitivity In temperate cereals, such as wheat and barley, sensitivity to photoperiod is mainly regulated by *Ppd* genes. Homologous genes have been identified in wheat and barley and have the function of a pseudo response regulator (*PRR*), most similar to the *Arabidopsis PRR7* [43]. The *PPR* proteins are characterized by a pseudo receiver domain near the amino-terminus and a CCT domain near the carboxy-terminus [44], which makes



Crop Development Related to Temperature and Photoperiod. Figure 6
Development rate responses to changes in day length at constant temperature. (**a**) Leaf appearance of plants grown in different day lengths and (**b**) development rate as a function of day length (Based on [41])

them distantly related to other CCT domains important in regulation of flowering such as *CONSTANS* [45] and *VRN-2* [35].

The photoperiod insensitive allele (*ppd-H1*) in barley slightly delays the gene expression of *CONSTANS* (*HvCO1*) that follows a circadian pattern [43] and is a transcriptional regulator of the *FT* gene [46]. In contrast, photoperiod insensitivity in wheat is regulated by a series of three homoeologous genes located in the colinear region on chromosome 2 group, which seem to be upstream of the *CONSTANS* gene in the photoperiod pathway (Fig. 7). The *Ppd-1 Da* allele confers insensitivity to photoperiod in a semidominant fashion, allowing wheat plants to flower regardless of the photoperiod [47]. Sequence analysis of wheat varieties known to have this mutation shows that there is a 2,089 bp deletion upstream of the coding region responsible for the photoperiod insensitivity phenotype. Other sequence variations producing nonfunctional proteins (*null* alleles) at the 2A and 2D genes in wheat have been observed; however their effects on photoperiod sensitivity are difficult to assess because they might be masked by functional proteins from other homoeologous genes. These, and other mutations, however, may have a quantitative effect in photoperiod sensitivity and flowering time [47].

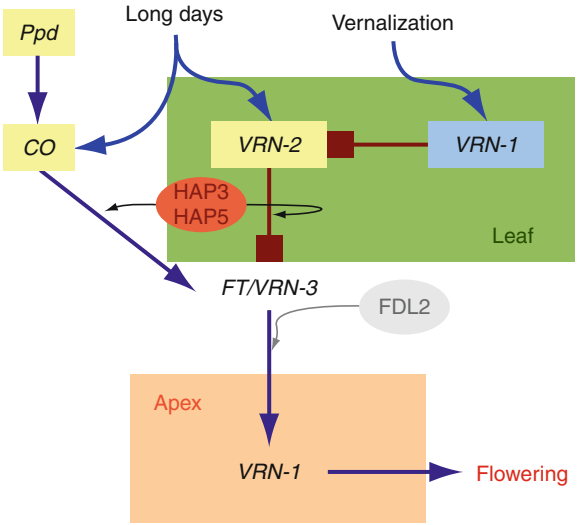


Crop Development Related to Temperature and Photoperiod. Figure 7
Model of the photoperiod pathway of flowering time. Arrows mean induction (or up-regulation) of gene expression; for example, CONSTANS is induced by the expression of *Ppd* genes

Coordinated Temperature and Photoperiod Regulation of Crop Development

The temperature and photoperiod regulation of crop development has been described in previous parts of this article, but both environmental factors act in coordination. It is clear that the phyllochron varies with planting date in temperate cereals and it has been suggested that the phyllochron is fixed by the rate of change of the photoperiod at crop emergence [48]. However, in general, temperature and photoperiod change together in the field. Results from experiments

in controlled environments show an effect of photoperiod on the temperature–response curve [49, 50]. The term "thermo–photo ratio" (the degree-days divided by day length in hours) has been used to study the coordinated effect of temperature and photoperiod on the phyllochron [49]. A linear relationship was found between the phyllochron and the thermo–photo ratio under both controlled environments and field conditions [49]. Slafer and Rawson [51] partitioned the photoperiod sensitivity of wheat phenophases into different parameters, which were affected by temperature, describing an interaction between genotype, photoperiod, and temperature. The effect of planting date on crop development has been studied extensively, yet it is difficult to draw conclusions of the coordinated effect of photoperiod and temperature on crop development. This is because when planting date is changed, both photoperiod and temperature are changed and their effects are difficult to separate.



Crop Development Related to Temperature and Photoperiod. Figure 8
Model of flowering related to photoperiod and vernalization. *Thick blue arrows* mean induction (or up-regulation) of gene expression or developmental process; for instance, photoperiod induces the expression of *VRN-2*. Square headed lines indicate repression (or down-regulation) of gene expression; for instance, VRN-3 up-regulates *VRN-1* at the apex

Genetic Framework of Flowering Time Several pathways regulate time to flowering in crops, namely, vernalization, photoperiod, autonomous, and gibberellic acid. The vernalization and photoperiod pathways have been described in parts 5.2 and 5.3 and *FT/VRN-3* gene is in both pathways, thus integrating the response to both vernalization and photoperiod factors (Figs. 5, 7, 8).

In the ancestral form of wheat and barley, after germination in the fall *VRN-2* is highly expressed by the long days and represses the expression of *FT/VRN-3*. As winter progresses, the photoperiod decreases and low temperatures induce the expression of *VRN-1* in the leaves, which represses the expression of *VRN-2*, allowing *FT* to be expressed by long days in the spring, a process regulated by photoperiod genes *Ppd* and *CO*. Then, the protein encoded by *FT/VRN-3* is translocated to the shoot apex, where it up-regulates *VRN-1*, which will induce the switch to reproductive phase. The HAP (HEME ACTIVATOR PROTEINS) complexes may mediate the transcriptional regulation of the CCT domain of *CO* and *VRN-2*. In plants, HAP subunits are encoded by multiple genes that, together with CCT domain proteins that can interact with HAP complexes, generate a large number of molecular combinations. These combinations provide a flexible signaling system that can integrate responses to environmental cues as photoperiod, vernalization, or stress (reviewed in [52]).

In environments where temperate cereals usually grow and were domesticated, vernalization requirements are met long before the photoperiod is inductive of the reproduction phase. This requires plants to "remember" they had been vernalized. The mechanism by which temperate crops "remember" vernalization is not known, but it has been studied in the model plant *Arabidopsis*. In addition to the genetic information carried on the DNA sequence (genes and alleles), chromatin structure is recognized as another source of genetic information. The chromatin can be highly condensed (heterochromatin) or more relaxed (euchromatin). The DNA is combined with proteins called histones and specific covalent histone modifications of histones favor the formation of chromatin structure that influences the level of gene expression. Other nongenetic mechanisms of gene expression are related to DNA

methylation. The DNA methylation at the promoter region of genes is generally related to lower levels of gene expression, and even gene silencing. Of the two mechanisms, it seems that histone modifications are involved in regulating the memory of vernalization (reviewed in [53]) in *Arabidopsis*. In *Arabidopsis*, the *FT* gene is repressed by *FLC*, which is down-regulated by vernalization. The stable repression of *FLC* involves de-acetylation of histone 3 (H3) upstream of *FLC*, methylation of H3 Lys9 and Lys27, which allows the binding of HP1 inducing the stable silencing of *FLC*.

Less known is the genetic basis of the quantitative response to temperature (thermal–response curve). The *Earliness* per se *A1* (*Eps^{m}A1*) gene affects time to flowering by reducing the vegetative phase and it has been associated with responses to temperature, probably by modifying the optimum temperature [54]. Two candidate genes for *Eps^{m}A1* are located in the genomic region where this gene has been located, *Mot1* and *FtsH4* [55]. The *Mot1* gene has features of the SNF2 family of transcriptional regulators. Other members of this family have been related to regulation of flowering in *Arabidopsis*, but the gene expression data do not show differences between the two alternative alleles [55]. The *FstH4* is a member of the FstH family of proteases and is homologous to the *Arabidopsis FstH4*, which has been found highly expressed in seed, and mutants in this gene show delayed germination that is carried over the growth cycle [55].

Modeling Approaches

For centuries, people have wanted to understand and predict aspects of crop development, particularly phenology. To do this, different conceptual, statistical, and mathematical models have been developed. Beginning in the 1970s, a variety of digital technologies began to emerge, one of which was crop simulation models for predicting growth, development, and yield. This section presents a broad overview of these crop simulation models, emphasizing wheat.

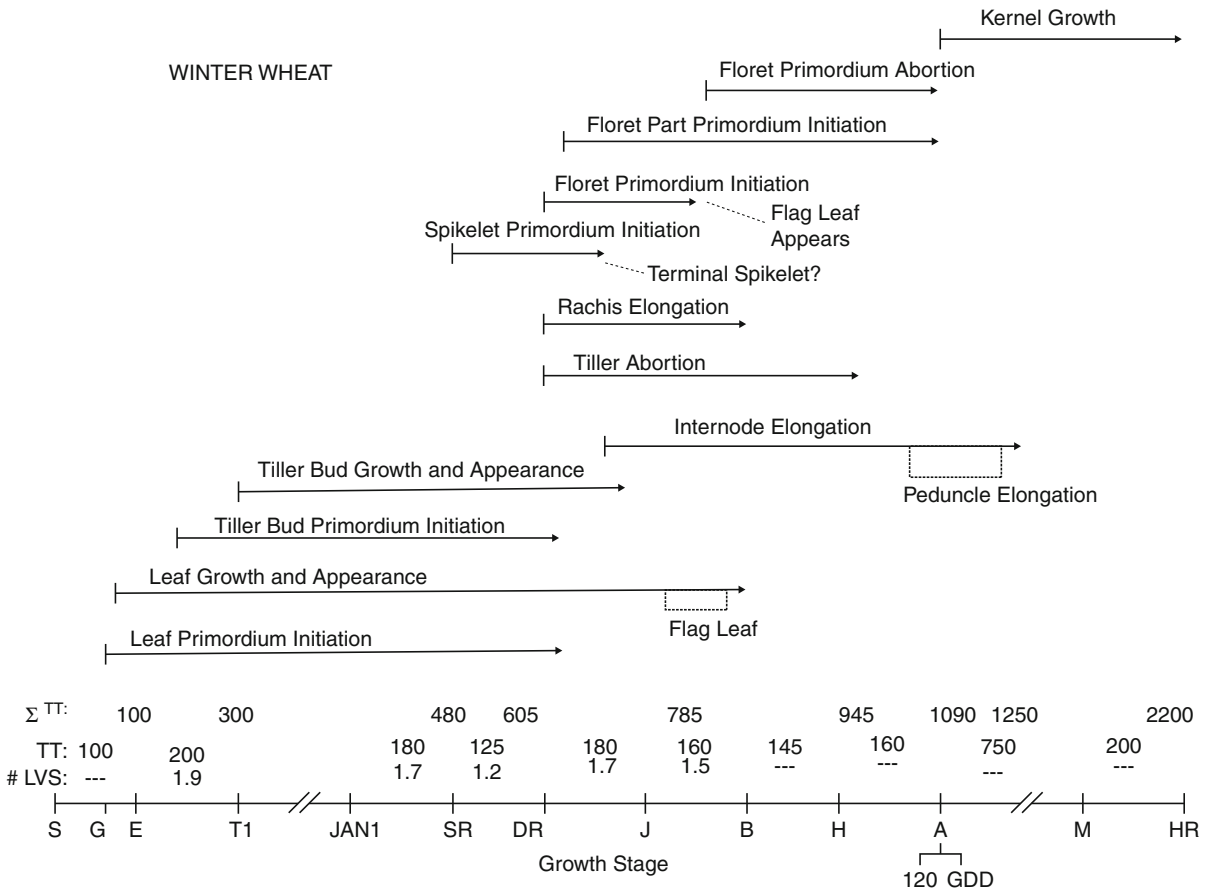
Many crop simulation models exist for simulating growth, development, and yield, and they cover scales from specific processes to the agroecosystem. Crop simulation models are a simplified mathematical representation of the plant. At the most fundamental level [56], crop simulation models generally simulate a trait, for example, yield (Y), as the function of daily growth rate (GR) that is partitioned to the yield component (P) and integrated over a daily time step from emergence (*emerge*) through physiological maturity (*maturity*):

$$Y = \int GR * P \tag{4}$$

Implementing Eq. 3 in a model usually begins assuming non-limiting conditions, thereby allowing for potential production to be estimated. The parameters can either be generic for a crop or adjusted to a specific genotype. By incorporating environmental variables (e.g., temperature, water, light, CO_2, and nutrients), crop simulation models can examine crop or genotype responses across a broad environmental range of limiting conditions, avoiding a common limitation of statistical (or regression) models. Depending on the purposes, including the role of biotic factors or management practices may also be important.

How a model implements Eq. 3 depends on model objectives and interests of the model developers. The earliest crop simulation models tended to focus on the scale of whole-plant growth and development, with little detail on processes at lower scales. These models use an energy- or light-driven approach to determine the growth rate, and this approach remains popular today. The basic approach simulates leaf area index on a daily time step, which is used to capture energy/sunlight and produce biomass that is then distributed to basic plant components of leaves (providing the feedback to the cycle), stems, roots, and seeds. Partitioning coefficients are often used to allocate the biomass produced, and phenology sub-models are essential in accurately predicting the timing when sources and sinks are present, and changing partitioning coefficients based on developmental stage.

As crop simulation modeling progressed, certain trends emerged. First, greater attention focused on representing plant processes below the whole-plant level. In general, energy- or light-driven modeling emphasized functional physiology, particularly for assessing energy balance and leaf functioning at the individual organ level (e.g., [57, 58]). Second, considerable research on crop development during the 1970s



Crop Development Related to Temperature and Photoperiod. Figure 9
Developmental sequence diagram of a generic winter wheat for optimal conditions. Question marks refer to uncertainty, important cultivar variation, or conflicting reports in the literature. Time line legend is TT is the thermal time for the interval; #LVS is number of leaves for the interval; S, sowing date; G, germination; E, seedling emergence; TI, tiller initiation/appearance; SR, single ridge stage; DR, double ridge stage; J, jointing; B, booting; H, heading; A, anthesis; and M, physiological maturity (From [59])

and 1980s undoubtedly spurred interest in including this new knowledge in the models. This led to alternative modeling approaches based on more developmentally driven approaches that recognized that plant development is orderly and predictable based on basic units (i.e., the phytomer) that dynamically appear, grow, and senesce over time as discussed earlier in this entry and shown in Fig. 9.

Early efforts beginning in the mid-1980s focused on developmental concepts such as leaf appearance (the phyllochron) and tillering that led to more accurate representation of canopy architecture. For instance, the AFRCWHEAT1/2 model developed in Europe [60–62] contained detailed tillering and leaf dynamics sub-models (e.g., appearance, growth, and senescence/abortion), and the resulting effect on canopy LAI was simulated and then used to estimate biomass. Simultaneously and independently, another effort was underway in the USA that resulted in the developmentally driven MODWht3 [63] and SHOOTGRO [http://arsagsoftware.ars.usda.gov, [59, 64–67]] models. SHOOTGRO is slightly more developmentally detailed for canopy processes than MODWht3, but less detailed in the root system and simulating biomass production.

SHOOTGRO provides the foundation to simulate the development and growth of individual phytomers (and phytomer components as shown in Fig. 9) on each morphologically identified shoot (main stem and tillers) on the median plant of up to six age classes, or cohorts, based on time of seedling emergence.

The Sirius model has one of the most developed and robust leaf appearance sub-models of any wheat simulation model [68]. As with the MODWht3 and SHOOTGRO models, the assumption used is that the developmental "clock" from emergence to anthesis is best represented by the rate of leaf appearance and final number of leaves, rather than thermal time. Based on vernalization requirement and photoperiod sensitivity of the variety being simulated and leaf ontogeny, the final leaf number is determined [69, 70]. This allows for an elegant quantitative description of both spring and winter wheat leaf appearance and integration with developmental events.

Regardless of modeling approach and goals, the ability to simulate genotype phenology across a broad range of environments for major crops such as wheat has been quite reliable. Many alternative approaches exist for predicting phenology, and approaches differ in input requirements and number of developmental stages simulated. Essentially all models are based on the thermal time approach, reflecting the importance of temperature discussed in Section Developmental Response to Temperature. An alternative to a strict thermal time approach, particularly for small-grain cereals, has been to use leaf numbers to estimate the time interval between developmental stages. In phenology sub-models, temperature effects are well considered, but rarely are the effects of water deficits (or nutrient availability) considered [71]. Exceptions include the SHOOTGRO model and PhenologyMMS (http://arsagsoftware/ars.usda.gov).

Determining plant parameters and how to address the genotype by environment interaction are common concerns for all models. With the explosion of genome mapping and molecular biology research, opportunities for understanding and resolving these issues are emerging [72–74]. For example, the presence or absence of known alleles influencing a trait can be used to determine the parameters used in the algorithm representing the process [75] or the response to environmental factors [76]. Clarification of gene networks controlling processes such as time of flowering has considerably advanced the understanding and simulation of these processes [77].

Crop simulation modeling is increasingly benefiting from the advent of object-oriented design and programming languages such as C++, C#, and Java, both in terms of developing and maintaining models as well as providing greater flexibility in representing plant processes within models. Initial object-oriented designs tended to view the plant as a collection of objects that equate to leaf, stem, root, and seed components. Recent attempts have begun to incorporate the phytomer approach of building plant canopies into the object-oriented design that can also be scaled up, or aggregated, into lower levels of resolution, such as the seed component of earlier designs [78–80].

Future Directions

Crop development is regulated by environmental factors that interact with the genetics of the plant. Many developmental responses related to temperature and photoperiod are well known and it is possible to predict them reasonably well at the crop level. Similarly, rapidly emerging knowledge from genomics research is helping to provide understanding of the genetic basis of certain aspects of crop development. Unfortunately, the quantitative integration of genetic and physiological knowledge is largely unknown, and both genetic and physiological models would benefit from better integration of knowledge. For example, the genetic basis of photoperiod and vernalization pathways is fairly well known and as new genomic studies are carried out, more complex models are being built [81] showing the complexity of flowering time. However, the genetic mechanisms described in this entry interact with responses to other environmental factors defining a network of signaling a highly complex response that is not fully understood.

Knowledge in crop development has greatly benefited from research in model organisms; however there are key differences, like the lack of the vernalization gene *FLC* in temperate cereals. For example, four vernalization genes have been described in wheat, but only three have been cloned and located in the vernalization pathway. Completing this pathway would greatly increase the understanding of how temperate crops respond to nonfreezing cold temperatures.

At the same time, the physiological response to temperature has been extensively studied, and genetic differences are well documented; however, the basis of genetic effects is not known. It is encouraging that new genes (e.g., *Eps-1*) are being identified, but the quantitative variation of crop development, once major processes (photoperiod and vernalization requirements) are solved, requires the identification of new genes, probably of small effect. For example, a recent study in maize found no large effect QTL for flowering time in a nested mapping population [82]. Although no major epistatic or environmental interactions were found, the individual QTL effect varied across founder lines of the population [82]. QTL or gene effects need to be accurately determined to build quantitative genetic models.

Bibliography

1. Heun M, Schafer-Pregl R, Klawan D, Castagna R, Accerbi M, Borghi B, Salamini F (1997) Site of einkorn wheat domestication identified by DNA fingerprinting. Science 278(80):1312–1314
2. Zohary D, Hopf M (2000) Domestication of plants in the old world. Oxford University Press, Oxford
3. Goethe JWV (2009) The metamorphosis of plants (re-print). MIT Press, Cambidge, MA, 123
4. Gray A (1879) Structural botany: or organography on the basis of morphology. Ivison Blakeman Taylor, New York/Chicago
5. Bateson W (1894) Materials for the study of variation treated with special regard to discontinuity in the origin of species. Macmillan, London
6. Wilhelm WW, McMaster GS (1995) Importance of the phyllochron in studying development and growth in grasses. Crop Sci 35:1–3
7. Jewiss O (1972) Tillering in grasses – its significance and control. J Br Grassl Soc 27:65–82
8. Klepper B, Rickman R, Belford R (1983) Leaf and tiller identification on wheat plants. Crop Sci 23:1002–1004
9. Klepper B, Rickman R, Peterson C (1982) Quantitative characterization of vegetative development in small cereal grains. Agron J 74:789–792
10. Haun JR (1973) Visual quantification of wheat development. Agron J 65:116–119
11. Klepper B, Tucker T, Dunbar B (1983) A numerical index to assess early inflorescence development in wheat. Crop Sci 23:206–208
12. Wilhem W, McMaster G (1996) Spikelet and floret naming scheme for grasses with spike inflorescence. Crop Sci 36:1071–1073
13. McMaster G (2005) Phytomers, phyllochrons, phenology and temperate cereal development. J Agric Sci 143:137–150
14. Askenasy E (1888) Über eine neue methode, um die vertheilung der wachstumsintensität in wachsenden theilen zu bestintaien. Verh Naturh Med Verl Heidelberg 2:70–153
15. Tesarová J, Nátr L (1990) Phyllochron and winter barley leaf growth rate. Biol Plant 32:450–459
16. Milthorpe F (1956) The relative importance of the different stages of leaf growth in determining the resultant area. In: Milthorpe F (ed) The Growth of Leaves. Proceedings of the 3rd Easter School in Agricultural Science, University of Nottingham, Nottingham, Butterwoths, London, pp 20–38
17. Esau K (1965) Plant anatomy. Wiley, New York
18. Bunting A, Drennan D (1966) Some aspects of the morphology and physiology of careal in the vegetative phase. In: Milthorpe F, Ivins J (eds) The Growth of Cereal and Grasses, Proceedings of 12th Easter School of Agricultural Science, University of Nottingham, Nottingham, Butterwoths, London, pp 20–38
19. Barthélémy D, Caraglio Y (2007) Plant architecture: a dynamic, multilevel and comprehensive approach to plant form, structure and ontogeny. Ann Bot 99:375–407
20. Large EC (1954) Growth stages in cereals illustration of the feekes scale. Plant Pathol 3:128–129
21. Zadoks J, Chang T, Konzak C (1974) A decimal code for the growth stages of cereals. Weed Res 14:415–421
22. Lancashire P, Bleiholder H, Boom T, Langelüddeke P, Strauss R, Weber E, Witzenberger A (1991) A uniform decimal code for growth stages of crops and weeds. Ann Appl Biol 119:561–601
23. McMaster G (2009) The development of the wheat plant. In: Carver B (ed) Wheat science and trade. Willey-Blackwell, Ames, pp 31–55
24. Reamur R (1735) Observations du thermomètre, faites è paris l'année 1735, comparées à celles qui ont été faites sous la ligne à l'Isle de france, à alger et en quelques-unes de nos isles de l'amérique. Mem Acad Roy Sci, Paris, France
25. Cao W, Moss DN (1989) Temperature effect on leaf emergence and phyllochron in wheat and barley. Crop Sci 29:1018–1021
26. Friend D, Helson V, Fisher J (1962) Leaf growth in marquis wheat, as regulated by temperature, light intensity, and daylength. Can J Bot 40:1299–1311
27. Jame YW, Cutforth HW, Ritchie JT (1998) Interaction of temperature and daylength on leaf appearance rate in wheat and barley. Agric For Meteorol 92:241–249
28. Yan W, Hunt L (1999) An equation for modelling the temperature response of plants using only the cardinal temperatures. Ann Bot 84:607–614
29. Chouard P (1960) Vernalization and its relations to dormancy. Ann Rev Plant Physiol 11:191–238
30. Flood R, Halloran G (1984) The nature and duration of gene action for vernalization response in wheat. Ann Bot 53:363–368
31. Ahrens J, Loomis W (1963) Floral induction and development in winter wheat. Crop Sci 3:463–466
32. Miao G, Zhang Y, Hou Y, Yin J, Wang S (1992) Effects of vernalization and photoperiod on leaf number of main stem in wheat. Acta Agron Sin 16:321–330

33. Wang S, Ward R, Ritchie J, Fischer R, Schulthess U (1995) Vernalization in wheat. I. A model based on the interchangeability of plant age and vernalization duration. Field Crop Res 41:91–100
34. Yan L, Loukoianov A, Tranquilli G, Helgera M, Fahima T, Dubkovsky J (2003) Positional cloning of the wheat vernalization gene VRN1. Proc Natl Acad Sci USA 100:6263–6268
35. Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. Science 303(80):1640–1644
36. Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene VRN3 is an orthologue of ft. Proc Natl Acad Sci USA 103:19581–19586
37. Kurup S, Jones HD, Holdsworth MJ (2000) Interactions of the developmental regulator abi3 with proteins identified from developing *Arabidopsis* seeds. Plant J 21:143–155
38. Yoshida T, Nishida H, Zhu J, Nitcher R, Distelfeld A, Akashi Y, Kato K, Dubcovsky J (2010) VRN-D4 is a vernalization gene located on the centromeric region of chromosome 5D in hexaploid wheat. Theor Appl Genet 120:543–552
39. Garner W, Allard H (1920) Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. J Agric Res 18:553–606
40. Borlaug NE (1983) Contributions of conventional plant breeding to food production. Science 219(80):689–693
41. Cao W, Moss DN (1989) Daylength effect on leaf emergence and phyllochron in wheat and barley. Crop Sci 29:1021–1025
42. Warrington IJ, Kanemasu ET (1983) Corn growth response to temperature and photoperiod. II. Leaf-initiation and leaf-appearance rates1. Agron J 75:755–761
43. Turner A, Beales J, Faure S, Dunford R, Laurie D (2005) The pseudo-response regulator ppd-h1 provides adaptation to photoperiod in barley. Science 310(80):1031–1034
44. Mizuno T, Nakamichi N (2005) Pseudo-response regulators (prrs) or true oscillator components (tocs). Plant Cell Physiol 46:677–685
45. Griffiths S, Dunford RP, Coupland G, Laurie DA (2003) The evolution of constans-like gene families in barley, rice, and *Arabidopsis*. Plant Physiol 131:1855–1867
46. Song YH, Ito S, Imaizumi T (2010) Similarities in the circadian clock and photoperiodism in plants. Curr Opin Plant Biol 13(5):594–603
47. Beales J, Turner A, Griffiths S, Snape JW, Laurie DA (2007) A pseudo-response regulator is misexpressed in the photoperiod insensitive Ppd-D1a mutant of wheat (triticum aestivum l.). Theor Appl Genet 115:721–733
48. Baker C, Gallagher J, Monteith J (1980) Daylength and leaf appearance in winter wheat. Plant Cell Environ 3:285–287
49. Cao W, Moss DN (1989) Temperature and daylength interaction on phyllochron in wheat and barley. Crop Sci 29:1046–1048
50. Ellis R, Roberts E, Summerfield R, Cooper J (1988) Environmental control of flowering in barley (Hordeum vulgare l). II. Rate of development as a function of temperature and photoperiod and its modification by low-temprateure vernalization. Ann Bot (Lond) 62:156–158
51. Slafer GA, Rawson HM (1996) Responses to photoperiod change with phenophase and temperature during wheat development. Field Crop Res 46:1–13
52. Distelfeld A, Li C, Dubcovsky J (2009) Regulation of flowering in temperate cereals. Curr Opin Plant Biol 12:178–184
53. Sung S, Amasino RM (2004) Vernalization and epigenetics: how plants remember winter. Curr Opin Plant Biol 7:4–10
54. Appendino ML, Slafer GA (2003) Earliness per se and its dependence upon temperature in diploid wheat lines differing in the major gene eps-A^m1 alleles. J Agric Sci 141:149–154
55. Faricelli M, Valárik M, Dubcovsky J (2010) Control of flowering time and spike development in cereals: the earliness per se eps-1 region in wheat, rice, and brachypodium. Funct Integr Gen 10:293–306
56. McMaster G, Ascough II J (2010) Crop management to cope with global change: a systems perspective aided by information technologies. In: Araus J, Slafer G (eds) Crop Stress Management & Climate Change. CAB International, Wallingford
57. Norman J (1979) Modeling complete crop canopy. In: Barfield B, Gerber J (eds) Modification of the aerial environment of plants. American Society of Agricultural Engineers, St. Joseph, pp 249–277
58. Grant R (2001) A review of the Canadian ecosystem model – ecosys. In: Shaffer M, Ma L, Hansen S (eds) Modeling carbon and nitrogen dynamics for soil management. Lewis, Boca Raton, pp 173–263
59. McMaster G, Wilhelm W, Morgan J (1992) Simulating winter wheat shoot apex phenology. J Agric Sci Cam 119:1–12
60. Weir A, Bragg P, Porter J, Rayner J (1984) A winter wheat crop simulation model without water or nutrient limitations. J Agric Sci Cam 102:371–382
61. Porter J (1984) A model of canopy development in winter wheat. J Agric Sci 102:383–392
62. Porter J (1993) Afrgwheat2: a model of the growth and development of wheat incorporating responses to water and nitrogen. Eur J Agron 2:69–82
63. Rickman R, Waldman S, Klepper B (1996) Modwht3: a development-driven wheat growth simulation. Agron J 88:176–185
64. Zalud Z, McMaster G, Wilhelm W (2003) Evaluating SHOOTGRO 4.0 as a potential winter wheat management tool in the Czech Republic. Eur J Agron 19:495–507
65. McMaster G, Klepper B, Rickman R, Wilhelm W, Willis W (1991) Simulation of shoot vegetative development and growth of unstressed winter wheat. Ecol Mod 53:189–204
66. Wilhelm W, McMaster G, Rickman R, Klepper B (1993) Aboveground vegetative development and growth as influenced by nitrogen and water availability. Ecol Mod 68:183–203

67. McMaster G, Morgan J, Wilhelm W (1992) Simulating winter wheat spike development and growth. Agric For Meteor 60:193–220
68. Jamieson P, Brooking I, Semenov M, McMaster G, White J, Porter J (2007) Reconciling alternative models of phenological development in winter wheat. Field Crop Res 103:36–41
69. Brooking I, Jamieson P, Porter J (1995) The influence of daylength on the final leaf number in spring wheat. Field Crop Res 41:155–165
70. Brooking I (1996) The temperature response of vernalization in wheat – a developmental analysis. Ann Bot 78:507–512
71. McMaster G, Wilhelm W (2003) Simulating wheat and barley phenological responses to water and temperature stress. J Agric Sci Cam 141:129–147
72. White J, Hoogenboom G (2003) Gene-based approaches to crop simulation: past experiences and future opportunities. Agron J 95:52–64
73. Edmeades G, McMaster G, White J, Campos H (2004) Genomics and the physiologist: bridging the gap between genes and crop response. Field Crop Res 90:5–18
74. White J (2006) From genome to wheat: emerging opportunities for modeling wheat growth and development. Eur J Agron 25:79–88
75. White J, McMaster G, Edmeades G (2004) Physiology, genomics and crop response to global change. Field Crop Res 90:1–3
76. Weiss A, Baenziger P, McMaster G, Wilhelm W, Al Ajlouni Z (2009) Quantifying phenotypic plasticity using genetic information for simulating plant height in winter wheat. Wagen J Life Sci 57:59–64
77. Welch S, Roe J, Dong Z (2003) A genetic neural network model of flowering time control in *Arabidopsis thaliana*. Agron J 95:71–81
78. Dingkuhn M, Luquer D, Quilot B, Reffye P (2005) Environmental and genetic control of morphogenesis in crops: towards models simulating phenotypic plasticity. Aus J Agric Res 56:1289–1302
79. Vos J, Marcelis L, de Visser P, Struik P, Evers J (2007) Functional-structural plant modelling in crop production. Springer, The Netherlands
80. McMaster G, Hargreaves J (2009) Canon in design: composing scales of plant canopies from phytomers to whole-plants using the composite design pattern. Wagen J Life Sci 57:39–51
81. Higgins JA, Bailey PC, Laurie DA (2010) Comparative genomics of flowering time pathways using *Brachypodium distachyon* as a model for the temperate grasses. PLoS ONE 5:e10065
82. Buckler ES, Holland JB, Bradbury PJ, Acharya CB, Brown PJ, Browne C, Ersoz E, Flint-Garcia S, Garcia A, Glaubitz JC et al (2009) The genetic architecture of maize flowering time. Science 325(80):714–718

Crop Diseases, Management and Control of

Dale R. Walters
Crop & Soil Systems Research Group, Scottish Agricultural College, Edinburgh, UK

Article Outline

Glossary

Biotroph A plant pathogenic microorganism which requires living host tissue in order to complete its life cycle. Rust and powdery mildew fungi are examples of biotrophs, as are viruses.

Oomycetes Also known as water molds, the Oomycetes are a large group of terrestrial and aquatic organisms. They superficially resemble fungi in mycelial growth and mode of nutrition, but molecular studies and distinct morphological characteristics place them in the kingdom Stramenopila (or Chromista) with brown and golden algae and diatoms.

Phytoalexin Antimicrobial substances synthesized de novo by plants and which accumulate rapidly at areas of infection by an incompatible pathogen. Phytoalexins are broad spectrum in action and are chemically diverse with different types characteristic of particular plant species. They can be grouped into several classes including terpenoids, alkaloids, phenolics.

Saprophyte An organism, e.g., a fungus or bacterium, that grows on and derives its nourishment from dead or decaying organic matter.

Virulence Refers to the relative ability of a pathogenic organism to cause disease.

Definition of the Subject

Plant diseases cause substantial crop losses every year and, historically, have led to considerable economic damage and human suffering. Controlling plant diseases is therefore vital to maintaining crop productivity and feeding the ever expanding human population. Crop diseases can be controlled using a variety of methods, notably cultural approaches, the use of resistance in the plant, and the application of chemicals (fungicides). However, the organisms that cause plant disease (plant pathogens) are genetically adaptable and can overcome plant resistance, and the toxic effects of fungicides. Ensuring that crops are adequately protected depends therefore on continually keeping one step ahead of the pathogens by improving existing control measures and developing new approaches. This article provides an overview of the various methods, traditional and novel, used to control crop diseases.

Introduction: The Need for Disease Control in Crops

Plant disease has plagued mankind ever since the beginnings of agriculture. Today, despite the many advances in crop protection technology, crop diseases continue to wreck havoc on crops, because of the genetic adaptability of the pathogens which cause plant disease. Crop losses at farm level can have serious implications for growers, but crop disease can inflict much more serious damage on a larger scale. A good example of just how devastating crop disease can be is the potato blight epidemic of the 1840s in Europe. This disease, caused by the Oomycete pathogen *Phytophthora infestans*, decimated crops across Europe and in Ireland, led to the death of some one million people and the emigration of several million more [1, 2]. Astonishingly, today, more than 170 years later, potato blight still poses a major problem for potato growers across the globe.

Crop losses as a result of disease can be expressed in various ways, such as potential losses and actual losses. Potential loss compares yields in a system without any form of crop protection treatment, with yields from a system with a similar intensity of crop production, but receiving crop protection treatments. Actual losses are those sustained despite the use of crop protection [3]. The efficacy of crop protection can be calculated as the percentage of potential losses prevented. Potential losses range from 8.5% for cotton to 21.2% for

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Crop Diseases, Management and Control of. Table 1 Estimated loss potential and actual losses due to pathogens (fungi and bacteria) in six major crops worldwide in 2001–2003 (Adapted from [3])

Crop	Crop losses (%) due to pathogens	
	Potential	Actual
Wheat	15.6 [12–20]	10.2 [5–14]
Rice	13.5 [10–15]	10.8 [7–16]
Maize	9.4 [8–13]	8.5 [4–14]
Potatoes	21.2 [20–23]	14.5 [7–24]
Soybeans	11.0 [7–16]	8.9 [3–16]
Cotton	8.5 [7–10]	7.2 [5–13]

potatoes, while actual losses range from 7.2% for cotton to 14.5% for potatoes, highlighting the importance of crop protection in reducing potential losses in all of these crops (Table 1).

Globally, agricultural production has grown faster than the human population over the past few decades [4]. In most parts of the world, this has been the result, not of increased area of cropped land, but of increased inputs, including pesticides [4]. In the period from 1963 to 2002, cereal yields increased by 114% globally, although the annual rate of growth fell from 3.14% in the period 1963–1976 to 0.84% in the period 1989–2002 [4]. In the period from 1960 to 2004, pesticide sales worldwide increased more than ten-fold to some $30 billion [3]. However, despite this increased pesticide use, crop losses as a result of pests, diseases, and weeds have not fallen significantly in the past 40 years.

Controlling Crop Diseases

Crop disease can be controlled using a variety of approaches. The first line of defense is the exclusion of the pathogen through plant quarantine and, for example, the use of pathogen-free propagating material. The next line of defense is to exclude, eliminate, or reduce pathogen inoculum. This can be achieved in various ways, including cultural control, use of host plant resistance, and chemical control. Finally, several of these approaches might be used together in an integrated program of disease control. In the sections below, these disease control options will be examined in more detail.

Cultural Control

Cultural control aims to prevent contact with the pathogen, to create environmental conditions unfavorable to the pathogen or at least to avoid favorable conditions, or to reduce the amount of pathogen inoculum available to infect crop plants. Methods used include host eradication, crop rotation, sanitation, irrigation, tillage, and improving crop growth conditions, for example, through appropriate fertilizer use. Cultural control provides the foundation for disease control in crops, and yet its important is often overlooked.

Host Eradication Host eradication refers to the removal and disposal of whole infected plants. This method is used routinely in nurseries, greenhouses, and fields to prevent the spread of pathogens, since it eliminates the infected plants that act as a source of inoculum. In potato cultivation, pathogens can overwinter in infected tubers left in the field and give rise to infected plants (known as volunteers) in the spring. These volunteers can acts as sources of inoculum, and their removal from the field and subsequent destruction will reduce levels of pathogen inoculum.

If a pathogen requires two hosts to complete its life cycle, control is possible by eradication of the less important host. The wheat stem rust fungus, *Puccinia graminis* f.sp. *tritici*, is a case in point. It requires two hosts, wheat and barberry, to complete its life cycle and until the 1950s was the most important pathogen of wheat in the United States [5]. Since the 1950s, however, stem rust has declined in importance in the United States, due in part to successful eradication of its alternate host, common barberry [6].

Sanitation Sanitation refers to eliminating or reducing the amount of inoculum present by various means, including removal of infected plant parts and plant debris. Destroying crop residues is an important practice, but how it is performed depends upon the type of crop and the type of pathogen. For example, burying crop debris can destroy certain pathogens, particularly if the residues are plowed in deeply enough, while

burning crop residue is common practice for cereal crops in some parts of the world and will destroy many pathogens. However, burning has some drawbacks, particularly loss of nutrients and increased soil erosion.

Crop Rotation Crop rotation is an ancient cultural practice and its benefits include maintenance of soil structure and organic matter, and a reduction in soil erosion that is often associated with continuous row crops [7]. The main purpose of rotating crops in conventional arable rotations is to reduce the incidence of diseases, pests, or weeds that are difficult to control with pesticides, and for this reason, short rotations of two to three crops are usually employed. In the United States, for example, the majority of the maize crop is grown on a 2–3 year rotation, while in the UK, barley and wheat usually form the main part of the rotation, with breaks of oilseed rape, beans, peas, or potatoes [8].

Continuous cropping with the same susceptible host plant will result in the establishment of a soil population of pathogenic microbes. Crop rotation avoids this and is often associated with a reduction in crop diseases caused by soilborne pathogens [7]. Using nonhost or less susceptible crop plants in the rotation can lead to a decline in specific populations of plant pathogens in the soil and is best suited for biotrophs, since they require the presence of the specific living host for survival, or pathogens with low saprophytic ability [9, 10]. Crop rotation is less suitable for controlling root-inhabiting pathogens that survive saprophytically or can exist for long periods in soil, e.g., pathogens with tough survival structures such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Pythium* spp. [11, 12].

Tillage Tillage has indirect effects on pathogen spread and can also be used to reduce pathogen inoculum in the soil. Conventional tillage uses primary and secondary cultivation to prepare a seed-bed for planting and results in considerable soil disturbance, while reduced tillage uses a single cultivation, or even no cultivation (no-tillage, zero tillage, direct drilling), and as a result leads to minimal soil disturbance. Minimum tillage and no-tillage practices can be grouped together under the generic term: conservation tillage [13].

Tillage can bury pathogens deeper in the soil where they are less likely to become a problem. It can alter soil texture, aeration, temperature, moisture, and density, and can also influence nutrient release in the soil, with benefits to the crop [8]. Tillage also leads to clear fluctuations in microbial activity and biomass in the soil [14]. Reduced tillage or no-till is often associated with higher microbial biomass and activity in upper soil layers compared to regular tillage (plowing) [15]. This concentration of crop debris in the top layers of the soil can promote the overwintering and survival of numerous pathogens and has prompted concern that increased disease and decreased yields will be the inevitable result of using conservation tillage practices. Although this has proved to be the case under some conditions, there have also been reports of decreases in the incidence of soilborne diseases. As suggested by Sturz et al. [13], such contradictory reports may reflect differences in root development and soil microbial biomass and activity under the different regimes. Thus, conservation tillage practices can lead to pathogen inoculum concentrations several orders of magnitude greater than those found under conventional tillage [16, 17] and, as a result, plant roots growing in the upper soil layers might be more prone to pathogen infection [13]. In contrast, increased microbial biomass and activity in the top soil layers can give rise to greater root density and root activity [18, 19], which may offset the damaging effects of disease on yield, and might also provide a highly competitive soil environment with resulting disease suppressive effects [20].

Severity of tan spot in wheat was found to increase under no-till conditions, but was reduced following reduced tillage [21]. To control blackleg (*Leptosphaeria maculans*) on canola (oilseed rape), it is recommended that crop debris is buried in the autumn and a nonhost crop be direct seeded the following spring to avoid reexposing the buried residue [22, 23]. Recent research suggests that inoculum production by *L. maculans* decreased with increasing duration of stubble burial in the field over 10 months before stopping completely [24]. This effect may be due to the mycobiota associated with the buried stubble, and these workers suggest that it might be possible to manipulate the population of saprophytic microbiota present on oilseed rape stubble to facilitate the decline of *L. maculans* [24].

Sowing Practices

Time of Sowing Altering the time of sowing to avoid high levels of pathogen inoculum or conditions conducive for development of a particular disease can lead to reduced severity of several crop diseases. For example, in the UK, sowing winter oilseed rape in August rather than September exposes the earlier sown crop to inoculum from stubble of the previous crop, resulting in more severe *Alternaria* infection on pods. In contrast, the risk of infection is reduced in the later sown crop because the stubble is buried by tillage [25]. Late sowing may also be recommended for autumn-sown barley crops, in order to decrease exposure of newly emerging seedlings to inoculum of *Rhynchosporium secalis* produced on previous barley crops in the area [26].

Depth of Sowing Sowing depth can influence the risk of infection, since the preemergence stage of the seedling, which is usually more susceptible to pathogen infection, is longer when seeds are sown deeper. In *Brassica rapa*, for example, rapid emergence of seedlings reduces preemergence damping-off because the period of contact between the emerging seedlings and *R. solani* in the soil is reduced [27]. Thus, significantly higher seedling emergence was reported for several cultivars of *B. rapa* sown at a depth of 1.5 cm compared to 3.0 cm [27].

Crop Density Crop density can exert considerable influence over disease incidence due to the ease with which pathogen inoculum can be transferred between closely spaced plants and alterations in crop microclimate. In densely planted crops, temperatures are more uniform, humidity is increased, and leaves are wet for longer, all of which provides favorable conditions for pathogen infection and subsequent development. Crop density can be manipulated in various ways, e.g., sowing, pruning, and fertilization.

Soil Amendments: Mulching, Fertilizers, and Organic Amendments

Mulches Mulches are used to conserve organic matter and moisture and to reduce soil erosion. A variety of materials can be used as mulches, including straw, manure, plastics, and paper. Mulching can lead to water retention and nutrient enrichment in the soil and can decrease soil temperature, all of which can influence pathogen infection and disease development in plants. Although mulching can reduce the spread of splash dispersed pathogens, by altering the environment, it could lead to increased severity of some diseases. Further, if crop residues are used in mulching, disease incidence could increase since the residues could be used as a food source by a range of pathogens.

Fertilizers Adequate mineral nutrition is central to crop production, and can also exert considerable influence on disease development [28, 29]. Below, the influence of nitrogen, phosphorus, potassium, calcium, and silicon on plant disease will be dealt with briefly.

Nitrogen Using nitrogen fertilizer above recommended rates can lead to increased disease incidence and lesion area. This has been shown for biotrophic fungal pathogens such as powdery mildews and rusts [30, 31] and necrotrophic pathogens such as *Magnaporthe grisea*, the cause of rice blast [32]. It is commonly thought that application of nitrogen fertilizer can increase disease severity via effects on crop canopy development. Thus, large canopies with high shoot densities may be more conducive to spore transfer and pathogen infection than sparse canopies. For example, nitrogen has been shown to increase the severity of *Fusarium* head blight in wheat, and it has been suggested that this might be the result of a nitrogen-induced increase in canopy size, leading to an altered microclimate [33]. In contrast, work on yellow rust on winter wheat suggested that the impact of nitrogen on disease was the result of effects of nitrogenous substances in wheat leaves on pathogen growth, rather than effects on canopy growth and microclimate [34].

But nitrogen is not always associated with increased crop disease. Indeed, various studies have reported no effect of nitrogen on disease severity (e.g., [35, 36]), while Hoffland et al. [31] found that the effect of nitrogen depended on the type of pathogen. Thus, nitrogen increased susceptibility of tomato to the powdery mildew pathogen *Oidium lycopersicum* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, while it had no effect on susceptibility to the vascular wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* [31]. In contrast, tomato plants were more susceptible to *Botrytis cinerea* when grown under low nitrogen conditions [37]. These results do not support the view that

nutrient-limited plants are better defended [38, 39]. It seems that generalizing about the effects of nitrogen on plant disease is unwise, and practically, although manipulation or assessment of crop nitrogen status might be used as part of disease control strategies, the approach adopted will depend on the crop and the pathogens from which it is most at risk [29].

Phosphorus In general, phosphorus fertilization tended to improve plant health, with reductions in disease recorded in 65% of cases studied by Perrenoud [40]. Nevertheless, phosphorus fertilization increased disease and pest problems in 28% of the cases examined [40]. As with nitrogen, the effects of phosphorus on plant disease might be the result of direct effects on the pathogen, host plant metabolism, leading to effects on pathogen food supply, and effects on plant defenses [29]. Indeed, foliar application of phosphate salts has been shown to induce resistance to pathogens in a range of crop plants, including cucumber [41], broad bean [42], grapevine [43], maize [44] and rice [45].

Clearly, an adequate phosphorus supply is important for crop growth and in turn, may well help to reduce disease. However, the regime of phosphorus fertilizer used will depend on a range of factors, including the crop and the pathogens likely to be important. Reuveni and Reuveni [46] suggested that foliar-applied phosphate might be used as part of an integrated disease control program. However, grower adoption of such an approach will depend on the existence of other, effective disease control measures and the economics of disease control in the particular crop.

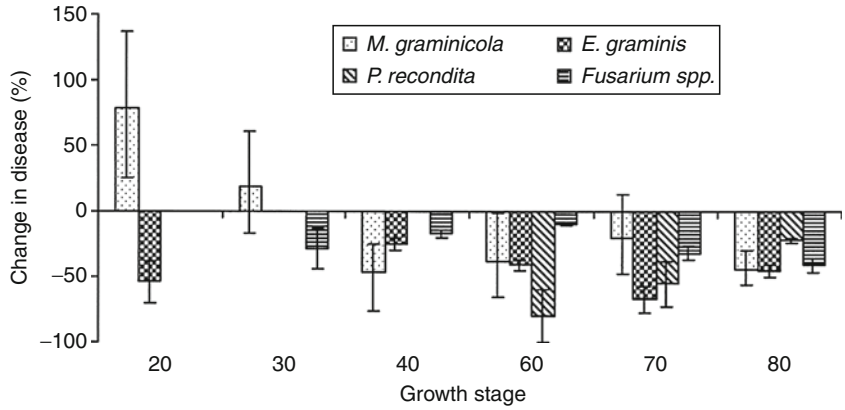
Potassium There are many reports that potassium is associated with disease reductions [47]. However, as these authors point out, inadequate consideration has been given to the effects of associated anions, nutrient balance, and nutrient status, to allow the definitive role of potassium to be determined. Thus, it has been suggested that in some cases, the effects of potassium, applied as potassium chloride fertilizer, might be due to the chloride ion rather than potassium [48]. Further, chloride fertilization has been shown to suppress disease in cereal crops [49].

There has been much interest in the application of fertilizers to crop foliage, including the effects of foliar fertilizer application for crop disease control [46, 50]. Foliar-applied potassium chloride has been shown to control *Blumeria graminis* and *Septoria tritici* on wheat in field studies [51, 52], probably due to osmotic effects on the fungal pathogens, disrupting pathogen development and subsequent infection [52, 53].

Application of potassium to deficient soils usually increases plant resistance to diseases [47]. This might be partly related to the effect of potassium in increasing epidermal cell wall thickness or disease escape as a result of vigorous crop growth [47], although the mechanisms by which potassium affects plant disease are not well understood.

Calcium There are many reports that application of calcium to soils, foliage, and fruit reduces the incidence and severity of a range of diseases of crops, including cereals, vegetable crops, legumes, fruit trees, as well as postharvest diseases of tubers and fruits [54]. For example, calcium has been shown to inhibit anthracnose (caused by *Colletotrichum gloeosporiodes* or *C. acutatum*) in apples [55] and to decrease postharvest disease development on strawberry [56], while treatment of tomato with calcium carbonate reduced fusarium crown rot disease [57]. In contrast, Nam et al. [58] could find no effect of calcium on anthracnose on strawberry. Because calcium increases resistance of plant cell membranes and cell walls to microbial enzymes, increasing calcium concentrations in storage organs could lead to enhanced resistance to pathogens [59, 60]. However, the form in which the calcium is applied can influence the mechanism by which calcium affects disease. For example, the addition of lime can affect disease by altering pH, while calcium salts (e.g., propionate) can be directly inhibitory to pathogens [54]. Making general recommendations for the use of calcium in plant disease control would be unwise, due to the range of crops and pathogens affected by calcium application. Instead, the appropriate amount and form of calcium to apply need to be determined for individual crop–pathogen interactions. The dwindling availability of fungicides, together with increasing public concern for the environment means that the use of calcium to control plant disease, especially postharvest, is attracting increased attention.

Silicon The effects of silicon in reducing disease severity have been known since 1940 [61]. However, it was not until the 1980s that more detailed work was



Crop Diseases, Management and Control of. Figure 1
Changes in disease in plots with incorporated straw. Bars show the difference between straw-treated and control plots as a percentage of the average in the control plots. For *Mycosphaerella graminicola,* the data are for severity on the top three living leaves; for other diseases, they refer to incidence. For *M.graminicola*, errors bars were calculated on the transformed scale and back-transformed; for other diseases, no transformation was needed (From [66])

carried out in this area. Thus, cucumbers grown in nutrient solutions supplemented with silicon were found to have significantly less powdery mildew infection than plants not receiving silicon supplementation [62, 63]. Indeed, silicon has been shown to suppress both foliar and soilborne pathogens in cucurbits [64] and to reduce susceptibility of rice to various pathogens [65]. Wheat grown in soil amended with silicon showed reduced infection by several pathogens, including *B. graminis* f.sp. *tritici, S. tritici,* and *Oculimacula yallundae* (Fig. 1) [66, 67].

It has been suggested that the effects of silicon in providing disease control are due to the creation of a mechanical barrier to penetration [68]. However, this has been disputed by studies which could find no evidence for the creation of a physical barrier following silicon treatment in wheat inoculated with powdery mildew and bitter gourd and tomato inoculated with *Pythium aphanidermatum* [69, 70]. Rather, several studies have suggested that silicon activates defenses in plants. For example, in wheat inoculated with *B. graminis* f.sp. *tritici*, epidermal cells of silicon-treated plants were shown to react to attempted infection with specific defenses, including papilla formation and callose production [71]. In the rice–*M. grisea* pathosystem – silicon-mediated resistance was found to be associated with accumulation of antimicrobial compounds at infection sites, including diterpenoid phytoalexins [72]. In fact, phytoalexin accumulation occurs in silicon-mediated resistance in both dicots and monocots and since phytoalexins are highly specific to plant species, it has been suggested that silicon might be acting on mechanisms shared by all plant species, e.g., those resulting in activation of plant stress genes [73].

Organic amendments Organic amendments include animal manure, solid wastes, and composts. Such amendments are often used to improve soil quality, usually by contributing to general suppressiveness through enhanced microbial biomass and activity [7]. Organic amendments are rich in labile carbon fractions which are an energy source for microorganisms, and moreover, they can themselves contain antagonistic microbes. A substantial body of data indicates that organic materials can reduce incidence of diseases caused by a range of plant pathogens [74, 75].

Irrigation Although an adequate water supply is vital to crop production, irrigation can play a detrimental rather than a beneficial role in managing plant diseases. For example, irrigation water can spread pathogen propagules and under dry conditions, can prevent desiccation of such propagules, thereby effectively increasing the level of inoculum in soil. Watering from overhead prolongs leaf wetness, thereby

providing favorable conditions for germination and infection by fungal spores. Overhead watering also increases the risk of splash-dispersal of spores, thus increasing pathogen spread. However, irrigation can be used to reduce the level of pathogen inoculum. Thus, the activity of microbes that destroy fungal sclerotia can be increased by alternate wetting and drying of the soil. Generally, drip or trickle irrigation, which delivers water directly to the root zone at a rate insufficient to lead to pathogen spread, is least likely to encourage disease development.

Use of Host Resistance

Most plants are resistant to most microbes. Thus, wheat plants are not affected by pathogens of tomato, and vice versa. This is known as nonhost resistance. However, every plant is attacked by its own pathogens, e.g., barley is attacked by the barley powdery mildew fungus, *B. graminis* f.sp. *hordei,* although it might not be able to defend itself equally well against all pathogens that are able to attack it. This ability of plants to resist attack by pathogens is genetically determined, but the diversity of types of genetic control of this resistance, and of conditions required for their expression, has led to a bewildering number of classifications of resistance types in plants. To complicate matters further, in some cases, different terms used by different authors are synonymous, while in other cases, the terms might be based on different criteria.

Types of Resistance

Seedling and Adult Plant Resistance Seedling resistance operates from the onset of plant growth and is effective throughout the life of the plant. It is generally controlled by single genes and is effective in the absence of matching virulence in the pathogen. In contrast, adult plant resistance (APR) covers a broad range of resistance types all of which are not effective at the plant's seedling stage. APR tends to be controlled by a number of genes, which might operate through a wide variety of mechanisms.

Polygenic and Oligogenic Resistance Resistance controlled by one, or at most two or three genes, is known as oligogenic. Here, a single gene can confer complete resistance. A gene conferring such resistance is often referred to as a major gene. Where resistance is controlled by a larger number of genes, it is known as polygenic. Genes conferring polygenic resistance are often referred to as minor genes.

Race-specific and Race-nonspecific Resistance The terms race-specific and race-nonspecific sought to differentiate between resistance that was subject to loss of effectiveness with the appearance of new virulent strains of a pathogen and resistance that was thought would never be lost because the pathogen was not capable of developing virulence to it. A major problem with these terms has been a lack of evidence to suggest that any particular resistance was race-nonspecific. The assumption was generally made that genes of large effect (major or seedling genes) were race-specific and genes of small effect (minor genes or APR) were race-nonspecific. This has subsequently and comprehensively been shown not to be the case in many host–parasite systems.

Vertical and Horizontal Resistance These terms were introduced to convey the difference between race-specific and race-nonspecific resistance [76]. In vertical resistance, there tends to be a high level of resistance by the host to some races of the pathogen and a low level of resistance to others, while in horizontal resistance, resistance tends to be exhibited equally to all races of the pathogen. Vertical resistance has also been denoted as specific resistance and horizontal resistance as general resistance. However, although the specific nature of some types of resistance can usually be established fairly easily, it is much more difficult to prove that resistance is truly general, since there is always the chance of a new pathogen race appearing with the ability to overcome the resistance.

Qualitative and Quantitative Resistance These terms are a rough match for seedling/APR and vertical/horizontal resistance, with qualitative resistance referring to a high level of resistance controlled by a single gene and quantitative resistance being controlled by several genes of smaller effect. The term "quantitative" subsequently came to be used to describe locations on chromosomes where genes of small effect were identified through mapping studies, hence "quantitative trait locus" or QTL. More recently the use of the term QTL has come to be used for major gene loci as well.

Partial Resistance Partial resistance was originally used in studies of the resistance in barley to leaf rust caused by *Puccinia hordei*. It describes resistance that reduced the rate of epidemic development despite having a susceptible reaction type [77]. Resistance to leaf rust was found to be governed by up to 6–7 minor genes with additive effects and was correlated with increased latency period and reduced infection frequency, pustule size, infectious period, and spore production. The term is now used more generally to describe any resistance that is only partially effective in reducing disease expression and is usually synonymous with minor gene resistance.

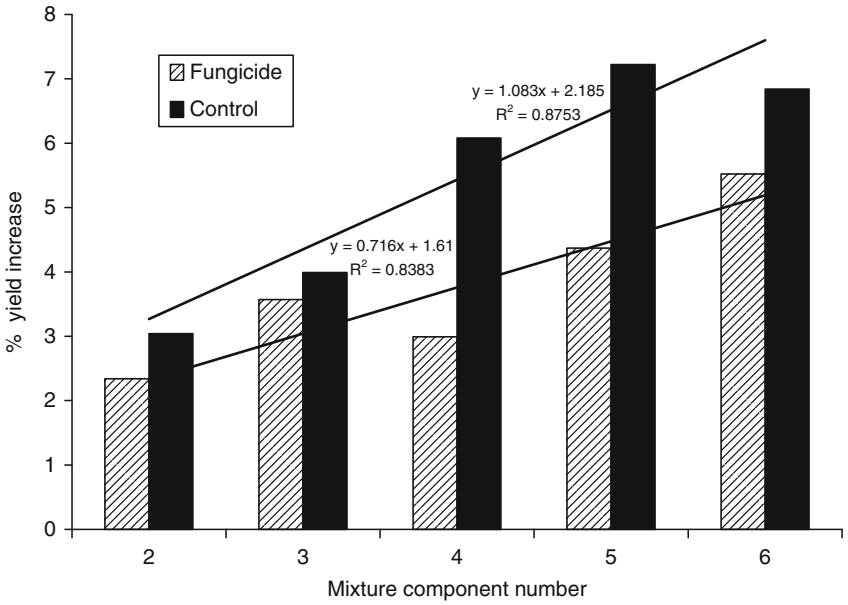
Durable Resistance Durable resistance was suggested for use by Johnson and Law [78] to refer to rust resistance in wheat that in practice had provided stable resistance in varieties that had been grown over a large area for many years. The term specifically avoided identifying the resistance with particular phenotypes or suggesting that the resistance would never be lost to a change in pathogen virulence. Specific well-studied examples are the *Sr2* and *Rpg1* genes for partial stem rust in wheat and barley, respectively, the genes *Lr34* and *Yr18* which provide partial resistance to leaf and stripe rust in wheat and which may in fact be the same gene and the *mlo* locus which provides resistance to powdery mildew in barley.

Genetic Engineering for Disease Resistance Using the techniques of genetic manipulation (GM), it is now possible to sequence and clone resistance genes from distantly related species. These genes could then be transferred into crop species, providing varieties with enhanced disease resistance. For example, the gene for resistance to bacterial blight of rice, *Xa21,* was sequenced, cloned, and transformed into rice, providing resistance against a range of pathogens [79]. This is a good example of how transgenic technologies could be used in a major crop and indeed, transgenic rice lines expressing *Xa21* are currently being tested in the field. The vision is to use GM technology to pyramid several different resistance genes in a rice variety, providing what is hoped will be durable resistance. However, unless there is coordination among plant breeders and the industry to prevent varieties being grown together, thereby allowing the pathogen to mutate one gene at a time, this strategy is unlikely to be successful.

One option for improving resistance to virus infection is to transform the gene that expresses the viral coat protein into the host plant. This approach was used to develop a line of papaya resistant to the very damaging papaya ringspot virus (PRSV). As a result of this work, two papaya varieties, Rainbow and SunUp, were commercialized. Rainbow was widely planted in Hawaii and was important in preventing the complete destruction of the papaya industry by PRSV [80].

Deployment of Resistance in Practice Many new crop varieties have been bred where resistance to a particular pathogen is based on the introduction of one gene. All too often history has shown that the introduction of such varieties is followed, within just a few years, by the appearance of a new race of the pathogen, able to overcome the "new" resistance. Such rapid breakdown of host resistance is favored by modern intensive agriculture, where crops are planted in monoculture covering huge areas. This favors any genetic variants of the pathogen with the ability to infect these new varieties. Breaking this "boom-and-bust" cycle of introducing a new resistant variety, followed by the rapid breakdown of resistance, can be achieved in various ways. One approach is to find durable sources of resistance. Another is to breed for combinations of race-specific genes in one crop variety. This is known as pyramiding and should be quite durable. However, it is controversial, since it might select for complex races of the pathogen possessing several matching virulences. Another approach is to diversify the deployment of resistance genes. This could involve spatial diversity across regions or fields, or the use of multilines or mixtures of cultivars.

A multiline is a series of near-isogenic (genetically identical) plant lines each differing in a single character, e.g., disease resistance. The plant lines can be grown together like a conventional crop, thereby retaining agronomic advantages such as crop uniformity, while confronting the pathogen with the problem of overcoming several different genes for resistance. A number of mechanisms have been proposed to account for disease control in multilines: (1) The high proportion of resistant plants in the multiline grown in the field reduces the amount of pathogen inoculums per unit



Crop Diseases, Management and Control of. Figure 2
Change in *R. secalis* infection of mixtures of winter barley cultivars compared with the mean of their components with different numbers of component cultivars (From [82])

area of crop, thereby reducing the amount of infection in the succeeding generation and the amount of inoculums produced by that generation. (2) Movement of pathogen inoculums between susceptible plants might be hampered by the presence of intervening resistant plants. (3) The average distance which the pathogen inoculums must travel in order to reach another susceptible plant is increased. (4) There might be induction of resistance, i.e., pre-inoculation with a race of the pathogen to which the line is resistant might protect it from a race to which it is normally susceptible [81]. However, the breeding program required for the development of a multiline is extensive. Similar in concept, but easier to put into practice, is the use of variety mixtures. Here, seed of a number of genetically distinct varieties of the crop are mixed and grown together as a single crop. Each variety possesses a different resistance gene(s), again presenting the pathogen with a genetic puzzle. But the use of mixtures requires careful choice of varieties, since they must possess similar characteristics, such as crop height and time to flowering. Nevertheless, mixtures have been shown to develop less disease than would be expected in component varieties grown alone. Mixtures can also provide yield increases, and the magnitude of disease control and effects on yield are dependent on the number of components in the mixture (Fig. 2). The mechanisms proposed to account for reductions in disease in mixtures include less efficient spread between plants and the induction of plant resistance by incompatible strains of the pathogen attempting to infect different hosts in the mixture [82].

Use of Chemicals to Control Crop Disease

Chemicals continue to play an important role in crop disease control. These chemicals act either by inhibiting germination, growth, and multiplication of the pathogen, or by killing the pathogen. Although chemicals can be used to control bacterial pathogens (bactericides) and nematodes (nematicides), this section will concentrate on chemicals with activity against fungal pathogens (fungicides).

Fungicides can be divided into several groups according to their mode of action. Protectant fungicides protect the plant against fungal propagules (e.g., spores) landing on the surface of leaves, stems, or fruit, but tend to be ineffective against established infections, since they do not enter the plant to any extent. To be effective therefore, protectant fungicides need to be

applied before the fungal pathogen enters the plant. In contrast, systemic fungicides enter the plant, and can become generally distributed within its tissues, thereby offering protection against fungal pathogens. Eradicant fungicides can enter plant tissues and can kill established infections. A number of protectant and systemic fungicides possess eradicant properties, while some systemic fungicides also have protectant activity.

Protectant Fungicides Protectant fungicides can be applied to the crop as high-volume or low-volume sprays, or occasionally as dusts. These fungicides tend to be broad spectrum, with activity against a range of different fungal pathogens. As a result, it is unlikely that pathogens would develop resistance to these chemicals. However, because they must be applied before the pathogen attempts to penetrate the plant, there is the need for reliable forecasting of infection risk. Protectant fungicides include the oldest and still widely used inorganic sulfur and copper compounds. Sulfur, applied as a dust, wettable powder, paste, or liquid, is used to control powdery mildews, some rusts, leaf blights, and fruit rots. Bordeaux mixture, made by mixing copper sulfate solution with calcium oxide or calcium hydroxide, consists mainly of colloidal hydrated cupric hydroxide stabilized by calcium sulfate. It has been replaced by copper oxychloride, which can be formulated by the manufacturer and simply diluted by the grower.

Organic sulfur compounds are an important and versatile group of fungicides and include thiram, ferbam, maneb, zineb, and mancozeb. These fungicides are all derivatives of dithiocarbamic acid, and because they are metabolized to isothiocyanate, they inactivate the sulfhydryl groups (-SH) in amino acids and enzymes. Thiram is used mostly for seed and bulb treatment for vegetables, flowers, and grasses, while ferbam is used to control foliage diseases, ornamentals, and fruit on trees. Maneb and mancozeb belong to a group of dithiocarbamic acid derivatives known as the ethylenebisdithiocarbamates. Maneb is a broad-spectrum fungicide for the control of foliage and fruit diseases of vegetables such as tomato, potato, and vine crops, as well as flowers, turf, and some fruit crops. Mancozeb is formed by the addition of zinc ion to maneb and a secondary effect of using mancozeb is the provision of manganese and zinc to plants deficient in these elements.

Various aromatic compounds have been developed as fungicides and include dichloran, used as a foliar or fruit fungicide, or as a postharvest spray for vegetables and flowers, and chlorothalonil, which is a broad-spectrum fungicide used on vegetables, field crops, ornamentals, and turf.

A number of very effective protectant fungicides belong to the rather heterogeneous group of heterocyclic compounds and include captan, iprodione, and vinclozolin. Captan is used for the control of leaf spots, blights, and fruit rots on various crops, as well as a seed treatment. It works by inhibiting thiol-containing enzymes in the fungal cell, and may also react with sulfhydryl groups.

Systemic Fungicides For a systemic fungicide to be effective, it must enter the plant and, to be translocated, it must be reasonably water-soluble. It must also be selective, possessing toxicity against the pathogen but not the host plant. Most systemic fungicides are xylem-mobile and as a result, tend to move from the base to the top of the plant, accumulating in leaves and shoot apices. As a result, such fungicides possess no activity against soilborne pathogens affecting roots. Phosphonate fungicides such as fosetyl-Al are also phloem-mobile and so can move down the plant to the roots, providing protection against, for example, root rots caused by *Phytophthora* species.

A wide range of systemic fungicides are now used to control plant pathogens. Unlike protectant fungicides, most systemic fungicides are site specific, and target only one, or just a few, specific steps in fungal metabolism. Although this was seen initially as a strength of systemic fungicides, it soon proved to be a weakness, since fungal pathogens were able to develop resistance to the chemicals, in some cases with alarming rapidity.

Acylalanine fungicides are effective against Oomycete pathogens such as *Phytophthora* and *Pythium* and include the widely used fungicide, metalaxyl. This is used as a soil or seed treatment and also possesses some curative activity. It moves readily from roots to shoots, although its lateral movement is slight. Some fungal pathogens have developed resistance to metalaxyl, and its use tends to be recommended in conjunction with a broad-spectrum fungicide.

Benzimidazole fungicides include some well-established and important chemicals, including benomyl, carbendazim, and thiabendazole. They are converted to methyl benzimidazole carbamate (MBC, carbendazim), which interferes with cell division. Benzimidazoles are broad spectrum in activity. Thus, benomyl is effective against a wide range of leaf spots, blights, rots, scabs, seedborne, and soilborne diseases. It can be applied to plants as a seed treatment, foliar spray, trunk injection, root dip, or fruit dip.

Oxanthiins, such as carboxin and oxycarboxin, possess the distinction of being the first chemicals with demonstrated systemic activity. They are active against some smut and rust pathogens, and also against *Rhizoctonia*. By inhibiting succinate dehydrogenase in fungal mitochondria, they affect respiration.

Organophosphate fungicides, such as foestyl-Al and phosphorous acid, are effective against Oomycete pathogens on a range of crops. Interestingly, fosetyl-Al has been reported to trigger plant defense mechanisms, e.g., phytoalexin biosynthesis and accumulation.

Other systemic fungicides include the pyrimidines, such as ethirimol and bupirimate, with activity against powdery mildews, and the triazoles, such as triadimefon, propiconazole, cyprodinil, and tebuconazole. The triazoles exhibit long-acting protective and curative activities against a broad spectrum of pathogens.

The most recently developed group of fungicides is the strobilurins. They are based on chemicals extracted from the wood rotting fungus *Strobilurus tenacelus*, and a range of highly effective strobilurins have been developed over the years. They work by blocking electron transfer at the site of quinol oxidation (Qo site, hence their description as QoI fungicides), thereby preventing ATP formation in respiration. These fungicides move trans-laminarly within leaves and some also move systemically through the vascular system. Important strobilurins include azoxystrobin, pyraclostrobin, and kresoxim methyl. They have broad-spectrum activity, and some also possess growth-promoting activity on treated plants, apparently by delaying senescence and altering plant–water relations.

Development of Resistance to Fungicides Until the advent of systemic fungicides, fungicide resistance was rare. The development of resistance in *Pyrenophora* to mercury used as a seed dressing, in the apple scab fungus *Venturia inaequalis* to dodine, and in *Penicillium* species to diphenyl compounds are exceptions. In contrast, the use of systemic fungicides soon led to the appearance of fungicide resistance. Thus, the pyrimidine fungicide dimethirimol was introduced in 1968, and by 1971, strains of the powdery mildew fungus *Sphaerotheca fuligenea* were detected on glasshouse cucumbers in the Netherlands. Similarly, following the introduction of QoI fungicides in the UK in 1996, resistance to powdery mildew in wheat was first recorded in 1998.

It seems that where a single site systemic fungicide is used intensively, there is the risk of fungal pathogens developing resistance to it. With single site fungicides, only a single mutation in the fungus might be required for resistance to develop. Some of the mechanisms by which a fungus might develop resistance following such a mutation include: (1) detoxification of the chemical, (2) decreased permeability of fungal cell membranes to the fungicide, (3) reduced affinity of the fungicide to the reactive site within the fungal cell, and (4) bypassing a blocked reaction via an alteration in fungal metabolism.

Given that fungicide resistance is now an accepted fact of life in crop protection, how can it be managed effectively? The first point to remember is that fungicides to which resistance has developed can still be useful in disease control if deployed sensibly. Strategies to minimize the risk of fungicide resistance developing include:

1. Reducing fungicide use
   - Applying fungicides only when necessary
   - Using fungicides as part of an integrated disease control program, including, for example, appropriate cultural control, and if available, resistant varieties
2. Diversifying fungicide treatments
   - Avoiding the repeated use of fungicides with the same mode of action
   - Instead, use mixtures of fungicides with different modes of action
   - During the growing season, alternating fungicides with different modes of action
   - Including a multisite fungicide in any fungicide mixture or spray program

Pesticides and Nontarget Organisms Pesticide use has greatly increased the quantity and improved the quality of food for the increasing world population. However, as pesticide use increased, so did concern about their adverse effects on nontarget organisms, including humans. Increasing public concern about the accumulation of pesticides in the environment and the impact on nontarget organisms has led to the introduction of rigorous regulatory processes [83, 84]. Nevertheless, there is continued concern over the impacts of pesticides on wildlife, including invertebrate populations, wild plants, and farmland birds [85]. These concerns have led to reviews of active substances used in plant protection, with the resulting withdrawal of an increasing number of crop protection products from the market [86]. This has created problems, and in some situations, effective control measures are no longer available to meet all the challenges posed by pathogens, pests, and weeds [86].

Biological Control of Plant Diseases Using Antagonistic Microorganisms

Biological control can be defined as the control of a plant pathogen using another living organism or organisms. This definition includes direct and indirect effects, as a result of either the introduction of antagonists, or manipulation of existing microbial populations to reduce disease. This section will deal with biological control using antagonistic microorganisms.

Antagonistic microorganisms control plant disease because they weaken or destroy the pathogen. They achieve this by various mechanisms, including: (1) directly parasitizing the pathogen, (2) producing antibiotics or toxins that exert an effect on the pathogen, (3) competing for space and nutrients with the pathogen, and (4) producing hydrolytic enzymes that destroy components of pathogen cells.

To be effective in disease control, a biological control agent (BCA) must be able to colonize a particular habitat or occupy a niche in sufficient numbers to disrupt the growth and survival of the target pathogen. For this reason, the most effective BCAs are likely to be found in the environment in which they are to be used. Thus, if the pathogen to be controlled infects plant roots, the best place to look for a potential BCA would be the rhizosphere. Finding appropriate BCAs is one thing, but using them in practice is quite another. For a start, as a living organism, the BCA must be formulated in such a way as to allow it to remain viable and survive following application [87]. Great advances have been made in the production and formulation of BCAs over the years, but despite the fact that 1,000 of microorganisms have been shown to interfere with growth and survival of plant pathogens under controlled and field conditions, relatively few have been registered and used in commercial practice. Currently, there are more than 50 bacterial products and 50 fungal products available commercially. The majority of both bacterial and fungal products are sold for control of seedborne or soilborne pathogens (Table 2), with fewer for foliar pathogens and timber decay fungi and even fewer for postharvest pathogens. Most contain individual microorganisms although there are some products that contain microorganism mixtures, and some individual microorganism strains are marketed in several different products expanding the potential market of a single active ingredient. Bacterial products are dominated by *Bacillus* species reflecting their ease of growth and production of long-lived spores mentioned earlier. Fungal products are dominated by *Trichoderma* spp. which are also easy to produce, generally have a low toxicity, and can sporulate well.

America's first biological fungicide seed treatment, Kodiak (marketed by Bayer), contains spores of *Bacillus subtilis* GB03 for control of *Alternaria*, *Aspergillus*, *Fusarium*, and *Rhizoctonia* spp. that attack root systems of a number of plants, including seed and pod vegetables, cotton, peanut, soybean, wheat, barley, and maize. The spore concentrate can be applied through standard slurry or mist seed treatment equipment, with fungicides if required, and the bacterium colonizes the root system providing control over the whole growing season.

Numerous products contain strains of *Trichoderma harzianum*, but one isolate, KRL-AG2 (T-22), sold by BioWorks Inc., USA, has been used and developed for several markets in US horticulture and agriculture in a number of different forms. When applied to soil, planting mixes, or turf, this BCA colonizes plant roots and provides protection against root pathogens such as *Cylindrocarpon*, *Fusarium*, *Pythium*, *Rhizoctonia*, and *Thielaviopsis*. RootShield granules are largely targeted at glasshouse and nursery use, and can be incorporated,

Crop Diseases, Management and Control of. Table 2 Examples of bacteria and fungi registered or commercially marketed as biological control agents for control of soilborne and seedborne plant pathogens (from [87])

Antagonist	Target pathogen(s)/ activity	Disease/host	Product name and source
BACTERIA			
Agrobacterium radiobacter	*Agrobacterium tumefaciens* causing root galls	Ornamentals and other plants sensitive to *A. tumefaciens* root galls	Norback 84-C, Galltrol-A, Nogall, Diegall, Dygall (Becker Underwood Pty Ltd, Australia; Bio-Care Technology Pty Ltd. Australia; New BiProducts, Inc. USA; AgBiChem Inc., USA; Agbioresearch Ltd. New Zealand)
Bacillus cereus BP01	Plant growth promotion	Cotton	Mepplus (MicroFlo Co. LLC, USA)
B. pumilus GB34	*Fusarium* spp., *R. solani*	Soybean	YieldShield concentrate; GB34 Biological Fungicide (Gustafson LLC, USA)
Bacillus licheniformis SB3086	Numerous pathogens, especially *Sclerotinia homeocarpa*	Ornamentals, turf	EcoGuard, Green Releaf (Novozymes Biologicals Inc., USA)
B. subtilis	*Pythium* damping-off	Tomato	Cillus, Green-all G (Greenbiotech Co, Korea)
	R. solani, Fusarium spp., *Alternaria* spp., and *Aspergillus* spp. pathogens	Root rots and seedling diseases generally	Kodiak, Epic, Concentrate, Kodiak HB, Quantum 4,000, System 3 (Gustafson LLC, USA)
	Fusarium spp., *Verticillium* spp., *R. solani,* and *Pythium* spp. pathogens	Various vegetable and field crops	PHYTOVIT WG (Prophyta Biologischer Pflanzenschutz GmbH, Germany)
B. subtilis GB03	*Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *R. solani*	Ornamentals, turf, dry and snap bean, cotton, peanut, soybean, wheat, and barley	Companion, Kodiak (Growth Products Ltd, USA; Gustafson LLC, USA; Bayer CropScience LP, USA)
B. subtilis MBI600	*Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Pythium* spp., *R. solani*	*Alfalfa*, dry/snap beans, peanut, soybean, field crops, turf, cotton	HiStick N/T, Pro-mix, Subtilex; Subtilex HB (Becker Underwood Inc., USA; Premier Horticulture Inc, Mexico)
B. subtilis subsp. *amyloliquefaciens* FZB24	*Fusarium* spp., *R. solani*	Various vegetables and ornamentals	Taegro, Tae-Technical (Earth Biosciences Inc. USA)
B. subtilis, *Bacillus circulans*, *Bacillus amyloliquefaciens*, *Paenibacillus polymyxa* (Mixture)	Damping-off (bacterial) diseases	All crops	Hydroguard (American Agritech, USA)
Burkholderia cepacia[a]	*Pythium* spp., *Fusarium* spp., *R. solani,* nematodes	*Alfalfa*, beans, clover, cotton, peas, wheat, vegetables, and others	Deny, Blue Circle (Stine Microbial Products, USA)
	Pythium spp., *Fusarium* spp., *R. solani,*	Maize, vegetables, cotton	Intercept (Soil Technologies Corp., USA)

Crop Diseases, Management and Control of. Table 2 (Continued)

Antagonist	Target pathogen(s)/ activity	Disease/host	Product name and source
Erwinia carotovora, nonpathogenic	Bacterial soft rots	Vegetables, cruciferous plants, rice	Biokeeper (Central Glass Co. Ltd., Japan)
P. polymyxa AC-1	Damping-off	Cucumber	NH, Topseed (Greenbiotech Co., Korea)
Pseudomonas sp.	Growth promotion, various seedborne and soilborne diseases	Vegetables, potato, cereals, etc.	Proradix (Sourcon-Padena, Germany)
Pseudomonas aureofaciens TX-1	Various turf grass pathogens	Turf grass diseases	Spotless (Turf Science laboratories Inc, USA)
Pseudomonas chlororaphis MA 342	*Drechslera* spp., *Septoria* spp., *Fusarium* spp.	Cereal seedborne diseases	Cedomon (BioAgri AB, Sweden)
P. chlororaphis 63-28	*Pythium* spp. *R. solani, F. oxysporum*	Stem and root rots, and wilt disease in various crop plants	AtEze (Eco Soil Systems Inc., USA)
Pseudomonas fluorescens	Frost damage, *E. amylovora*	Fruit, potato, tomato, berries	BlightBan A506 (NuFarm Inc., USA)
Pseudomonas solanacearum, nonpathogenic	*P. solanacearum*	*P. solanacearum* rots in vegetables	PSSOL (Natural Plant Protection, France)
P. syringae	*Botrytis* spp., *Penicillium* spp., *Mucor* spp.	Fruit, potato	Bio-save (EcoScience Corp., USA)
Streptomyces colombiensis WYE20	*R. solani*	Turf	Mycocide (KIBC Co., Korea)
Streptomyces goshikiensis WYE324	*R. solani*	Rice, turf	Safegrow (KIBC Co, Korea)
Streptomyces griseoviridis K61	Various soilborne pathogens	Vegetable and ornamental soilborne diseases	Mycostop (Verdera Oy, Finland)
Streptomyces lydicus WYCD108	Various soilborne pathogens	Root rots in many crops, turf, and ornamentals	Acinovate (Natural Industries Inc. USA)
FUNGI			
Aspergillus flavus AF36	*A. flavus* (aflatoxin +)	Cotton	AF36 (Arizona Cotton Research and Protection Council, USA)
A. flavus NRRL 21882	*A. flavus* (aflatoxin +)	Peanut	Afla-guard (Circle One Global Inc, USA)
Coniothyrium minitans CON/M/91-08	*Sclerotinia minor, S. sclerotiorum*	Protected vegetable and field crops	Contans WG (Prophyta Biologischer Pflanzenschutz GmbH, Germany; Sylvan Bio Products Inc, USA) and Intercept WG (Encore Technologies, MN, USA)
C. minitans KONI	*S. minor, S. sclerotiorum*	Glasshouse crops and amenity areas	KONI (Bioved Ltd., Szigetszentmiklos, Hungary)

Crop Diseases, Management and Control of. Table 2 (Continued)

Antagonist	Target pathogen(s)/ activity	Disease/host	Product name and source
F. oxysporum Fo47	*F. oxysporum*	Asparagus, basil, carnation, cyclamen, gerbera, tomato	Fusaclean (Natural Plant Protection, France)
Gliocladium catenulatum J1446	*Pythium* spp., *R. solani*, and numerous other pathogens	Damping-off of vegetables, herbs, ornamentals, and numerous other plants	Prestop Mix, Prestop WP, Primastop (Verdera Oy, Finland)
Gliocladium (*Trichoderma*) *virens* G-21	*Pythium ultimum, R. solani*	Damping-off of bedding plants, greenhouse crops, and ornamentals	SoilGard 12 G formerly GlioGard (Certis Inc, Columbia, MD, USA)
Pythium oligandrum	Numerous diseases	Numerous crops	Polyversum (Biopreparaty Ltd, Czech Republic)
Trichoderma spp.	Soilborne fungal pathogens	Turf, glasshouse crops, and field crops	Trich-A-Soil (Becker Underwood Pty Ltd, Australia)
	Sclerotium cepivorum, Pyrenochaeta	Onion	Tenet (Agrimm Tecnologies, New Zealand)
	Soilborne plant pathogens	Ornamentals, fruit, turf, olive, vine	Trichomic (AMC Chemical, Spain)
	Armillaria	Tree seedlings	Arborguard (Biodiscovery Ltd, New Zealand)
T. harzianum T-22 (KRL-AG2)	*Fusarium* spp., *Pythium* spp., *Cylindrocarpon* spp., *Thielaviopsis* spp., *R. solani, S. homeocarpa*	Range of crops, ornamentals, and turf	T-22 HC, T-22 Planter Box, T-22 Granules PlantShield HC, RootShield drench and granules, TurfShield, TRIANUM-P, TRIANUM-G (Bio-Works Inc, Fairport, NY, USA; Koppert, the Netherlands)
T. harzianum	Various fungi	Legumes and leaf vegetables	Supresivit (Borregaard and Reitzel, Denmark or Fytovita, Czech Republic)
	Pythium spp., *R. solani* seedling diseases	Numerous crops	Eco-T (Plant health Products, South Africa)
T. harzianum GBF-0208	Numerous pathogens	Vegetables, bulbs, turf	Green-all T WP (Green Biotech Co. Ltd., Korea)
T. harzianum + *Trichoderma viride*	*Fusarium* spp., *Phytophthora* spp., *Pythium* spp., and *R. solani*	Field crops, vegetables, ornamental and turf	Trichodry, Trichoflow, Trichogrow, Trichopel R Trichopel (Agrimm Technologies, New Zealand)
T. harzianum T 35 + *T. harzianum* TH315	*Fusarium* spp., *Pythium* spp., *R. solani, S. rolfsii*	Seedlings diseases on a range of crops and potato	Root Pro and Root-Protato (Mycontrol, Hamovil, Israel)

Crop Diseases, Management and Control of. Table 2 (Continued)

Antagonist	Target pathogen(s)/ activity	Disease/host	Product name and source
T. harzianum + *T. polysporum*	Various root-infecting fungi	Glasshouse crops	BINAB-T W P(Bio-Innovation Eftr AB, Bredholmen, Sweden; or Svenska Predator AB, Sweden; or Bayer, Sweden)
T. viride	*Fusarium* spp., *Pythium* spp., *R. solani*	Damping-off, foot rots and collar rots of several plants	Ecoderma (Margo Biocontrols Private Ltd., Bangalore, India)

[a]*B. cepacia* is no longer available in North America because of concerns over the potential for human pathogenicity of some strains of this species

top-dressed, broadcast, or applied in-furrow or to planting holes for use on flowers, bedding plants, ornamentals, vegetables, pome and stone fruit, trees, and tree nuts. For agriculture, T-22 Planter Box is applied as a coating on seeds and seed pieces, an in-furrow spray, and as a transplant starter and T-22 HC as a broadcast or in-furrow treatment, both for use on field and row crops, hay and forage crops, bulbs and vegetables. In Europe, T-22 (Koppert, the Netherlands) is available as TRIANUM-G and TRIANUM-P, wettable granule and wettable powder formulations. Much of the scientific background concerning the use of this isolate has been extensively reviewed [88].

In terms of the future, advances that can improve quantity, quality, and shelf life of inocula would be welcome, particularly for bacteria such as *Pseudomonas* that do not form spores. In recent work, both *Pseudomonas* and *Trichoderma* isolates have been simultaneously applied to seed via drum priming and found to survive and proliferate on roots similarly to when applied individually [89]. This procedure may be a way to apply and maintain multiple BCAs in a commercially relevant process. Further approaches include integration with other control strategies such as cultural methods and chemical treatments [87]. A better understanding of the natural ecology of any potential BCA might facilitate a more rational approach to production and use.

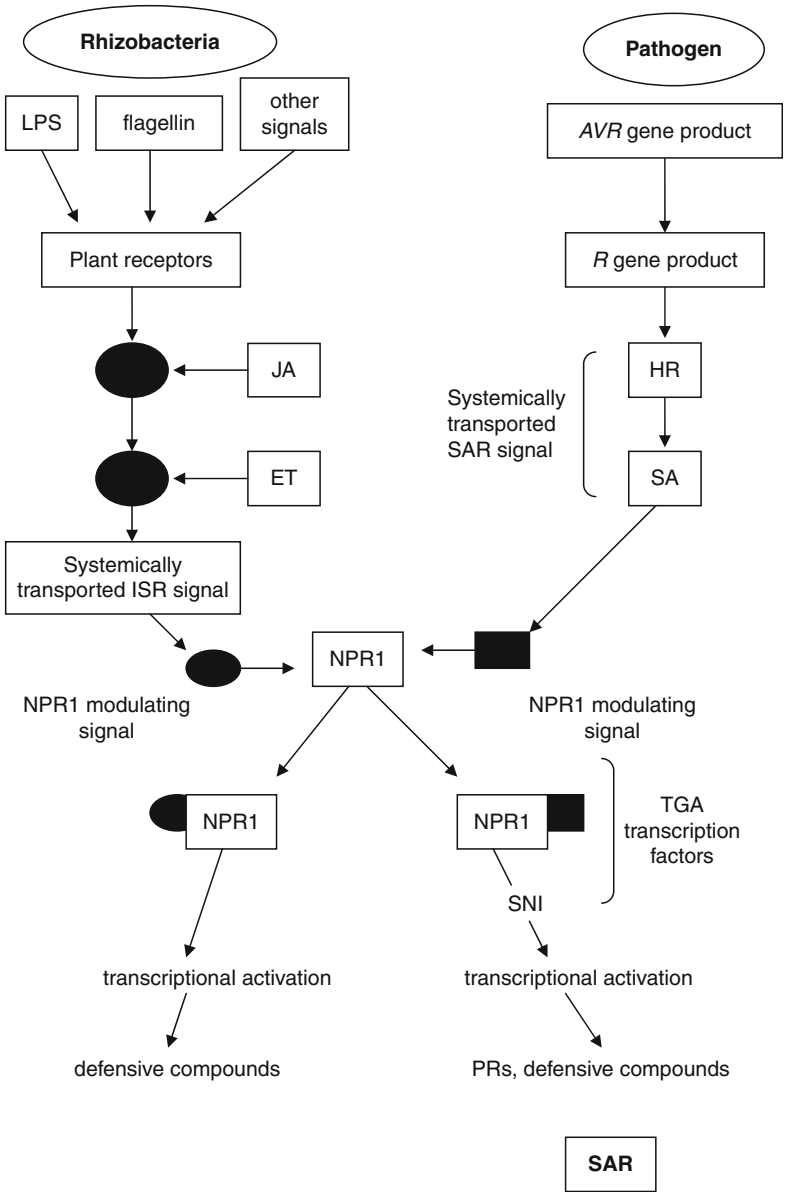
Induced Resistance to Control Crop Diseases

Following infection by a microbial pathogen, susceptible plants can develop an enhanced resistance to further infection [90]. This is known as induced resistance and can be split broadly into two types: systemic acquired resistance (SAR) and induced systemic resistance (ISR).

In SAR plants, develop a broad-spectrum systemic resistance to pathogen infection following a localized infection by a necrotizing pathogen or treatment with various agents, e.g., acibenzolar-S-methyl (ASM) or Probenazole (Oryzemate®). SAR is associated with increased levels of salicylic acid (SA) and with the coordinate expression of a specific set of genes encoding PR proteins ([91]; Fig. 3). Moreover, application of SA or one of its functional analogues, such as ASM, induces SAR and activates the same set of PR genes [92]. Indeed, expression of a set of PR genes, and PR-1 in particular, is used as a marker for SAR induction, although it is important to note that the induction of resistance is not always accompanied by PR-1 expression [93, 94].

In contrast to SAR, ISR develops as a result of colonization of plant roots by certain strains of plant growth–promoting rhizobacteria (PGPR) and is mediated by jasmonic acid– (JA) and ethylene (ET)-sensitive pathways ([91]; Fig. 3). Phenotypically, ISR is similar to SAR in that it acts unspecifically against taxonomically different pathogens [91, 95].

The resistance responses described above can be associated with direct activation of defenses. However, such responses can also be associated with an ability to "recall" previous infection, root colonization, or chemical treatment. This phenomenon is known as priming and results in plants responding more rapidly and



Crop Diseases, Management and Control of. Figure 3
A model for the signal transduction network controlling induced systemic resistance (ISR) mediated by plant growth–promoting rhizobacteria and pathogen-induced systemic acquired resistance (SAR) in *Arabidopsis thaliana*. LPS, lipopolysaccharide; PRs, pathogenesis-related proteins; *AVR*, avirulence gene product; *R*, resistance gene product; HR, hypersensitive response; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; NPR1, a regulatory protein involved in signaling in SAR and ISR in *A. thaliana*; SNI, transcriptional repressor of SAR genes; TGA transcription factors, family of transcription factors interacting with SA-induced NPR1 (From [91])

effectively when exposed to subsequent pathogen attack ([96]; Fig. 3). Usually, no changes in gene expression or in the levels of resistance traits are detectable in response to the priming agent alone, which might be a chemical elicitor such as ASM or a challenging pathogen. Interestingly, priming of resistance is usually caused by agents that fully induce resistance when applied at higher doses [97, 98] and suggests

that direct resistance induction and priming might differ from one another quantitatively rather than qualitatively.

A wide range of microbes and chemicals are known to induce resistance and it seems likely that other forms of induced resistance exist. Thus, it is well known that the nonprotein amino acid, β-aminobutyric acid (BABA), can induce resistance in a variety of crop plants [99]. BABA-induced resistance (BABA-IR) has been used as a model for the study of priming and in *Arabidopsis*, it is based on various mechanisms. Thus, BABA-IR against *P. syringae* and *B. cinerea* functions via priming for SA-inducible defenses, while against a different set of pathogens (*Hyaloperonospora parasitica*, *Plectosphaerella cucumerina* and *Alternaria brassicicola*), it is based on priming for resistance through the formation of callose-rich papillae [100–102].

Since the introduction of the first chemical resistance activator Probenazole (registered in Japan as Oryzemate®, Meiji Seika Kaisha Ltd) in 1975, a number of other chemical and microbial activators have been developed, including ASM, registered as Bion® and Actigard® (Syngenta), Milsana® (*Reynoutria sacalinensis* extract, KHH BioScience Inc. USA), Elexa® (chitosan, SafeScience, USA), and Messenger® (harpin protein, Eden Bioscience, USA). However, although high levels of disease control can be achieved with plant activators in controlled environments, their performance under field conditions has been less impressive. In fact, the moderate levels of disease control and high levels of variability exhibited by plant activators in the field have been instrumental in the very slow uptake of induced resistance in crop production systems. In the following paragraphs, the performance of selected plant activators under field conditions will be discussed.

Probenazole (3-allyloxy-1,2-benziothiazole-1,1-oxide) was first introduced for the control of rice blast disease (*Pyricularia oryzae*) and bacterial blight (*Xanthomonas oryzae*). It is widely used in Asia where it is applied as a granular treatment either to paddy fields or as a seedling box treatment. Following application, the compound is absorbed by the roots, then systemically transferred to the rest of the plant and can control rice blast disease for between 40 and 70 days post application [103]. However, despite continuous use since its introduction, there have been no reports of pathogen insensitivity to probenzole and indeed, it still accounted for 53% of the chemicals used for seedling box treatments on rice in 2005 [103]. It is believed this is because the compound is only weakly toxic to fungi and activates disease defense systems in rice [104, 105].

A large body of data has accumulated on the efficacy of ASM against a range of diseases under field conditions [106, 107]. Most studies report disease control, although the level of control ranges from 4% to 99%. High levels of disease control were achieved on tobacco, where infections by *P. syringae* pv. *tabaci, Cercospora nicotianae,* and *Alternaria alternata* were reduced by 99%, 91%, and 89%, respectively [108, 109]. In wheat, the crop that ASM was originally aimed at, disease control was not so impressive, ranging from 35% for *Puccinia recondita* and *Septoria* spp., to 77% for *B. graminis* f.sp. *hordei* [110, 111]. ASM even increased disease levels in peanut, where infection by *Cercosporidium personatum* was greater than untreated controls by 52% [112]. Working on oilseed rape, Liu et al. [113] found that pretreatment with ASM in October/November decreased the number of leaf lesions caused by the Phoma stem canker pathogen *L. maculans* in the autumn/winter, as well as the severity of stem canker in the subsequent spring/summer. In this work, reductions in numbers of leaves with lesions were between 25% and 55% [113]. More recently, ASM was shown to reduce infection of barley by the leaf scald pathogen *R. secalis* by 45% [114].

Chitosan is a common polymer in shells of crustaceans, exoskeletons of insects, and cell walls of fungi [115]. A commercial formulation, Elexa®, contains 4% chitosan as its active ingredient and has been shown to protect a range of crops against pathogens. For example, when used as a seed treatment, it reduced downy mildew severity on pearl millet by 58%, and when used as a foliar spray, it reduced infection by 75% [116]. When used on grapevines, eight applications of Elexa® applied over the season reduced the incidence of downy mildew by 50% and powdery mildew by 75% compared to untreated controls [117].

A number commercially available products are based on microbial proteins. One such is Messenger®, which is based on the protein harpin obtained from *Erwinia amylovora* [118]. Used as a crop protectant, Messenger® has had mixed success. For example, although it possessed good efficacy against blue mold

in apples [119], its efficacy against gray mold in strawberry [120] and target spot of tomato [121] was poor. In some interesting recent work, Chen et al. [122] generated specific fragments of $HpaG_{Xooc}$, a harpin from *X. oryzae* pv. *oryzicola*, and found that one of these fragments, $HpaG_{10–42}$, stimulated growth of rice plants and provided enhanced resistance to *X. oryzae* pv. *oryzae* and *M. grisea*. $HpaG_{10–42}$ was also shown to control bacterial blight, rice blast, and sheath blight, and to increase grain yields, under field conditions [123]. Here the level of disease control depended on the cultivar, with greater control obtained with *indica* compared to *japonica* cultivars.

ISR was first shown to be effective under field conditions in the mid-1990s, when application of PGPR as a seed treatment followed by soil drench application led to a reduction in severity of bacterial wilt [124], and control of bacterial angular leaf spot and anthracnose [125]. Subsequent research by Raupach and Kloepper [126] showed that treatment of cucumber seed with PGPR led to increased plant growth and control of angular leaf spot and anthracnose. Field experiments in Thailand in 2001 and 2002 studied the effects of PGPR, used alone or as mixtures, on control of southern blight of tomato caused by *Sclerotium rolfsii*, anthracnose of long cayenne pepper caused by *Colletotrichum gloeospoiroides*, and mosaic disease of cucumber caused by cucumber mosaic virus (CMV) [127]. Mixtures of PGPR (all *Bacillus* spp.) were found to suppress disease more consistently than the PGPR strain *Bacillus pumilus* IN937b, used alone.

Induced resistance offers the prospect of durable, broad-spectrum disease control using the plants own resistance. However, it is plagued by inconsistency and relatively poor disease control compared with fungicides. These problems relate to the fact that induced resistance is a host response and as such is greatly influenced by genotype and environment. Unfortunately, the understanding of the impact of these influences on induced resistance is poor, as is the understanding of how best to use induced resistance in crop protection practice. Understanding in these areas needs to improve, and answers to several important, practical questions are required, for example: (1) Should resistance inducing agents be applied early or late in the season? (2) Is induced resistance effective against pathogens with long periods of asymptomatic growth in plant tissue, e.g., *R. secalis* on barley? (3) Can resistance inducers be used as a means of reducing fungicide applications to crops, e.g., can resistance inducers be applied early to reduce pathogen infection and colonization, thereby allowing less fungicide to be used? What is required is research related to specific crops aimed at trying to determine how best to fit induced resistance into disease control programs. Farmers and crop protectionists have grown accustomed to high levels, or even complete, disease control. Ultimately, for induced resistance to gain more widespread acceptance in crop protection, there will need to be a lowering of expectation in terms of levels of disease control.

Other Approaches to Controlling Crop Diseases

Biofumigation Biofumigation involves the suppression of plant pests and diseases by biocidal hydrolysis products, most notably the isothiocyanates released by glucosinolate (GSL)-containing plants in soil. It can involve GSL-containing plants as rotation crops, or intercrops by incorporating fresh plant material as green manure, or by utilizing processed plant products high in GSLs, e.g., seed meals. Strategies for the field implementation of biofumigation are described in detail by Kirkegaard [128].

Soil Solarization Soil solarization, or soil heating, harnesses solar energy in order to increase soil temperature. This is commonly achieved by mulching (covering, tarping) the soil with transparent polyethylene or other transparent plastic sheets. The following are a few of the important principles of soil solarization: (1) It should be carried out during periods of high temperature and intense solar radiation, with low levels, or no precipitation. (2) Soil needs to be moist in order to increase the thermal sensitivity of resting structures and improve heat conduction. (3) The mulching period needs to be sufficiently long (~4 weeks or greater) in order to achieve disease control at all desired depths. The efficacy of soil solarization in controlling crop diseases and the mechanisms involved are discussed in detail by Gamliel and Katan [129].

Integrated Control of Plant Diseases

As pointed out by Agrios [130], plant disease control is most effective when all of the relevant information

regarding the crop, potential pathogens, previous disease history, availability of host resistance, environmental conditions, etc., are taken into account in devising the disease control program. An integrated program of disease control aims to (1) eliminate or reduce the initial pathogen inoculum, (2) reduce the effectiveness of the initial pathogen inoculum, (3) maximize host plant resistance, (4) delay the onset of disease in the plant/crop, and (5) slow down the development and progression of secondary cycles [130].

The precise approach adopted will depend on the crop and its disease spectrum, but would probably include several of the following: (1) appropriate hygiene/sanitation, e.g., using disease-free seed/planting material, (2) using appropriate cultural measures, (3) using resistant plant varieties, (4) using appropriate fungicides, and (5) using biologically based methods, if available and appropriate, e.g., biological control, induced resistance.

Control of late blight (*P. infestans*) on potato provides a good example of an integrated approach to disease control (see www.endure-network.eu). For late blight, the first step in an integrated strategy is reducing primary sources of inoculum, such as avoiding infected seed tubers, using certified potato seed, preventing or reducing oospore production by controlling volunteer potatoes, for example. The next step is variety choice, since use of a variety expressing some resistance to late blight offers the potential to reduce fungicide inputs as part of an integrated strategy. Nevertheless, fungicides play a crucial role in integrated control of late blight. Although control measures are aimed mainly at preventing infection, if late blight appears in a crop, the control strategy switches to stopping or reducing the epidemic. Effective control of late blight depends on access to information such as phase of crop growth, fungicide products available, dose rates, timings, weather conditions, as well as access to tools, such as an appropriate decision support system (DSS). The DSS can integrate all relevant information to generate spray recommendations, thereby increasing the efficacy of the control strategy without increasing risk.

Future Directions

High crop yields are maintained in most developed countries through the use of improved varieties, together with fertilizers and pesticides. In these countries, farmers and growers are accustomed to achieving high levels of disease control with fungicides, although, as discussed above, the development of fungicide resistance can erode fungicide efficacy. Levels of disease control obtained with many biologically based control methods are lower than those achieved using fungicides, and moreover, many biologically based methods tend to provide inconsistent disease control. Thus, although induced resistance can provide high levels of disease control on some crops, with many crops, disease control is less impressive. Expression of induced resistance in crop plants can also be variable, depending, for example, on genotype and environment. Problems also exist with variability and inconsistency of disease control provided by some BCAs. Farmer perceptions of inadequate and inconsistent disease control will not persuade them to adopt biologically based approaches. Minimizing the effects of these problems clearly requires further research.

In spite of the huge effort by researchers to develop novel biologically based solutions for disease control (such as BCAs, plant-derived substances, induced resistance agents), few products have reached the marketplace. The relatively high cost of registration, together with the limited market size for some products, has been identified as a major barrier. However, this problem has been recognized by regulatory authorities, and in the UK, for example, the Chemicals Regulation Directorate (Pesticides) has launched a scheme that encourages applicants to register their products. Under this scheme, the requirements for registration can be tailored to the product type and importantly, the application fee can be reduced [86].

The continued ability of pathogens to overcome host resistance genes and to develop resistance to fungicides seriously erodes the ability to provide effective, lasting disease control on important crops. These problems combined with the withdrawal of active substances from the market and increasing public concern with the effects of pesticides on the environment create a huge challenge for plant pathology in the future. Plant disease control has an important role to play in efforts to feed the world's increasing population. However, providing effective and lasting disease control, without harming the environment, will require not just a sensible approach to the use of host resistance and fungicides, but also a range of innovative approaches. In some situations, innovative

control methods might be used to complement existing approaches. In other cases, for example for those diseases for which no adequate control measures exist, innovative control options might provide the only solution.

Bibliography

Primary Literature

1. Large EC (1940) The advance of the fungi. Jonathan Cape, London
2. Strange RN (2003) Introduction to plant pathology. Wiley, Chichester
3. Oerke E-C (2006) Crop losses to pests. J Agric Sci 144:31–43
4. Hazell P, Wood S (2008) Drivers of change in global agriculture. Philos Trans R Soc B 363:495–515
5. Leonard KJ (2001) Stem rust – future enemy? In: Peterson P (ed) Stem rust of wheat: from ancient enemy to modern foe. APS, St. Paul, pp 119–146
6. Campbell CL, Long DL (2001) The campaign to eradicate the common barberry in the United States. In: Peterson P (ed) Stem rust of wheat: from ancient enemy to modern foe. APS, St. Paul, pp 16–50
7. Janvier C, Villeneuve F, Alabouvette C, Edel-Hermann V, Mateille T, Steinberg C (2007) Soil health through soil disease suppression: which strategy descriptors to indicators? Soil Biol Biochem 39:1–23
8. Ball BC, Bingham I, Rees RM, Watson CA, Litterick A (2005) The role of crop rotations in determining soil structure and crop growth conditions. Can J Soil Sci 85:557–577
9. Bailey DJ, Duczek LJ (1996) Managing cereal diseases under reduced tillage. Can J Plant Pathol 18:159–167
10. Peters RD, Sturz AV, Carter MR, Sanderson JB (2003) Developing disease suppressive soils through crop rotation and tillage management practices. Soil Tillage Res 72:181–182
11. Sumner DR (1982) Crop rotation and plant productivity. In: Rechcigl J (ed) Handbook of agricultural productivity, vol I. CRC, Boca Raton, pp 273–315
12. Umaerus VR, Scholte K, Turkensteen LJ (1989) Crop rotation and the occurrence of fungal diseases in potatoes. In: Vos J, Van Loon CD, Bollen GJ (eds) Effects of crop rotation on potato productions in the temperate zones. Academic, Dordrecht, pp 171–189
13. Sturz AV, Carter MR, Johnston HW (1997) A review of plant disease, pathogen interactions and microbial antagonism under conservation tillage in temperate humid agriculture. Soil Tillage Res 41:169–189
14. Van Bruggen AHC, Semenov AM, van Diepeningen AD, de Vos OJ, Blok WJ (2006) Relation between soil health, wave-like fluctuations in microbial populations, and soil-borne plant disease management. Eur J Plant Pathol 115: 105–122
15. Van Diepeningen AD, de Vos OJ, Zelenev VV, Semenov AM, van Bruggen AHC (2005) DGGE fragments oscillate with or counter to fluctuations of cultivable bacteria along wheat roots. Microb Ecol 50:506–517
16. Khan AG (1975) The effect of vesicular arbuscular mycorrhizal associations on growth of cereals II. Effects on wheat growth. Ann Appl Biol 80:27–36
17. McFadden AG, Sutton JC (1975) Relationships of populations of *Trichoderma* spp. in soil to disease in maize. Can J Plant Sci 55:579–586
18. Lynch JM, Panting LM (1980) Cultivation and the soil biomass. Soil Biol Biochem 12:29–33
19. Carter MR, Rennie DA (1984) Nitrogen transformations under zero and shallow tillage. Soil Sci Soc Am J 48:1077–1081
20. Chen W, Hoitink HAJ, Madden LV (1988) Microbial activity and biomass in container media for predicting suppressiveness to damping-off caused by *Pythium ultimum*. Phytopathology 78:1447–1450
21. Carignano M, Staggenborg SA, Shroyer JP (2008) Management practices to minimize tan spot in a continuous wheat rotation. Agron J 100:145–153
22. Gladders P, Musa TM (1980) Observations of the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. Plant Pathol 29:28–37
23. Kolte SJ (1985) Diseases of annual edible oilseed crops, vol II, Rapeseed-mustard and sesame diseases. CRC, Boca Raton
24. Naseri B, Davidson JA, Scott ES (2008) Survival of *Leptosphaeria maculans* and associated mycobiota on oilseed rape stubble buried in soil. Plant Pathol 57:280–289
25. Humpherson-Jones FM (1992) Epidemiology and control of dark leaf spot of Brassicas. In: Chelkowski J, Visconti A (eds) Alternaria: biology, plant diseases and metabolites. Elsevier, Amsterdam, pp 267–288
26. Zhan J, Fitt BDL, Pinnschmidt HO, Oxley SJP, Newton AC (2008) Resistance, epidemiology and sustainable management of *Rhynchosporium secalis* populations on barley. Plant Pathol 57:1–14
27. Nuttall WF (1982) The effect of seeding depth, soil moisture regime, and crust strength on emergence of rape cultivars. Agron J 74:1018–1022
28. Datnoff LE, Elmer WH, Huber DM (2007) Mineral nutrition and plant disease. APS, St. Paul
29. Walters DR, Bingham IJ (2007) Influence of nutrition on disease development caused by fungal pathogens: implications for plant disease control. Ann Appl Biol 151:307–324
30. Mascagni HJ Jr, Harrison SA, Russin JS, Desta HM, Colyer PD, Habetz RJ, Hallmark WB, Moore SH, Rabb JL, Hutchinson RL, Boquet DJ (1997) Nitrogen and fungicide effects on winter wheat produced in the Louisiana Gulf Coast region. J Plant Nutr 20:1375–1390
31. Hoffland E, Jeger MJ, van Beusichem ML (2000) Effect of nitrogen supply rate on disease resistance in tomato depends on the pathogen. Plant Soil 218:239–247
32. Talukder ZI, McDonald AJS, Price AH (2005) Loci controlling partial resistance to rice blast do not show marked QTL

X environment interaction when plant nitrogen status alters disease severity. New Phytol 168:455–464
33. Lemmens M, Buerstmayr H, Krska R, Schumacher R, Grausgruber H, Rackenbauer P (2004) The effect of inoculation treatment and long term application of moisture on Fusarium head blight symptoms and deoxynivalenol contamination in wheat grains. Eur J Plant Pathol 110:299–308
34. Neumann S, Paveley ND, Beed FD, Sylvester-Bradley R (2004) Nitrogen per unit area affects the upper asymptote of *Puccinia striiformis* f.sp. *tritici* epidemics in winter wheat. Plant Pathol 53:725–732
35. Buschbell T, Hoffmann GM (1992) The effects of different nitrogen regimes on the epidemiologic development of pathogens on winter-wheat and their control. J Plant Dis Prot 99:381–403
36. Olesen JE, Mortensen JV, Jorgensen LN, Andersen MN (2000) Irrigation strategy, nitrogen application and fungicide control in winter wheat on a sandy soil. I. Yield, yield components and nitrogen uptake. J Agric Sci (Cambridge) 134:1–11
37. Hoffland E, van Beusichem ML, Jeger MJ (1999) Nitrogen availability and susceptibility of tomato leaves to *Botrytis cinerea*. Plant Soil 210:263–272
38. Bryant JP, Chapin FS III, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40:357–368
39. Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or to defend. Q Rev Biol 67:283–335
40. Perrenoud S (1990) Potassium and plant health, 2nd edn. International Potash Institute, Bern
41. Mucharromah E, Kuc J (1991) Oxalates and phosphates induce systemic resistance against disease caused by fungi, bacteria and viruses in cucumber. Crop Prot 10:265–270
42. Walters DR, Murray DC (1992) Induction of systemic resistance to rust in *Vicia faba* by phosphate and EDTA: effects of calcium. Plant Pathol 41:444–448
43. Reuveni M, Reuveni R (1995) Efficacy of foliar application of phosphates in controlling powdery mildew fungus on field-grown winegrapes: effects on cluster yield and peroxidase activity. J Phytopathol 143:21–25
44. Reuveni R, Agapov V, Reuveni M (1994) Foliar spray of phosphates induces growth increase and systemic resistance to *Puccinia sorghi* in maize. Plant Pathol 43:245–250
45. Mandahar HK, Lyngs Jorgensen HJ, Mathur SB, Smedegaard-Petersen V (1998) Resistance to rice blast induced by ferric chloride, di-potassium hydrogen phosphate and salicylic acid. Crop Prot 17:323–329
46. Reuveni R, Reuveni M (1998) Foliar fertilizer therapy – a concept in integrated pest management. Crop Prot 17:111–118
47. Prabhu AS, Fageria NK, Huber DM, Rodrigues FA (2007) Potassium and plant disease. In: Datnoff LE, Elmer WH, Huber DM (eds) Mineral nutrition and plant disease. APS, St. Paul, pp 57–78
48. Fixen PE, Gelderman RH, Gerwing J, Cholick FA (1986) Response of spring wheat, barley and oats to chloride in potassium chloride fertilizers. Agron J 78:664–668
49. Engel RE, Eckhoff J, Berg RK (1994) Grain yield, kernel weight and diseases responses of winter wheat cultivars to chloride fertilization. Agron J 86:891–896
50. Ehret DL, Menzies JG, Bogdanoff C, Utkhede RS, Frey B (2002) Foliar applications of fertiliser salts inhibit powdery mildew on tomato. Can J Plant Pathol 24:437–444
51. Cook JW, Kettlewell PS, Parry DW (1993) Control of *Erysiphe graminis* and *Septoria tritici* on wheat with foliar-applied potassium chloride. J Sci Food Agric 63:126
52. Mann RL, Kettlewell PS, Jenkinson P (2004) Effect of foliar-applied potassium chloride on septoria leaf blotch of winter wheat. Plant Pathol 53:653–659
53. Kettlewell PS, Cook JW, Parry DW (2000) Evidence for an osmotic mechanism in the control of powdery mildew disease of wheat by foliar-applied potassium chloride. Eur J Plant Pathol 106:297–300
54. Rahman M, Punja ZK (2007) Calcium and plant disease. In: Datnoff LE, Elmer WH, Huber DM (eds) Mineral nutrition and plant disease. APS, St. Paul, pp 79–94
55. Biggs AR (1999) Effects of calcium salts on apple bitter rot caused by two *Colletotrichum* spp. Plant Dis 83: 1001–1005
56. Cheour F, Willemot C, Arul J, Desjardins Y, Makhlouf J, Charest PM, Gosselin A (1990) Foliar application of calcium chloride delays postharvest ripening of strawberry. J Am Soc Hortic Sci 115:89–792
57. Woltz SS, Jones JP, Scott JW (1992) Sodium chloride, nitrogen source and lime influence Fusarium crown rot severity in tomato. HortScience 27:1087–1088
58. Nam MH, Jeong SK, Lee YS, Choi JM, Kim HG (2006) Effects of nitrogen, phosphorus, potassium and calcium nutrition on strawberry anthracnose. Plant Pathol 55:246–249
59. Conway WS, Sams CE (1984) Possible mechanisms by which postharvest calcium treatment reduces decay in apples. Phytopathology 74:208–210
60. Biggs AR, Peterson CA (1990) Effect of chemical applications to peach bark wounds on accumulation of lignin and suberin and susceptibility to *Leucostoma persoonii*. Phytopathology 80:861–865
61. Wagner F (1940) Die bedeutung der kieselsnure far das wachstum einiger kulturpflanzen, ihren nnhrstoffhaushalt und ihre anfnlligkeit gegen echte mehltaupilze. Phytopathol Z 12:427–479
62. Miyake Y, Takahashi E (1983) Effect of silicon on the growth of solution cultured cucumber plant. Soil Sci Plant Nutr 29:71–83
63. Adatia MH, Besford RT (1986) The effects of silicon on cucumber plants grown in recirculating nutrient solution. Ann Bot 58:343–351
64. Belanger RR, Bowen P, Ehret DL, Menzies JG (1995) Soluble silicon: its role incrop and disease management of greenhouse crops. Plant Dis 79:329–336
65. Datnoff LE, Rodrigues FA, Seebold KW (2007) Silicon and plant disease. In: Datnoff LE, Elmer WH, Huber DM (eds) Mineral nutrition and plant disease. APS, St. Paul, pp 233–246

66. Rodgers-Gray BS, Shaw MW (2000) Substantial reductions in winter wheat diseases caused by addition of rice straw but not manure to soil. Plant Pathol 49:590–599
67. Rodgers-Gray BS, Shaw MW (2004) Effects of straw and silicon soil amendments on some foliar and stem base diseases in pot-grown winter wheat. Plant Pathol 53:733–740
68. Kim SG, Kim KW, Park EW, Choi D (2002) Silicon-induced cell wall fortification of rice leaves: a possible cellular mechanism of enhanced host resistance to blast. Phytopathology 92:1095–1103
69. Samuels AL, Glass ADM, Ehret DL, Menzies JG (1991) Mobility and deposition of silicon in cucumber plants. Plant Cell Environ 14:485–492
70. Heine G, Tikum G, Horst WJ (2007) The effect of silicon on the infection by and spread of *Pythium aphanidermatum* in single roots of tomato and bitter gourd. J Exp Bot 58:569–577
71. Belanger RR, Benhamou N, Menzies JG (2003) Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f.sp. *tritici*). Phytopathology 93:402–412
72. Rodrigues FA, McNally DJ, Datnoff LE, Jones JB, Labbe C, Benhamou N, Menzies JG, Belanger RR (2004) Silicon enhances the accumulation of diterpenoid phytoalexins in rice: a potential mechanism for blast resistance. Phytopathology 94:177–183
73. Fauteux F, Remus-Borel W, Menzies JG, Belanger RR (2005) Silicon and plant disease resistance against pathogenic fungi. FEMS Microbiol Lett 249:1–6
74. Bailey KL, Lazarovits G (2003) Suppressing soil-borne diseases with residue management and organic amendments. Soil Tillage Res 72:169–180
75. Litterick A, Wood M (2009) The use of composts and compost extracts in plant disease control. In: Walters D (ed) Disease control in crops: biological and environmentally-friendly approaches. Wiley, Oxford, pp 93–121
76. Vanderplank JE (1968) Disease resistance in plants. Academic, New York
77. Parlevliet JE, Van Ommeren A (1975) Partial resistance of barley to leaf rust, *Puccinia hordei*. II. Relationship between field trials, micro plot test and latent period. Euphytica 24:293–303
78. Johnson R, Law CN (1975) Genetic control of durable resistance to yellow rust (*Puccinia striiformis*) in the wheat cultivar Hybride de Bersée. Ann Appl Biol 81:385–391
79. Wang GL, Song WY, Ruan DL, Sideris S, Ronald PC (1996) The cloned gene, *Xa21,* confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. Mol Plant Microbe Interact 9:850–855
80. Gonsalves D (2004) Transgenic papaya in Hawaii and beyond. AgBioForum 7(1/2):36–40, http://www.agbioforum.org
81. Burdon JJ (1978) Mechanisms of disease control in heterogeneous plant populations: an ecologist's view. In: Scott PR, Bainbridge A (eds) Plant disease epidemiology. Blackwell, Oxford, pp 193–200
82. Newton A (2009) Plant disease control through the use of variety mixtures. In: Walters D (ed) Disease control in crops: biological and environmentally friendly approaches. Wiley, Oxford, pp 162–171
83. Holm RE, Baron JJ, Kunkel D (2005) The IR-4 programme and its cooperation with the crop protection industry to provide new pest control solutions to US specialty crop growers. Proc BCPC Int Congr Crop Sci Technol 1:239–250
84. Stark G (2008) The EU thematic strategy for pesticides and the UK national pesticides strategy. In: Proceedings crop protection in northern Britain 2008, Dundee, pp 23–26
85. Best J, Burn A (2005) 'Polluter pays' or 'public good': tackling indirect effects of pesticides. Proc BCPC Int Congr Crop Sci Technol 2:1097–1102
86. Richardson DM (2005) The registration process, its effect on active substance availability, and initiatives to reduce the impact on minor crops at both UK and EU level. Proc BCPC Int Congr Crop Sci Technol 1:231–238
87. Whipps JM, McQuilken MP (2009) Biological control agents in plant disease control. In: Walters D (ed) Disease control in crops: biological and environmentally friendly approaches. Wiley, Oxford, pp 27–61
88. Harman GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96:190–194
89. Bennett AJ, Whipps JM (2007) Dual application of beneficial microorganisms to seed during drum priming. Appl Soil Ecol 38:83–89
90. Hammerschmidt R (2007) Introduction: definitions and some history. In: Walters D, Newton A, Lyon G (eds) Induced resistance for plant disease control: a sustainable approach to crop protection. Blackwell, Oxford, pp 1–8
91. Pieterse CMJ, Van Loon LC (2007) Signalling cascades involved in induced resistance. In: Walters D, Newton A, Lyon G (eds) Induced resistance for plant disease control: a sustainable approach to crop protection. Blackwell, Oxford, pp 65–88
92. Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner H (1996) Systemic acquired resistance. Plant Cell 8:1809–1819
93. Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185–209
94. Sparla F, Rotino L, Valgimigli MC, Pupillo P, Trost P (2004) Systemic resistance induced by benzothiadiazole in pear inoculated with the agent of fire blight (*Erwinia amylovora*). Sci Hortic 101:269–79
95. Zehnder GW, Murphy JF, Sikora EJ, Kloepper JW (2001) Application of rhizobacteria for induced resistance. Eur J Plant Pathol 107:39–50
96. Goellner K, Conrath U (2008) Priming: it's all the world to induced resistance. Eur J Plant Pathol 121:233–242
97. Kohler A, Schwindling S, Conrath U (2002) Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPR1/NIM1* gene in *Arabidopsis*. Plant Physiol 128:1046–1056
98. van Hulten M, Pelser M, Van Loon LC, Pieterse CMJ, Ton J (2006) Costs and benefits of priming for defense in *Arabidopsis*. Proc Natl Acad Sci USA 103:5602–5607

99. Jakab G, Cottier V, Toquin V, Rigoli G, Zimmerli L, Metraux J-P, Mauch-Mani B (2001) β-aminobutyric acid-induced resistance in plants. Eur J Plant Pathol 107:29–37
100. Zimmerli L, Jakab G, Métraux J-P, Mauch-Mani B (2000) Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by beta-aminobutyric acid. Proc Natl Acad Sci USA 97:12920–12925
101. Zimmerli L, Metraux J-P, Mauch-Mani B (2001) β-aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic pathogen *Botrytis cinerea*. Plant Physiol 126:517–523
102. Ton J, Mauch-Mani B (2004) β-aminobutyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J 38:119–130
103. Iwata M (2001) Probenazole – a plant defence activator. Pestic Outlook 12:28–31
104. Watanabe T, Igarashi H, Matsumoto K, Seki S, Mase S, Sekizawa Y (1977) The characteristics of probenazole (oryzemate) for the control of rice blast. J Pesticide Sci 2:291–296
105. Watanabe T (1997) Effect of probenazole (Oryzemate) on each stage of rice blast fungus (*Pyricularia oryzae Cavara*) in its life cycle. J Pesticide Sci 2:395–404
106. Vallad GE, Goodman RM (2004) Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Sci 44:1920–1934
107. Walters D, Walsh D, Newton A, Lyon G (2005) Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathology 95: 1368–1373
108. Cole DL (1999) The efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance, against bacterial and fungal diseases of tobacco. Crop Prot 18:267–273
109. Perez L, Rodriguez ME, Rodriguez F, Roson C (2003) Efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance, against tobacco blue mold caused by *Peronospora hyoscyami* f.sp. *tabacina*. Crop Prot 22:405–413
110. Gorlach J, Volrath S, Knauf-Belter G, Hengy G, Beckhove U, Kogel K-H, Oostendorp M, Staub T, Ward E, Kessmann H, Ryals J (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8:629–643
111. Stadnik MJ, Buchenauer H (1999) Control of wheat diseases by a benzothiadiazole-derivative and modern fungicides. J Plant Dis Prot 106:466–475
112. Zhang S, Reddy MS, Kokalis-Burelle N, Wells LW, Nightengale SP, Kloepper JW (2001) Lack of induced systemic resistance in peanut to late leaf spot disease by plant growth-promoting rhizobacteria and chemical elicitors. Plant Dis 85:879–884
113. Liu SY, Liu Z, Fitt BDL, Evans N, Foster SJ, Huang YJ, Latunde-Dada AO, Lucas JA (2006) Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. Plant Pathol 55:401–412
114. Paterson L, Walsh DJ, Walters DR (2008) Effect of resistance elicitors on *Rhynchosporium secalis* infection of barley. In: Proceedings crop protection in northern Britain 2008, Dundee, pp 163–168
115. Hadwiger LA (1999) Host-parasite interactions: elicitation of defence responses in plants with chitosan. In: Jolles P, Muzzarelli RAA (eds) Chitin and chitinases. Birkhauser, Switzerland, pp 185–200
116. Sharathchandra RG, Niranjan Raj S, Shetty NP, Amruthesh KN, Shekar Shetty H (2004) A chitosan formulation Elexa™ induces downy mildew disease resistance and growth promotion in pearl millet. Crop Prot 23:881–888
117. Schilder A, Gillet J, Sysak R, Wise J (2002) Evaluation of environmentally friendly products for control of fungal diseases of grapes. In: Proceedings of the 10th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing and Viticulture. Weinsberg, Germany, pp 163–167
118. Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer A, Beer SV (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85–88
119. de Capdeville G, Beer SV, Watkins CB, Wilson CL, Tedeschi LO, Aist JR (2003) Pre- and post-harvest harpin treatments of apples induce resistance to blue mold. Plant Dis 87:39–44
120. Meszka B, Bielenin A (2004) Possibilities of integrated grey mold control on strawberry plantations in Poland. Bull OILB SROP 27:41–45
121. Pernezny K, Stoffella P, Collins J, Carroll A, Beaney A (2002) Control of target spot of tomato with fungicides, systemic acquired resistance activators, and a biocontrol agent. Plant Prot Sci 38:81–88
122. Chen L, Qian J, Qu S, Long J, Yin Q, Zhang C, Wu X, Sun F, Wu T, Hayes M, Beer SV, Dong H (2008) Identification of specific fragments of $HpaG_{Xooc}$, a harpin from *Xanthomonas oryzae* pv. *oryzicola,* that induce disease resistance and enhance growth in plants. Phytopathology 98:781–791
123. Chen L, Zhang S-J, Zhang S-S, Qu S, Ren X, Long J, Yin Q, Qian J, Sun F, Zhang C, Wang L, Wu X, Wu T, Zhang Z, Cheng Z, Hayes M, Beer SV, Dong H (2008) A fragment of *Xanthomonas oryzae* pv. *oryzicola* Harpin $HpaG_{Xooc}$ reduces disease and increases yield of rice in extensive grower plantings. Phytopathology 98:792–802
124. Wei G, Yao GW, Zehnder S, Tuzun S, Kloepper JW (1995) Induced systemic resistance by select plant growth promoting rhizobacteria against bacterial wilt of cucumber and the beetle vectors. Phytopathology 85:1154
125. Wei G, Kloepper JW, Tuzun S (1996) Induced systemic resistance to cucumber diseases and increased plant growth by plant growth promoting rhizobacteria under field conditions. Phytopathology 86:221–224

126. Raupach GS, Kloepper JW (2000) Biocontrol of cucumber diseases in the field by plant growth promoting rhizobacteria with and without methyl bromide fumigation. Plant Dis 84:1073–1075
127. Jetiyanon K, Fowler WD, Kloepper JW (2003) Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. Plant Dis 87: 1390–1394
128. Kirkegaard J (2009) Biofumigation for plant disease control – from the fundamentals to the farming system. In: Walters D (ed) Disease control in crops: biological and environmentally friendly approaches. Wiley, Oxford, pp 172–195
129. Gamliel A, Katan J (2009) Control of plant disease through soil solarisation. In: Walters D (ed) Disease control in crops: biological and environmentally friendly approaches. Wiley, Oxford, pp 196–220
130. Agrios GN (2005) Plant pathology, 5th edn. Elsevier, Burlington

Books and Reviews

Cheatham MR, Rouse MN, Esker PD, Ignacio S, Pradel W et al (2009) Beyond yield: plant disease in the context of ecosystem services. Phytopathology 99:1228–1236
Lucas JA (1998) Plant pathology and plant pathogens. Blackwell, Oxford
Pollock C, Pretty J, Crute I, Leaver C, Dalton H (2008) Sustainable agriculture I. Philos Trans R Soc B 363:443–680
Pollock C, Pretty J, Crute I, Leaver C, Dalton H (2008) Sustainable agriculture II. Philos Trans R Soc B 363:681–913
Strange RN (1993) Plant disease control: towards environmentally acceptable methods. Chapman & Hall, London
Walters D, Newton A, Lyon G (2007) Induced resistance for plant defence: a sustainable approach to crop protection. Blackwell, Oxford
Walters D (2009) Disease control in crops: biological and environmentally friendly approaches. Wiley, Oxford

Crop Plants Transformation Methods

Dawei Yuan[1], Svetlana Dashevskaya[1], Richard M. Twyman[2], Teresa Capell[1], Paul Christou[3,4]
[1]Department of Vegetal Production and Forestry Science, ETSEA, University of Lleida, Lleida, Spain
[2]Department of Biological Sciences, University of Warwick, Coventry, UK
[3]Department de Produccio Vegetal i Ciencia Forestal, Universitat de Lleida/ICREA, Lleida, Spain
[4]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Article Outline

Glossary

Acetosyringone A phenolic compound that activates the *Agrobacterium tumefaciens vir* genes and thus initiates DNA transfer.

Agrobacterium-mediated transformation The introduction of DNA into plants by the bacterium *Agrobacterium tumefaciens*.

Bactofection Gene transfer to animal cells mediated by bacteria.

BIBAC A binary vector for plant transformation based on the high-capacity bacterial artificial chromosome (BAC).

Binary vector A plant transformation vector in two parts, one carrying the T-DNA and one carrying the *vir* genes.

Cassette A modular DNA sequence designed for portability between different vectors.

Chimeric (transformed plants) Comprising cells with different genotypes, usually a first-generation transformant (T_0) where some cells are transgenic and others are not.

Concatemer A DNA molecule containing multiple contiguous copies of the same sequence.

Conjugation DNA transfer between cells through a specialized conduit known as a pilus.

Counterselectable marker A selectable marker gene whose absence is required for cells to survive.

Destination vector The vector designated to receive a DNA cassette during LR recombination in the Gateway system.

Direct DNA transfer DNA transfer mediated by physical or chemical means rather than by bacteria or viruses.

Entry clone The vector holding the DNA cassette that needs to be transferred to the Destination vector during LR recombination in the Gateway system.

Episomal A genetic entity that replicates independently of the host chromosome, such as a plasmid or nonintegrating virus.

Explant A piece of plant tissue transferred to culture and propagated independently.

Filler DNA Extra DNA added at junctions during illegitimate recombination, either by synthesis across a template or random addition of nucleotides.

Gateway A proprietary cloning system based on LR recombination, a form of site-specific recombination using the *att*B and *att*P sites of *Escherichia coli* and bacteriophage λ.

Gene targeting Disruption of a preselected gene by homologous recombination with a DNA cassette.

Helper plasmid A plasmid that is not used as a cloning vehicle, but which supplies necessary functions in *trans*.

Homing endonuclease A specialized type of restriction enzyme encoded by introns and inteins with a long asymmetric recognition sequence.

Homologous recombination Recombination requiring long regions of homology but no sequence specificity.

Illegitimate recombination Recombination requiring neither long regions of homology nor specific sequences; it can occur by direct nonhomologous end-joining (all sequences conserved, sometimes with added filler DNA) or at regions of microhomology.

***In planta* transformation** Transformation methods that can be used with intact plants rather than tissue explants, and which therefore require no regeneration.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Integration The insertion of one DNA sequence in the midst of another.

Minichromosome A vector comprising the minimal elements that allow it to function as a chromosome.

Minimal cassette The minimal sequences required to achieve transformation and transgene expression, typically a promoter, gene, and terminator.

Multigene transfer The simultaneous transfer of more than one gene into a plant.

Particle bombardment A physical transformation method based on the acceleration of DNA-coated metal particles into intact plant cells.

Promoter The DNA sequence in front of a gene which controls its spatiotemporal expression profiles and whether its expression is sensitive to external stimuli.

Reporter gene A gene whose function is to yield an easily detectable product that can be used to measure or delimit the activity of a regulatory element such as a promoter.

Screenable marker Another name for a reporter gene.

Selectable marker A gene whose function is to confer on transformed cells some property that allows them to be propagated in conditions under which nontransformed cells either die or cannot grow efficiently.

Southern blot (DNA blot) A method for the detection of DNA sequences using specific labeled complementary probes.

Stable transformation Transformation followed by integration of DNA into the host genome such that the transgene becomes a permanent new genomic locus.

Superbinary vector A binary vector in which the *vir* gene functions are enhanced.

Supervirulent An *Agrobacterium tumefaciens* strain with a broader host range or more efficient DNA transfer ability than normal strains.

T-DNA The section of DNA within a binary vector which is transferred to the host plant.

Transactivation domain The part of a transcription factor that activates transcription.

Transient expression The expression of introduced DNA for a short time before the DNA is diluted and degraded.

Transfection (a) In bacteria, the transfer of phage DNA into bacterial cells by chemical or physical means; (b) in animals and plants, the transfer of any DNA into cultured cells by chemical or physical means.

Transformant A plant that has been transformed with exogenous DNA.

Transformation (a) In bacteria, the transfer of plasmid or genomic DNA into bacterial cells by chemical or physical means; (b) in animal cells, the spontaneous or induced change from a normal to an oncogenic phenotype; (c) in plants, any means of DNA transfer that does not involve the use of a virus.

Transgenic Containing integrated exogenous DNA in the nuclear genome.

Transplastomic Containing integrated exogenous DNA in the plastid genome.

Terminator The sequence following the gene that is required to terminate transcription.

***vir* Gene** One of several genes on binary vectors that are required in *trans* to mediate T-DNA transfer.

Zinc finger A zinc-coordinating protein motif usually associated with DNA binding, often found in sequence-specific DNA binding proteins such as transcription factors.

Definition of the Subject and Its Importance

The term **transformation** (or more properly "genetic transformation") was first coined to describe the natural process by which bacteria take up free DNA from their surroundings. The term was required to distinguish this process from two other ubiquitous natural gene transfer mechanisms in bacteria: **conjugation** (direct transfer of DNA from cell to cell through a connecting tube called a pilus) and **transduction** (transfer of DNA between cells carried by the capsid of a virus). All three of these natural processes have been exploited in the laboratory as ways to introduce exogenous DNA into bacterial cells, and in this context the definition of transformation was later refined to take into account the different consequences of gene transfer depending on the origins of the transforming DNA. The meaning of **transformation** was thus restricted to the uptake of naked plasmids or genomic DNA fragments, whereas an alternative term – **transfection** – was coined to describe the uptake of naked phage DNA, the distinction required because only the latter can initiate a phage infection.

Unsurprisingly, the same terms have been adopted to describe analogous processes in other organisms, but the precise definitions vary due to preexisting conventions. Cultured animal cells are said to undergo transformation when they change from the normal phenotype (cessation of growth when confluence is achieved) to an oncogenic phenotype (continued growth, formation of foci) so "transformation" is usually avoided in the context of gene transfer to prevent ambiguity. Therefore, transfection is used to describe the introduction of any naked DNA into animal cells regardless of its origin. The transfer of DNA from bacteria to animal cells (including the use of conjugation-like mechanisms) is unusual and has only recently been adopted as a gene transfer procedure in the laboratory, so a new term has been coined for this process – **bactofection**. The introduction of DNA into animal cells using a virus as a carrier is known as transduction, just as it is in bacteria.

The terminology for plants is a hybrid of the conventions used for bacteria and animal cells. Transformation refers to any gene transfer process where a virus is not used as a carrier (otherwise transduction is the correct term, as in bacteria and animal cells). This means that **plant transformation** includes both the uptake of naked DNA (**direct DNA transfer**) and the transfer of DNA by the conjugation-like method adopted by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (***Agrobacterium*-mediated transformation**). However, due to operational analogies with cultured animal cells, the introduction of DNA into cultured plant protoplasts is also termed transfection, so in this specific context the terms transformation and transfection are synonymous.

The fate of the introduced DNA depends on many factors. If it does not contain an origin of replication that is active in plant cells it is usually maintained in the nucleus for a short period of time (hours to days), eventually being diluted and degraded. If the introduced DNA contains an active expression cassette, then a product may be expressed during this time, a process known as **transient expression**. In a very small number of cells, the DNA integrates into the genome and becomes a permanent new locus. This is known as **stable transformation**. It is a rare event (approximately one in every 10,000 cells that takes up exogenous DNA integrates it into the genome) and selectable marker genes are usually employed to ensure that the small number of transformed cells can grow at the expense of their nontransformed peers, allowing the recovery of transgenic tissues and whole plants. Cells, tissues, and whole plants that are stably transformed with a particular fragment of exogenous DNA are termed **transgenic** if the DNA integrates into the nuclear genome or **transplastomic** if it integrates into the plastid genome. The integrated DNA is known as the **transgene** (depending on context this term can refer to a single gene or to the integrated segment of exogenous DNA which may contain one or more genes).

If the introduced DNA does have an origin of replication that functions in plants, then it may be maintained **episomally** (as an extrachromosomal replicon). This occurs when the introduced genetic material is part of a replication-competent recombinant plant virus, or when it includes the components of a plant centromere, allowing it to function as a minichromosome. Viral vectors are generally short-lived, so they are also used for transient expression. However, the ability of viruses to spread systemically means that the transgene may be expressed at very high levels throughout the plant, and the infection may last days or weeks. Minichromosome vectors are a relatively new development in plants but as with equivalent "artificial chromosome" vectors in yeast and animal cells, the idea is that they should facilitate stable transformation without the need for integration into the genome.

Plant transformation is a fundamental component of both basic and applied plant biology. For basic research, transformation allows scientists to study how genes function and allows the expression of both endogenous genes and transgenes to be controlled. This has increased our understanding of how plants grow, develop, and defend themselves against pests, diseases, and harsh environments, how photosynthesis is controlled, and the basis of primary and secondary metabolism. For applied research, transformation can be used to improve the agronomic performance of crops, making them hardier, more nutritious, more productive, or converting them from conventional crops into green factories producing chemical precursors, novel oils, industrial enzymes, and

pharmaceuticals. Plants provide human beings with all types of useful products: food and animal feed, fibers and structural materials, and small molecules that can be used as dyes, scents, and medicines. Plants have been cultivated for these products since the dawn of history, and for the same length of time people have sought to improve plants by breeding them and selecting the better-performing and most useful varieties. The limitations of this approach, i.e., the fact that breeders are restricted to the existing gene pool in each group of sexually compatible species, and that breeding takes a long time to achieve its goals, can be overcome by plant transformation, thus accelerating the development of plants with novel, beneficial traits.

Introduction

Plant transformation became a routine process in the 1980s but several attempts to transfer DNA into plant tissues were reported in the previous 2 decades, although stable transformation was never confirmed. The first deliberate transformation of plant tissue with laboratory-created recombinant DNA was achieved in 1983 when several researchers reported the introduction of recombinant plasmids, including backbone sequences from cloning vectors and selectable marker genes [1–3]. This marked the first creation of transgenic plant cells in the currently accepted meaning of the word. In the intervening 30 years, heterologous genes have been introduced into well over 100 different plant species either through the use of the soil bacterium *A. tumefaciens* or alternative strategies involving direct DNA transfer to plant cells and tissues. In addition, many plant viruses have been developed as episomal vectors, allowing high-level transient gene expression, although because of their inability to achieve stable transformation they are not considered further in this article.

Principles and Methods of Plant Transformation

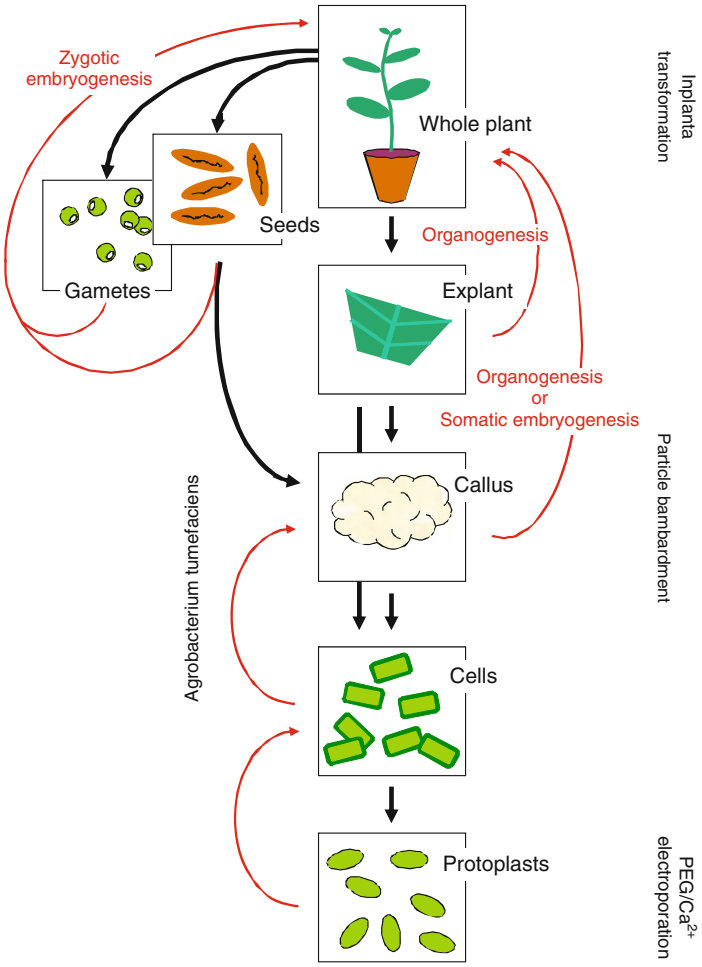
A fundamental difference between animals and plants is that differentiated plant tissue shows a high degree of developmental plasticity. Depending on the species, isolated stem segments, leaf disks, and seed-derived callus tissue may be able to regenerate an entire new plant under appropriate culture conditions. For most plant species, some form of tissue culture step is therefore used for the successful production of transgenic plants after cells or small tissue explants have been subjected to the actual transformation procedure. However, it should be noted that whole-plant (*in planta*) transformation strategies are also available in some species, in which the need for tissue culture is minimized or eliminated.

The ease with which plant material is manipulated and interconverted in culture provides many opportunities for the development of techniques for gene transfer and the recovery of transgenic plants (Fig. 1). DNA can be introduced into most types of plant material – protoplasts, cell suspensions, callus, vegetative tissue explants, gametes, seeds, zygotes, embryos, organs, and whole plants; so, the ability to recover fertile plants from such material is often the limiting step in plant genetic engineering rather than the DNA transfer process itself. It is also possible to maintain transformed plant cell lines or tissues (e.g., root cultures) rather than regenerating whole plants as these can often be sustained indefinitely in culture.

Gene transfer to plants can be achieved through three types of mechanism – viral transduction, bacterial gene delivery, and chemical/physical direct DNA transfer. Bacterial gene delivery using the soil pathogen *A. tumefaciens* is the most widely used method for dicotyledonous plants, but increasingly also for monocots. Physical methods are the next most popular, especially particle bombardment for the transformation of more recalcitrant monocots such as cereals. Chemical transfection methods are little used these days, and are only suitable for protoplasts. Each of the above methods can be used to achieve either transient expression or stable transformation of the nuclear genome, while direct gene transfer can also be used to achieve chloroplast transformation. As stated earlier, viral transduction does not lead to stable transformation and virus vectors are not discussed in this article.

Principles of *Agrobacterium*-Mediated Transformation

Gene transfer from bacteria to plants occurs naturally and is responsible for crown gall disease. This is a plant tumor that can be induced in a wide variety of gymnosperms and dicotyledonous angiosperms by



Crop Plants Transformation Methods. Figure 1

Strategies for the transformation of higher plants [4]. *The boxes* show the various different targets for transformation and how they are obtained from whole plants (*black arrows*). The methods used to transform each of these targets are shown in *black text*. *The red arrows* show the routes used to obtain whole transgenic plants

inoculating wound sites with the Gram-negative soil bacterium *A. tumefaciens*. The tumor produces plant hormones responsible for the proliferation of undifferentiated plant tissue, and specialized amino acid derivatives known as opines that the bacteria use as a carbon source. The entire system has therefore evolved as a way for the bacteria to exploit plants for accommodation and food.

Even so, the continued presence of the bacteria is not required to maintain the tumor, indicating that some "tumor-inducing principle" is transferred from the bacterium to the plant at the wound site. Research in the late 1970s identified the principle as a small segment of DNA that is transferred to the plant genome (hence T-DNA, for transferred DNA). The source of the T-DNA is a large plasmid, the tumor-inducing (Ti) plasmid, resident in the bacterium.

Agrobacterium-mediated transformation was developed as a platform technology following the dissection and functional analysis of two key components of natural Ti-plasmids: the T-DNA, which contains DNA sequences required in *cis* for DNA transfer, and the *vir* (virulence) region, which contains genes whose products are required in *trans* for DNA transfer.

The resulting transformation system is known as a binary vector system because the T-DNA and *vir* genes do not need to be present on the same vector. This means that the T-DNA can be housed on a small shuttle vector suitable for cloning in bacteria, while the *vir* genes are provided on a second "helper" plasmid.

Natural T-DNA carries genes encoding enzymes for plant hormone synthesis and enzymes for opine synthesis, and these are the factors that drive the formation of the crown gall tumor. The hormone genes are often called oncogenes because they lead to tumor formation. However, neither the oncogenes nor the opine genes are necessary for DNA transfer. If these genes are removed, leaving an "empty" T-DNA cassette, the "disarmed" T-DNA can still be transferred to the plant genome, but the transformed cells at the wound site no longer proliferate and form a tumor. All that is required for transfer is the *vir* region, the T-DNA border sequences which are targets for excision from the Ti-plasmid, and a small number of loci on the *A. tumefaciens* genome. The T-DNA border sequences are 25-bp imperfect direct repeats which define the boundaries of the T-DNA. An enhancer, sometimes called the *overdrive* sequence, is located external to the right-hand repeat and is also required for high-efficiency transfer [5, 6]. Disarming the T-DNA does not improve the efficiency of transformation but it does improve the efficiency of regeneration, since the oncogenes produce phytohormones that interfere with normal plant development. At the same time, however, this removes any visual confirmation that transformation has taken place, and selectable or screenable marker genes must be included in the T-DNA to allow the identification of transformants.

The *vir* genes are organized into several operons. Two of these genes, *vir*A and *vir*G, are constitutively expressed at a low level and control the plant-induced activation of the other *vir* genes. The VirA protein is a kinase that spans the inner bacterial membrane, and acts as the receptor for certain phenolic molecules that are released by wounded plant cells. Many such compounds have been characterized, but acetosyringone is the most widely used in the laboratory to induce *vir* gene expression [7]. These phenolic compounds do not actually attract bacteria to wounded plant cells (the bacteria are attracted by simple molecules such as sugars and amino acids) but the *vir* genes are induced after bacterial attachment. Activated VirA transphosphorylates the VirG protein, which is a transcriptional activator of the other *vir* genes. Further genes on the bacterial chromosome also encode transcription factors that regulate *vir* gene expression (reviewed in [8, 9]). The induction of *vir* gene expression results in the synthesis of proteins that form a conjugative pilus through which the T-DNA is transferred to the plant cell. The components of the pilus are encoded by genes in the *vir*B operon (reviewed in [10]). DNA transfer itself is initiated by an endonuclease formed by the products of the *virD1* and *virD2* genes. This introduces either single-strand nicks or a double-strand break at the 25-bp borders of the T-DNA, a process enhanced by the VirC12 and VirC2 proteins, which recognize and bind to the overdrive enhancer element. The VirD2 protein remains covalently attached to the processed T-DNA. Recent studies have suggested that the type of T-DNA intermediate produced (single- or double-stranded) depends on the type of Ti-plasmid, with double-stranded T-DNA favored by nopaline plasmids (where the T-DNA is a single element) and single "T-strands" favored by octopine and succinopine plasmids, where the T-DNA is split into noncontiguous sections. T-strands are coated with VirE2, a single-stranded DNA-binding protein. The whole complex, sometimes dubbed the firecracker complex because of its proposed shape, is then transferred through the pilus and into the plant cell. The VirD2 protein may protect the T-DNA against nucleases, target the DNA to the plant cell nucleus, and help integrate it into the plant genome. The protein has two distinct nuclear localization signals, with the C-terminal signal thought to play the major role in targeting the T-DNA [11]. Once in the nucleus, the T-DNA is thought to integrate through a process of illegitimate recombination, perhaps exploiting naturally occurring chromosome breaks [12–14].

Contemporary binary vectors have all the conveniences of bacterial cloning vectors such as multiple unique restriction sites in the T-DNA region to facilitate subcloning, the *lac*Z gene for blue-white screening and a λ *cos* site for preparing cosmid libraries. A popular binary vector in current use is pGreen, which is <5 kbp in size, has 18 unique restriction sites in the T-DNA, a *lac*Z gene for blue-white screening of recombinants, and a selectable marker that can be used both in bacteria and in transformed plants

[15]. The progressive reduction in size has been made possible by removing essential genes required for replication in *A. tumefaciens* and transferring those genes to the bacterium's genome, or onto a helper plasmid. The pGreen plasmid, for example, contains the Sa origin of replication, which is much smaller than the more traditional Ri and RK2 regions. Furthermore, an essential replicase gene is housed on a second plasmid called pSoup resident within the bacterium. All conjugation functions have also been removed [15].

More recent innovations have also been incorporated into binary vectors, such as the inclusion of Gateway technology, a proprietary technology developed by Invitrogen (now part of Life Sciences, Carlsbad, California, USA) which is based on the *att*B and *att*P site-specific recombination sites and associated enzymes employed by bacteriophage λ to integrate into the *Escherichia coli* chromosome during lysogeny (the process by which the bacteriophage genome integrates into the bacterial chromosome and becomes dormant). Under normal circumstances, the *att*P site in the phage genome and the *E. coli att*B site undergoes site-specific recombination catalyzed by a phage enzyme, resulting in integration and the formation of two hybrid sites flanking the prophage, *att*L, and *att*R. To develop this as a cloning system, sequences are prepared with flanking *att*B sites (typically generated using extended PCR primers) and these undergo recombination with *att*P sites in a Gateway vector to generate an Entry Clone in which the sequence is flanked by *att*L sites. The Entry Clone can then be mixed with a plant-specific Destination Vector, which contains *att*R sites flanking a marker gene, such that the cloned gene is transferred into the destination vector to replace the marker (LR recombination). A large number of Gateway-compatible binary vectors have been developed [16] allowing universal cloning independent of the presence of restriction endonuclease sites and the assembly of multiple genes on one plasmid, a modification known as MultiRound Gateway [17].

Methods of *Agrobacterium*-Mediated Transformation

Many dicotyledonous plants can be transformed using variations of the simple protocol developed by Horsch et al. [18] in which small disks punched from leaves are surface-sterilized and inoculated in a medium containing *A. tumefaciens* harboring the recombinant binary vector. The disks are cultured for 2 days, during which T-DNA transfer takes place, and are then transferred to a medium containing the selection agent and carbenicillin to kill the bacteria. After 2–4 weeks, developing shoots are excised from the callus and transplanted to root-inducing medium, and thereafter into soil.

This leaf disk method is unsuitable for most monocotyledonous plants because they lie outside the *Agrobacterium* host range, and do not respond to wounding in the same way as dicots. In order to transform staple crops such as cereals, modified culture conditions were developed involving explants containing a high proportion of actively dividing cells, such as embryos or apical meristems. In dicots, cell division is often induced by wounding, whereas wound sites in monocots tend to become lignified. This probably explains why traditional procedures such as the leaf disk method are inefficient in monocots. Hiei et al. [19] showed that the cocultivation of *Agrobacterium* and rice embryos in the presence of 100 mM acetosyringone was a critical factor for successful transformation. Efficiency could be improved further by using so-called "supervirulent" strains like AGL-1, which incorporate modifications to boost gene transfer activity, such as overexpressed *vir*G (switching on the expression of the other *vir* genes) and/or *vir*E1, which is a major limiting factor in T-DNA transfer (reviewed in [20]). Komari et al. [21] used a different strategy, in which a portion of the virulence region from the Ti-plasmid of supervirulent strain A281 was transferred to the T-DNA-carrying plasmid to generate a so-called superbinary vector. The advantage of the latter technique is that the superbinary vector can be used in any *Agrobacterium* strain.

Principles of Direct DNA Transfer

Direct DNA transfer, as the name suggests, involves the introduction of DNA directly into the cell without using a biological carrier. This has two important advantages over *Agrobacterium*-mediated transformation. First, there is no dependency on the biological properties of the carrier, so any species and variety is

suitable for transformation. Second, there is no need to use specialized vectors for transformation – indeed transformation is possible without any vectors at all. The principles of direct DNA transfer are therefore much more straightforward than those for *Agrobacterium*-mediated transformation. All that is required is a mechanism for getting DNA into the plant cell, and ensuring it reaches the nucleus. This can be achieved using physical or chemical means.

Chemical Methods for Direct DNA Transfer Historically, the first direct transfer methods were chemical, and were closely related to the (at the time) newly devised methods for gene transfer to animal cells. Animal cells lack a cellulose wall and are protected by a simple plasma membrane. Therefore, the methods devised for animal cells would not work directly on plant cells, but were suitable for protoplasts. Gene transfer across the protoplast membrane is promoted by a number of chemicals, of which polyethylene glycol has become the most widely used due to the availability of simple transformation protocols [22]. Alternatively, DNA uptake may be induced by electroporation [23]. Putative transformants are transferred to selective medium, where surviving protoplasts regenerate their cell walls and commence cell division, producing a callus. Subsequent manipulation of the culture conditions then makes it possible to induce shoot and root development, culminating in the recovery of fertile transgenic plants. The major limitation of protoplast transformation is not the gene transfer process itself, but the ability of the host species to regenerate from protoplasts. Protoplast transformation was also the first method developed for gene transfer to the chloroplast genome of higher plants (see below).

Physical Methods for Direct DNA Transfer There is a great diversity of physical approaches for gene transfer to plants, including electroporation of walled plant cells, perforation of the cell with silicon carbide whiskers, microinjection, and poration with a finely focused laser beam. In most of these cases, only transient expression of the introduced DNA has been achieved, although transgenic corn plants have been recovered following whisker-mediated transformation [24]. Particle bombardment (microprojectile bombardment, biolistics) is a more robust and reliable method for stable transformation which has been successful for the transformation of cereals, soybean, cotton, phaseolus, and other recalcitrant crops. Initially, a modified shotgun was used to accelerate small (1–4 μm) metal particles into plant cells at a velocity sufficient to penetrate the cell wall (∼250 m/s). In the initial test system, intact onion epidermis was bombarded with tungsten particles coated in tobacco mosaic virus (TMV) RNA. Three days after bombardment, approximately 40% of the onion cells that contained particles also showed evidence of TMV replication [25]. A plasmid containing the *cat* (chloramphenicol acetyltransferase) reporter gene driven by the CaMV35S promoter was then tested to determine whether DNA could be delivered by the same method. Analysis of the epidermal tissue 3 days after bombardment revealed high levels of transient CAT activity [26]. The stable transformation of explants from several plant species was achieved soon after these initial experiments. Early reports included the transformation of soybean [27], and corn [28, 29]. The ability to stably transform plant cells by this method offered the exciting possibility of generating transgenic plants representing species that were, at the time, intractable to other transformation procedures. Early successes included soybean, cotton, papaya, and tobacco (reviewed in [30]). Particle bombardment has also been pivotal in the development of chloroplast transformation technology (see below).

There is no intrinsic limitation to the potential of particle bombardment since DNA delivery is governed entirely by physical parameters [31]. Many different types of plant material have been used as transformation targets, including callus, cell suspension cultures, and organized tissues such as immature embryos, meristems, and leaves. The number of species in which transgenic plants can be produced using variants of particle bombardment has therefore increased dramatically over the last 20 years including rice [32], wheat [33], and oat [34], as well as many other crops (reviewed in [30]). The original gunpowder-driven device has been improved and modified resulting in greater control over particle velocity and hence greater reproducibility of transformation conditions. An apparatus based on electric discharge has been used for the development of variety-independent gene transfer methods for the more recalcitrant cereals and

legumes [35], while several instruments have been developed where particle acceleration is controlled by pressurized gas (reviewed in [36]). Physical parameters such as particle size and acceleration (which affect the depth of penetration and the amount of tissue damage) as well as the amount and conformation of the DNA used to coat the particles must be optimized for each species and type of explant [30, 37]. However, the nature of the transformation target is probably the most important single variable in the success of gene transfer.

In Planta Transformation Methods

Most transformation methods for plants require some form of tissue culture step. This is because the fundamental principle of most transformation methods is that plants are regenerated from a small number of transformed cells that can survive under selection. Experiments using the model dicot *Arabidopsis thaliana* have led the way in the development of so-called *in planta* transformation techniques, where the need for tissue culture is minimized or eliminated altogether. Such methods involve the introduction of DNA, either using *A. tumefaciens* or direct transfer, into intact plants [38, 39].

The procedure is carried out at an appropriate time in the plant's life cycle so that the DNA becomes incorporated into cells that will contribute to the germ line, directly into the germ cells themselves (often at around the time of fertilization), or into the very early plant embryo. Generally, *in planta* transformation methods have a very low efficiency, so the small size of Arabidopsis and its ability to produce over 10,000 seeds per plant is advantageous. This limitation has so far prevented *in planta* techniques from being widely adopted for crop species, although radish, pak choi, and *Medicago truncatula* are exceptions [40–42]. The first *in planta* transformation system involved imbibing Arabidopsis seeds overnight in an *A. tumefaciens* culture, followed by germination [43]. A large number of transgenic plants containing T-DNA insertions were recovered but in general this technique has a low reproducibility. A more reliable method has been described by Bechtold et al. [38] in which the bacteria are vacuum infiltrated into Arabidopsis flowers. An even simpler technique called floral dip has become widely used [39]. This involves simply dipping Arabidopsis flowers into a bacterial suspension at the time of fertilization. In both these methods, the transformed plants are chimeric, but give rise to a small number of transgenic progeny. It has been established that T-DNA is transferred into the ovule during the transformation procedure [44].

An alternative to the direct transformation of germ line tissue is the introduction of DNA into meristems *in planta* followed by the growth of transgenic shoots. In Arabidopsis, this has been achieved simply by severing apical shoots at their bases and inoculating the cut tissue with *A. tumefaciens* suspension [45]. Using this procedure, transgenic plants were recovered from the transformed shoots at a frequency of about 5%. In rice, explanted meristem tissue has been transformed using *A. tumefaciens* and particle bombardment, resulting in the proliferation of shoots that can be regenerated into transgenic plants. Such procedures require only a limited amount of tissue culture.

Vectors for Plant Transformation

Components of Plant Transformation Vectors

The vectors used for plant transformation are usually designed with four purposes in mind – the ability to replicate in both *E. coli* and *A. tumefaciens* (i.e., shuttle vector capability), suitability for subcloning (multiple restriction enzyme sites and/or Gateway compatibility), the ability to confer a selectable phenotype on transformed cells (selectable marker genes), and the ability to drive transgene expression (an expression cassette, consisting minimally of a promoter, site for transgene insertion and a terminator/polyadenylation site).

For *Agrobacterium*-mediated transformation, the binary vector system comprises one transformation vector and one helper plasmid containing the *vir* genes (see above). The transformation vector contains the T-DNA, with the selectable marker gene and expression cassette housed within (reviewed in [46]). The replication functions are not required for DNA transfer and are found on the plasmid backbone, but they are required for maintenance in *A. tumefaciens* and cannot be dispensed with entirely, nor moved to the helper plasmid because the replication functions are required in *cis*. The *E. coli* replication functions (generally the ColE1 origin) are not required in

A. tumefaciens but are needed for basic cloning operations prior to transformation (reviewed in [47]) and these are also found on the plasmid backbone.

In contrast to *Agrobacterium*-mediated transformation, direct transfer (e.g., particle bombardment) is an entirely physical process with no dependence on biological functions. Therefore, replication functions on vectors used for direct DNA transfer are solely present to facilitate cloning in *E. coli*. They are entirely dispensable for the transformation process and can indeed be a nuisance if integrated into the plant genome since they encourage recombination and may in some cases promote transgene silencing. Only the expression cassette and selectable marker are required *in planta*, and therefore the plasmid backbones can be removed prior to transformation, leaving the small, linear cassettes as the substrate (reviewed in [31]). Although this is functionally equivalent to the linear T-DNA which is excised from the binary vector during *Agrobacterium*-mediated transformation, it should be noted that the T-DNA excision process is often imprecise, resulting in varying amounts of backbone sequences being cotransferred to the plant genome.

The Development of Binary Vectors

One of the first binary vectors for *Agrobacterium*-mediated transformation was pBIN19 [48] although this has fallen out of favor because of its low copy number in *E. coli*, which makes it difficult to obtain large amounts of DNA for cloning (Table 1). Another disadvantage of pBIN19 is that the selectable marker is next to the right border. Because T-DNA transfer is directional, with the right border being transferred first, it is better to have the marker next to the left border to ensure that resistant plants have received a complete (or nearly complete) copy of the T-DNA. These two disadvantages were addressed in more recent vectors such as pPZP and pBINPLUS, which contained a high-copy-number origin of replication for *E. coli* and a selectable marker gene at the left T-DNA border [49, 50]. Two rare restriction sites were also provided for cloning convenience.

As discussed above, the successful *Agrobacterium*-mediated transformation of monocots rested on the development of superbinary vectors with extra copies of some of the *vir* genes to enhance transformation efficiency. In the first instance, the *vir*B, *vir*C, and *vir*G genes were transferred from Ti-plasmid pTiBo542 [52, 53] carried by supervirulent strains of the bacterium, such as A281 or EHA101. Hiei et al. [19] constructed a new superbinary vector called pTOK233 by adding the *vir*B, *vir*G, and *vir*C genes of pTiBo542 to achieve rice transformation. The T-DNA in this case carried the *npt*II selectable marker under the control of the *nos* promoter, the *hpt* selectable marker driven by the CaMV 35S promoter and an intron-*gusA* fusion gene also driven by the CaMV 35S promoter. A comparison between the binary vector pIG121Hm in the supervirulent strain EHA101 and the superbinary vector pTOK233 in the regular strain LBA4404 showed that pTOK233 was slightly more efficient [19]. Similar combinations have been used to achieve the transformation of many monocot species (reviewed in [54, 55]). In corn and sorghum, efficient transformation systems were established only with superbinary vectors in LBA4404, whereas a standard binary vector in a supervirulent strain was inefficient even with improved co-culture conditions [56].

One disadvantage of the superbinary system is the large size of all the vector components, reducing the convenience of cloning by standard methods. Therefore, the final construction step of a superbinary vector involves the cointegration of an intermediate vector such as pSB11 and an acceptor vector such as pSB1 via homologous recombination between the shared DNA segments [21]. The intermediate vector is a small plasmid containing the T-DNA and ColE1 origin for replication in *E. coli*. The acceptor vector is an IncP plasmid, which can be replicated in *E. coli* and *A. tumefaciens*, and carries the 14.8-kb *KpnI* fragment. If a gene of interest is to be introduced into plants in tandem with a marker gene, the two genes are first inserted into an intermediate vector, which is introduced into a strain of *A. tumefaciens* that carries an acceptor vector so that the cassette is integrated into the acceptor to generate the final superbinary vector. This approach also facilitates the transfer of large segments of DNA with minimal rearrangement and favors a low number of integrated copies [46].

Other improvements have been made to reduce the frequency of vector backbone transfer. For example, Hanson et al. [57] placed the lethal *barnase* gene outside the left T-DNA border so that any transgenic plants

Crop Plants Transformation Methods. Table 1 Development of binary vectors for *Agrobacterium*-mediated transformation

Vector	Category	Details	References
pBIN19	Binary vector	Low copy number in *Escherichia coli,* plant resistance marker is next to the right border	[48]
pPZP	Binary vector	ColE1 origin of replication, plant marker is adjacent to the left border of T-DNA	[49]
pCAMBIA	Binary vector	Modification of pPZP	[49]
pBINPLUS	Binary vector	Selectable marker gene at the left T-DNA border, a higher copy number in *E. coli,* and two rare restriction sites for easier cloning	[50]
pIG121Hm	Binary vector	A derivative of pBIN19	[19]
pTOK233	Superbinary vector	Contains *vir*B, *vir*C, and *vir*G genes from pTiBo542	[19, 51]
pTiBo542	Ti-plasmid	Has *virB, virC,* and *virG*	[52, 53]
pSB11	Intermediate vector of superbinary system	ColE1 origin of replication, multiple cloning sites within T-DNA, replicates only in *E. coli*, has a fragment homologous to the acceptor vector pSB1	[21]
pSB1	Acceptor vector of pSB11 of superbinary system	Replicates in *E. coli* and *Agrobacterium*	[21]

containing sequences beyond the left border were counterselected. Another way to reduce backbone cotransfer is to insert additional left border sequences, increasing the likelihood of recognition by the corresponding Vir proteins and thus suppressing transfer, as has been demonstrated in rice [58] and Arabidopsis [59]. Improvements have also been made to facilitate rapid and efficient subcloning. The zero background TA cloning system [60] was developed for *Agrobacterium*-mediated transformation and uses restriction enzyme *XcmI* to generate 3′-T overhangs in the linearized vector within the counterselectable marker gene *ccd*B, which ensures that self-ligated vectors are not propagated after transformation, but that PCR products with 3′-A overhangs can be inserted without further modification.

Multiple Gene Transfer (MGT)

In the early years of plant biotechnology, most transgenic plants contained two transgenes – one selectable marker under the control of a constitutive promoter to facilitate the selective propagation of transformed cells, and a "primary transgene" or "gene of interest" which could be under the control of any sort of promoter and was intended to alter the plant's phenotype in a specific manner. MGT is now being embraced as an approach to generate plants with more ambitious phenotypes [61]. MGT allows goals that were once impossible to be achieved, e.g., the import of complex metabolic pathways, the expression of entire protein complexes, and the development of transgenic crops simultaneously engineered to produce a spectrum of added-value compounds [61, 62].

Essentially there are two MGT methods known as the linked and unlinked cotransformation strategies. In the linked strategy, all the different genes are linked on the same piece of transforming DNA. This is the normal approach chosen with *Agrobacterium*-mediated transformation where several genes are carried within the same T-DNA borders. However, it is quite possible to transform plants efficiency with (a) an *Agrobacterium* strain carrying a binary vector that has two separate T-DNAs, and (b) different *Agrobacterium* strains carrying different T-DNAs, although this becomes increasingly complex as the number of genes increases. There are several reports of a mixed strategy where two *Agrobacterium* strains carrying different T-DNAs each with multiple genes has been used to achieve MGT (reviewed in [63]).

MGT is more easily achieved by direct gene transfer because there is no need to combine multiple genes on the same length of DNA. When the genes are supplied separately as a mixed preparation of plasmids, there appears to be no bias in the process of integration. Tandem cotransformation can be undertaken, although it becomes more cumbersome as larger numbers of genes are required, but cotransformation with discrete, unlinked genes is just as efficient, and the genes tend to cointegrate at the same locus. As many as 12 transgenes have been integrated using this unlinked cotransformation approach [64].

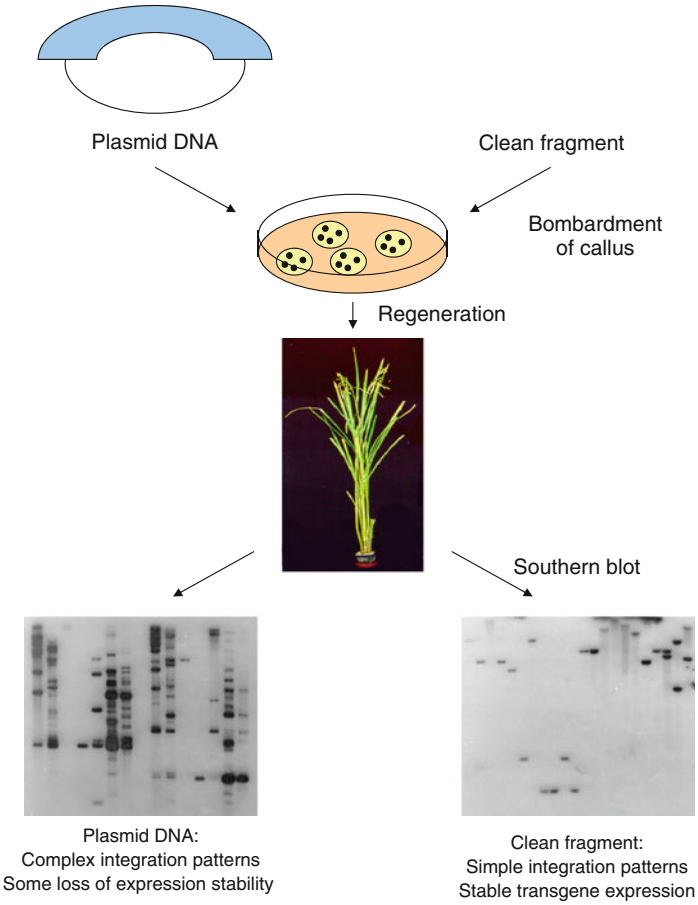
The main issue with linked MGT is the inconvenience of serial cloning. Very rare restriction sites can help to address this challenge, as shown by Goderis et al. [65] who developed a binary vector for MGT incorporating 13 hexanucleotide restriction sites, six octanucleotide restriction sites, and five sites for homing endonucleases, which are extremely rare in natural sequences and allow unidirectional cloning. Six different expression cassettes in auxiliary vectors with different promoter and terminator sequences were cut with the five different homing endonucleases plus an additional octanucleotide restriction endonuclease and transferred into the homing endonuclease sites of the binary vector. Modified auxiliary vectors also facilitate N- or C-terminal fusions to five different autofluorescent tags (EGFP, EYFP, Citrine-YFP, ECFP, and DsRed2) expressed from the tandem CaMV 35S constitutive promoter [66]. Similar systems have been developed by Thomson et al. [67], with the pUGA vectors for direct transfer and the pUGA2 vectors for *Agrobacterium*-mediated transformation. A series of unidirectional shuttle vectors containing various combinations of homing endonuclease sites was constructed and used to create artificial gene clusters in the pUGA or pUGA2 vectors, allowing the simultaneous transfer of up to six genes. Versatile systems now exist that offer a large number of promoters, terminators, and autofluorescent tags (reviewed in [68]).

A further strategy for linked MGT is to use the Cre/*lox*P recombination system together with homing endonucleases. In this method, two donor vectors are needed to introduce the expression cassettes into the acceptor vector using Cre recombinase [69]. After each round of recombination, the unnecessary backbone sequence from the donor vector is cleaved out using the homing endonucleases leaving just one *lox*P site for the next step. In this manner, ten transgenes were inserted in the acceptor vector and introduced into rice by *Agrobacterium*-mediated transformation.

The most recent generation of vectors are modular and multifunctional, such as the pCLEAN-G/pCLEAN-S dual binary vectors for *Agrobacterium*-mediated transformation [70] and the pORE vector system that can be adapted for both *Agrobacterium*-mediated transformation and direct DNA transfer [71]. The pORE vector series consists of "open" vectors for general plant transformation, "reporter" vectors for promoter analysis, and "expression" vectors for transgene expression. The sets comprise various combinations of promoters (PHPL, PENTCUP2, and PTAPADH), selectable markers (*npt*II and *pat*), and reporter genes (*gusA* and *smgfp*), and any element can be modified independently.

Direct DNA Transfer Using Minimal Expression Cassettes

As discussed above, vector backbone sequences are not required for direct DNA transfer and they may also promote recombination within the transgenic locus and/or act as triggers for de novo DNA methylation. To determine whether minimal linear cassettes (promoter, transgene, and terminator) could be used for direct DNA transfer, Fu et al. [72] separated the expression cassettes for the marker genes *gusA* and *hpt* from the parent vector, and used these as substrates for coating the microprojectiles used in particle bombardment, with the intact plasmids as a control. They found not only that the linear cassettes were equally efficient for transformation, but also that the elimination of the plasmid backbone had a remarkable positive effect on the resulting transgenic rice plants (Fig. 2). The cassette transformants generally contained fewer transgene copies than those transformed with intact plasmids, and they showed stronger and more stable expression. Further analysis of the minimal cassette population showed that the transgenes were expressed in most of the transgenic plants and that, for all the transgenes, overall expression levels and coexpression frequencies were higher than previously reported for whole plasmid transformants. These results were confirmed using the *yfp* (yellow



Crop Plants Transformation Methods. Figure 2
The clean DNA transformation system compared to particle bombardment with whole plasmid DNA [4]. The transformation strategy is shown, and two representative DNA blots are compared to demonstrate the simpler integration patterns resulting from transformation with linear minimal cassettes

fluorescent protein) and *hpt* markers, with concatemers forming only rarely in such plants [73].

Promoters Used for Plant Transformation

The promoters used in plant biotechnology are traditionally divided into three categories – constitutive (active continuously in most or all tissues), spatiotemporal (tissue-specific or stage-specific activity), and inducible (regulated by the application of an external chemical or physical signal) [74]. Most basic transformation vectors incorporate strong constitutive promoters because it is assumed that the objective is to express the transgene(s) at the highest possible level. Initially, the *A. tumefaciens nos* (nopaline synthase), *ocs* (octopine synthase), and *mas* (mannopine synthase) promoters were popular because they were already present in the natural T-DNA sequences from which the early binary vectors were developed, and had evolved to be active in many plant species (at least those within the *A. tumefaciens* host range). However, the CaMV 35S promoter was found to be stronger and unaffected by wounding, and its modular nature made it easy to modify [75, 76]. The activity of the CaMV 35S promoter can be increased by duplicating the enhancer up to four times [77] and the enhancer can also increase the activity of heterologous promoters to which it is attached [78].

Although widely used, the CaMV 35S promoter has certain limitations such as its poor performance in monocots, its suppression by feeding nematodes, and

the intellectual property issues affecting its commercial deployment (reviewed in [79]). For this reason, alternative virus promoters with similar or improved properties have been sought. Thus far, however, the only virus promoters that have been developed into established expression vectors are those from *Cestrum yellow leaf curling virus* (CmYLCV) [80], which can be licensed from Syngenta Biotechnology, Inc. for limited research purposes, and from *Subterranean clover stunt virus* (SCSV), which has been used to construct the pPLEX series of expression vectors for use in both dicots and monocots [81, 82].

Constitutive expression in monocots is usually achieved with housekeeping promoters, particularly those from the rice *actin1* and corn *ubiquitin1* genes (reviewed in [83, 84]). In both cases, the presence of the first intron of the gene is required for high-level expression [83, 84], and the addition of this intron to the CaMV 35S promoter also enhances its activity in monocots, e.g., 40-fold in corn [85]. In contrast, the first intron of the recently characterized rice *actin2* gene contains a negative regulatory element whose removal is required for high-level promoter activity [86].

Many different plant promoters have been described that restrict expression to particular cells, tissues, organs, or developmental stages, and seed-specific promoters are probably the most diverse. Seeds are a frequent target for genetic engineering in plants because they can accumulate recombinant proteins to levels that would be lethal in vegetative tissues but can do so without compromising plant growth and development; they are also a harvestable product and thus the target for nutritional improvement. Many promoters have been identified that target gene expression specifically to the seed, or to a particular region of the seed such as the endosperm, embryo, or aleurone [74, 79, 87]. Anther-specific promoters are also very useful because they can be used to control male fertility, an important trait in crop breeding, while fruit- and tuber/root-specific promoters are valuable for the nutritional improvement of fruit and root vegetable crops, pest/disease resistance, and the use of staple crops as factories for the production of novel proteins and metabolites.

Inducible promoters are also highly valued in plants because they allow transgenes to be controlled by internal and external physical or chemical cues. Many different inducible promoters have been identified in plants and these generally fall into three categories – (1) those responsive to endogenous signals (plant hormones); (2) those responsive to external physical stimuli (abiotic and biotic stresses); and (3) those responsive to external chemical stimuli. Such promoters provide immense scope for the precise regulation of transgene expression through external control, ranging from the precise control of transgene activation/inactivation in experimental settings to the ability to activate transgenes on an agricultural scale by the application of chemical sprays. Examples of commonly used promoters include those responsive to phytohormones (particularly auxin, abscisic acid, gibberellin, and ethylene), heat-shock promoters responsive to raised temperatures, light-inducible promoters, promoters induced by wounding or by exposure to elicitors produced by pathogens, and promoters that respond to specific metabolites [74, 79]. Sugar responsive promoters fall into the latter category and the *cis*-acting elements that confer sensitivity to sugar are particularly useful for controlling gene expression in cultured plant cells. For example, elements from sporamin and amylase promoters have been studied in detail and the minimal α-amylase 3 promoter makes the normally constitutive rice *actin1* promoter sensitive to the presence of sugar [88]. Inducible promoters that respond to xenobiotic signals are also valuable because transgenes can be activated without affecting endogenous genes. Martinez et al. [89] developed a hybrid system consisting of the tobacco budworm ecdysone receptor ligand-binding domain fused to the mammalian glucocorticoid receptor DNA-binding domain and the VP16 transactivation domain. The receptor responds to tebufenozide (an insecticide better known by its trade name CONFIRM). Similarly, Padidam et al. [90] have developed a system that is based on the spruce budworm ecdysone receptor ligand-binding domain, and responds to another common insecticide, methoxyfenozide (INTREPID).

Selectable and Screenable Markers

As stated above, selectable marker genes provide a phenotype that allows transformed cells to be propagated under conditions where nontransformed cells cannot survive, such as in the presence of an otherwise

Crop Plants Transformation Methods. Table 2 Selectable markers used for plant transformation (Data compiled and updated from [91–93])

Gene (Product)	Source	Phenotype and other comments
aad (aminoglycoside adenyltransferase)	*Shigella flexneri*	Provides resistance to trimethoprim, streptomycin, spectinomycin, and sulphonamides. Used mainly for chloroplast transformation
bar (phosphinothricin acetyltransferase)	*Streptomyces hygroscopicus*	Resistance to phosphinothricin (PPT), which is a component of the herbicides bialophos, Basta, and glufosinate
ble (glycopeptide-binding protein)	*Streptalloteichus hindustantus*	Resistance to the glycopeptide antibiotics bleomycin and pheomycin (and the derivative Zeocin)
dhfr (dihydrofolate reductase)	Mouse	Resistance to methotrexate
sul1 (dihydropteroate synthase)	*Escherichia coli*	Resistance to sulfonamides (Asulam)
epsps (enolpyruvylshikimate phosphate synthase)	*Petunia hybrida*	Resistance to the herbicide glyphosate
hpt (hygromycin phosphotransferase)	*Klebsiella* spp.	Resistance to the aminoglycoside antibiotic hygromycin B
manA (mannose-6-phosphate isomerase, MIP)	*E. coli*	Ability to grow on mannose as sole carbon source
neo/nptII/aphII (neomycin phosphotransferase)	*E. coli*	Resistance to the aminoglycoside antibiotics neomycin, kanamycin, and geneticin (G148)

toxic or growth-disrupting reagent (positive selection) or in the absence of an otherwise essential nutrient (negative selection). This is necessary to isolate the small number of transformed cells from the overwhelming majority of their nontransformed peers which, without selection, would quickly outcompete them. Although a wide range of selectable markers has been tested in plants, only a few are used routinely (reviewed in [47, 91]). The broadest markers are suitable in most plants and are expressed using the most active constitutive promoters to ensure that all cell types are protected under selection (the CaMV35S promoter in dicots and the actin or ubiquitin promoters in monocots, as discussed earlier). Most selectable markers are described as conditional because an external reagent must be applied to facilitate selection, whereas others are nonconditional, i.e., they work without any external selection reagent. The typical selectable markers used in plant transformation are positive and conditional, and work by conferring resistance to a toxic substance such as an antibiotic or herbicide that has a very specific intracellular target (Table 2).

Marker genes that confer antibiotic resistance originate from bacteria but have been modified to function well in plants. The first marker to be used in plants was neomycin phosphotransferase (*npt*II, *aph*II), which confers resistance to the aminoglycoside antibiotics neomycin, kanamycin, and geneticin (G148) [94]. This is probably still the most widely used marker in the laboratory but some plants are naturally resistant to kanamycin, and the antibiotic can also interfere with normal development in some species. An alternative is hygromycin phosphotransferase (*hph, hpt, aph*IV), providing resistance to the antibiotic hygromycin through the ATP-dependent phosphorylation of a 7-hydroxyl group [95]. Other antibiotic-resistance markers used less frequently include those conferring resistance to bleomycin [96], gentamycin [97], and methotrexate [98].

Marker genes that confer herbicide resistance may originate from bacteria or plants, and those conferring resistance to the broad-spectrum herbicides phosphinothricin (PPT)/glufosinate and glyphosate are used the most widely. PPT/glufosinate is a competitive inhibitor of glutamine synthetase (GS), the only

enzyme that can catalyze the assimilation of ammonia into glutamic acid in plants. Inhibition of GS therefore results in the accumulation of toxic levels of ammonia. The enzyme phosphinothricin *N*-acetyltransferase (PAT) encoded by either the *bar* or *pat* genes (these are genes from different microbial species) can be used to provide PPT/glufosinate resistance in transformed plant cells [99]. Glyphosate, the active ingredient of Roundup, inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is required for the synthesis of aromatic amino acids. Glyphosate resistance can be conferred by markers encoding a modified EPSPS that is not affected by the herbicide, or those encoding a bacterial enzyme that breaks down the herbicide (glyphosate oxidoreductase, GOX) [100]. Markers providing resistance against sulfonamindes such as Asualam [101] and chlorsulfuron [102] are also used occasionally.

Negative selectable markers are useful because they allow transformed cells to be selected based on the absence of something that is necessary for nontransformed cells to grow or regenerate efficiently. The *E. coli manA/pmi* gene confers the ability to use mannose as a sole carbon source so that only transformed cells survive on media containing mannose but lacking sucrose [103]. The gene is a negative conditional marker because mannose itself is not toxic to nontransformed plants, but rather the absence of sucrose (nontransformed plants grow perfectly well in the presence of both sugars). Another example is the *A. tumefaciens ipt* gene, which promotes the synthesis of cytokinins [104]. This is a nonconditional marker because it confers the ability to produce shoots in growth medium lacking exogenous cytokinins, i.e., nothing has to be added to the medium to facilitate selection. Simply, transformed tissues are placed on medium lacking cytokinins and only those tissues that produce shoots are transgenic.

The other major class of marker genes are known as screenable, scorable, or visible markers (or reporter genes) because rather than providing cells with a selective advantage they confer a phenotype that can easily be detected without interfering with other cellular processes, allowing transformed cells to be identified and studied (Table 3). Although screenable markers have been used for the prosaic purpose of identifying transformed cells and manually separating them from nontransformed cells, they tend to be used for more sophisticated purposes, such as reporter assays and tracing experiments. Some screenable marker genes only provide their visual signal when provided with a particular substrate, i.e., they are conditional (e.g., *gusA*, *luc*). Others have an intrinsic ability to yield a visible signal, i.e., they are nonconditional (e.g., *gfp*, *DsRed*), and these are the most useful since they can be used in living organisms.

The *E. coli gusA* (*uid*A) gene encodes the enzyme β-glucuronidase (GUS), the most widely used conditional screenable marker in plants. As well as its endogenous substrates, GUS can process a range of chromogenic and fluorescent derivatives in a range of assays that allow the quantification or in situ localization of reporter gene activity. The most common substrate for GUS histochemical staining is 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), a clear substrate that yields a blue product. Other common substrates include p-nitrophenyl β-D-glucuronide, which is used for spectrophotometric quantitative assays, and 4-methylumbelliferyl-beta-D-glucuronide (MUG), which produces a quantitative fluorescent signal. GUS is preferred in plants over the very similar reporter GAL (encoded by *lacZ* and widely used in microbes and animals) because of its stability in plants and its lack of toxicity and background activity. The main disadvantage of GUS is that it cannot be conveniently used for in vivo imaging because plant cells must be fixed or destroyed to visualize the reaction. Its stability can also be problematic if the aim of an experiment is to study fluctuations, since the longevity of the protein can mask transient decreases in expression.

Both the disadvantages of GUS are addressed by luciferase (LUC) an enzyme from the firefly (*Photinus pyralis*) which catalyzes the ATP-dependent oxidative decarboxylation of luciferin, producing light in the process. LUC allows nondestructive qualitative and quantitative assays to be carried out both in vitro and in vivo, and because the reaction has a short half-life, it can be used to monitor fluctuating activity. A series of vectors that incorporate the *luc* gene have been developed for plants, including the LucTrap series that allow targeted and random transcriptional and translational fusions of a transgene with a *luc* gene optimized for plant cells [106]. One drawback of *luc* is that it is still

Crop Plants Transformation Methods. Table 3 Screenable markers (reporter genes) used in plants (Data compiled and updated from [83, 105])

Gene (product)	Comments
gusA (β-glucuronidase)	Source: *Escherichia coli gusA/uidA* gene
	Activity: catalyzes the hydrolysis of β-glucuronides
	Assays: nonisotropic; in vitro assays are colorimetric or fluorometric; also histochemical assay format using X-gluc
	Advantages: simple, sensitive, quantitative, many assay formats available, inexpensive
	Disadvantages: assays are destructive; enzyme is stable so unsuitable for studies of downregulation
cat (chloramphenicol acetyltransferase)	Source: *E. coli* Tn*9*
	Activity: catalyzes the transfer of acetyl groups from acetyl coenzyme A to chloramphenicol
	Assays: in vitro assays only, isotropic
	Advantages: simple to perform
	Disadvantages: low sensitivity, expensive, low resolution in vivo, reliance on isotopic assay format
luc (luciferase)	Source: The firefly *Photinus pyralis*
	Activity: light produced in the presence of luciferase, its substrate luciferin, oxygen, Mg^{2+}, and ATP
	Assays: nonisotopic bioluminescent assays in vitro and in vivo
	Advantages: sensitive, rapid turnover, quantitative
	Disadvantages: Expensive detection equipment, limited reproducibility of some assay formats
Anthocyanin regulators	Source: corn (*Zea mays*)
	Activity: induces pigmentation
	Assays: visual screening for pigmented cells in vivo
	Advantages: simple, inexpensive, nondestructive
	Disadvantages: low sensitivity, not quantitative, background expression, adverse effects on transgenic plants
GFP (green fluorescent protein)	Source: the jellyfish *Aequorea victoria*
	Activity: intrinsic fluorescence under blue/UV light
	Assays: nonisotopic, in vivo assays in live plants
	Advantages: intrinsic activity (no substrate requirements), sensitivity, use in live plants; many variants with modified absorption and emission spectra available, and different subcellular targeting signals; several variants can be used simultaneously
	Disadvantages: weak signal in some systems (this is being addressed through the use of modified GFPs with stronger emission and reduced photobleaching); no variants that emit in the orange-red part of the spectrum

Crop Plants Transformation Methods. Table 3 (Continued)

Gene (product)	Comments
DsRed (red fluorescent protein)	Source: coral reef *Discosoma* spp.
	Activity: intrinsic fluorescence under blue/UV light
	Assays: nonisotopic, in vivo assays in live plants
	Advantages: intrinsic activity (no substrate requirements), sensitivity, use in live plants; many variants with modified absorption and emission spectra available, and different subcellular targeting signals; several variants can be used simultaneously
	Disadvantages: weak signal in some systems, no variants that emit in the violet-blue-green part of the spectrum

a conditional reporter, requiring the substrate luciferin and the presence of oxygen, ATP, and magnesium ions. In contrast, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a nonconditional reporter allowing the direct, noninvasive visualization of fluorescence in vivo in real time merely by exposure to blue/UV light. The original *gfp* gene was nonfunctional in plants because of a cryptic splice site, but this has been corrected and the protein has been widely deployed as a vital marker [107]. One of the major advantages of GFP is that its spectral qualities can be modified by mutation, giving rise to a whole family of derivatives with enhanced brightness, less susceptibility to quenching, and a range of excitation/emission wavelengths allowing different reporters to be used in vivo simultaneously. In combination with variants that allow targeting to different compartments within the plant cell, many sophisticated forms of analysis become straightforward to implement, e.g., allowing the real-time monitoring of protein processing, trafficking and protein–protein interactions [108]. As well as GFP and its derivatives, other bioluminescent proteins have also been identified, including DsRed from *Discosoma* spp. This is similar to GFP but covers a different spectral range (red GFP is not available) but there is little background fluorescence and it can be visible under white light [109].

The useful properties of selectable and screenable markers can also be combined into one protein. For example, Ochiai-Fukuda et al. [110] developed a fusion marker incorporating enhanced green fluorescent protein and blasticidin deaminase, conferring resistance to the aminoacylnucleoside antibiotic blasticidin S. The *gfbsd* marker was introduced into rice callus and allowed the rapid and efficient selection and visual confirmation of transformed cells.

Consequences of Nuclear Transformation

Integration of Nuclear Transgenes

As discussed above, plant transformation is a multistep process, the first step involving the transfer of DNA into the plant cell, which can be achieved either by direct transfer or by *Agrobacterium*-mediated transformation. Once the DNA reaches the nucleus, the next step (transgene integration) is dependent predominantly on that DNA and on factors provided by the plant cell, although in the case of *Agrobacterium*-mediated transformation it is possible that the Vir proteins complexed with the T-strand may facilitate the integration process (see below). A number of groups have investigated the structure of genomic/T-DNA and T-DNA/T-DNA junctions in plants and have concluded that integration occurs by illegitimate recombination (see [111, 112]). A strand invasion mechanism has been proposed (reviewed in [12]), in which the 3′ end of the T-strand initiates the integration process by hybridizing to a short region of homology in the plant genome, the second strand being completed by primer extension of the plant DNA. Other models suggest conversion of the T-strand into a double-stranded intermediate, which integrates at the site of naturally occurring chromosome breaks via double-strand DNA break repair. This is supported by experiments that show transformation efficiency increases following UV irradiation, which generates nicks and breaks in

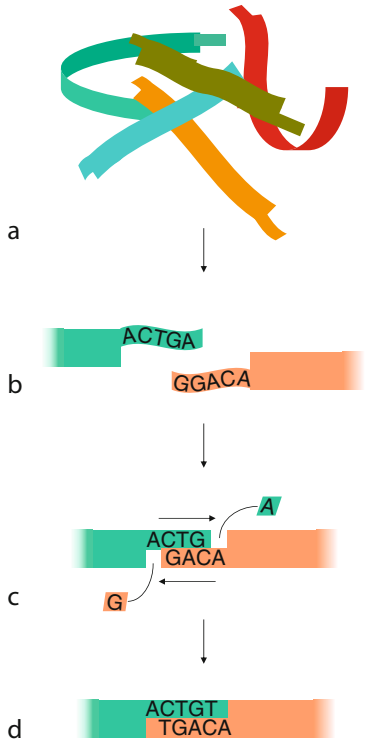
genomic DNA. However, since T-DNA integration still occurs in DNA repair mutants, it is possible both mechanisms occur simultaneously albeit with different efficiencies.

DNA repair models argue that proteins encoded by the host plant have a much more important role in T-DNA integration than *Agrobacterium* proteins, such as VirD2, which are imported into the plant with the T-DNA. However, since the VirD2 protein remains covalently attached to the 5′ end of the T-strand during transfer it is also likely to influence integration [113]. In an in vitro assay, VirD2 can ligate together a cleaved T-DNA border sequence but cannot ligate T-DNA to other genomic targets unless plant cell extracts are also present [114], a phenomenon supported by the identification of Arabidopsis mutants impaired for T-DNA integration [115].

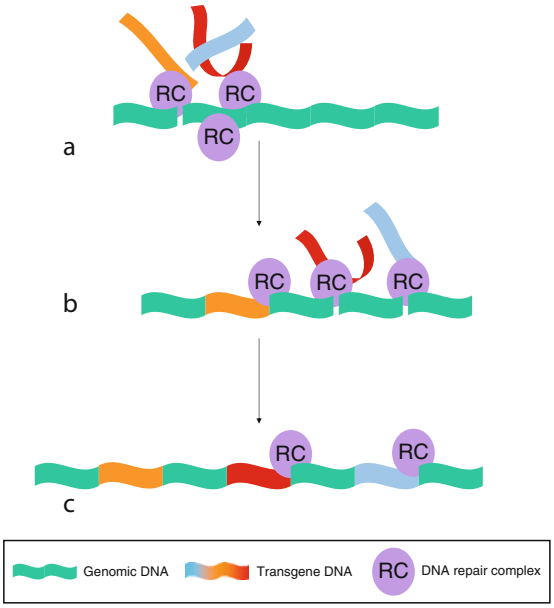
Much can be learned about the T-DNA integration mechanism by the inspection of borders, especially the borders between adjacent T-DNA sequences in multicopy insertions. The formation of heterodimers during cotransformation argues in favor of T-DNA concatemerization prior to integration. Although inverted repeats around the right border are often precise, those around the left border and those separating direct T-DNA repeats are often characterized by the insertion of variable-sized regions of filler DNA, which may be derived from the T-DNA sequence or from plant genomic DNA [116]. This suggests either the simultaneous integration of multiple T-DNAs at a single locus, or a two-phase mechanism, in which a primary T-DNA integration event stimulates further secondary integrations in the same area, similar to those proposed for particle bombardment (see below). Zhu et al. [117] carried out a comprehensive study of T-DNA border characteristics in a population of transgenic rice plants including 156 T-DNA/genomic DNA junctions, 69 T-DNA/T-DNA junctions, and 11 T-DNA/vector backbone junctions, which included 171 left borders and 134 right borders. Conserved cleavage was observed in 6% of left and 43% of right borders, microhomology was observed in 58% of T-DNA/genomic DNA, 43% of T-DNA/T-DNA, and 82% of T-DNA/vector junctions, mostly at left borders, and about one third of the T-DNA/genomic DNA and T-DNA/T-DNA junctions showed evidence of filler DNA (up to 344 bp). This was derived mainly from the T-DNA region adjacent to the breakpoint and/or from the rice genomic DNA flanking the T-DNA integration site, with T-DNA/T-DNA filler DNA showing the greatest complexity. Interestingly, when two T-DNAs were integrated in the inverted repeat configuration, significant truncation was always observed in one of the two T-DNAs whereas with the direct repeat configuration, large truncations were rare. These data suggest that no single integration mechanism can account for all observations but the presence of filler DNA at many of the junctions argues that a template-driven DNA synthesis mechanism must be involved.

The analysis of plasmid/plasmid and plasmid/genomic junctions in transgenic plants generated by particle bombardment reveals features characteristic of illegitimate recombination similar to those seen for T-DNA junctions, suggesting that the same overall integration mechanisms may be involved [118]. For example, such junctions are characterized by regions of microhomology, filler DNA, trimming of the DNA ends so sequences are lost, and AT-rich elements surrounding the junction site, with similarity to topoisomerase I binding/cleavage sites (Fig. 3). In the analysis of multiple plasmid/plasmid junctions in 12 transgenic rice lines, Kohli et al. [120] observed 10 plants with microhomology at the junctions and 2 plants where junctions appeared to be generated by blunt ligation, with no overlap. A similar ratio of conserved end-joining to microhomology-mediated recombination was observed by Gorbunova and Levy [121] and Salomon and Puchta [111]. Topoisomerase I sites were also observed adjacent to 10 out of 12 junctions characterized in transgenic Arabidopsis plants generated by particle bombardment [122] and in four of the six junctions in the commercial SUNUP variety of papaya [123]. Illegitimate recombination therefore appears to be responsible both for the integration of foreign DNA into the plant genome, and the linking of multiple plasmid copies, which is similar to the mechanism proposed for T-DNA integration (see above).

When nuclei from the cells of transgenic cereal plants generated by particle bombardment are analyzed by fluorescence in situ hybridization (FISH) using a transgene-specific probe, a curious phenomenon in often observed in which a single fluorescent spot in the interphase nucleus separates into multiple signals along a metaphase chromosome [124]. Any model for

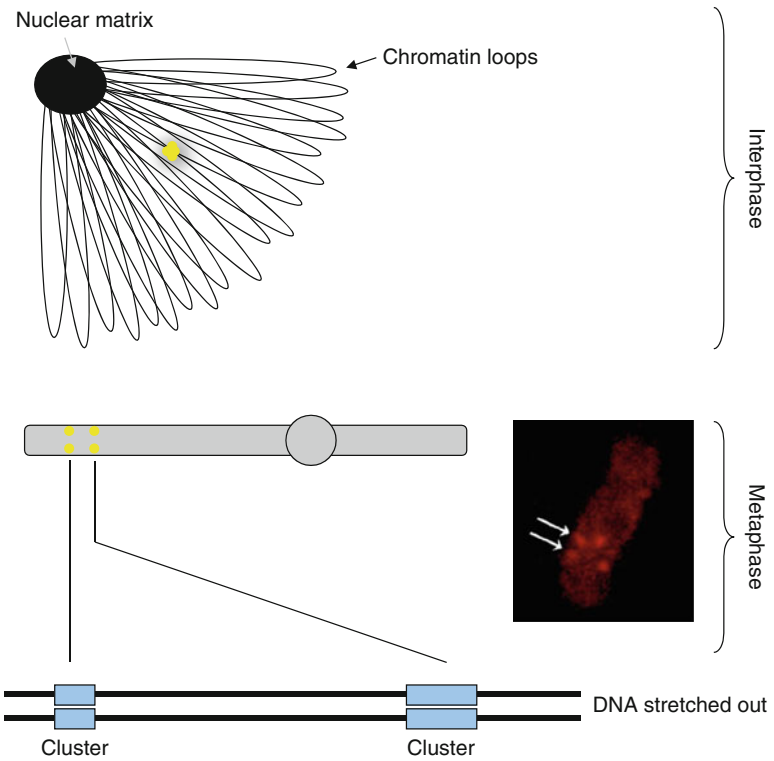


Crop Plants Transformation Methods. Figure 3
Mechanism for transgene integration at regions of microhomology [119]. A mixture of DNA fragments with ragged ends (**a**) interacts with a double-stranded DNA break with partially complementary ragged ends (**b**). Repair synthesis across the gap (**c**) generates a recombination junction (**d**) which may be completely conserved if the homology is precise, or may involve either the loss of terminal sequences or the insertion of filler DNA if the homology is partial



Crop Plants Transformation Methods. Figure 4
Explanation for the formation of transgene arrays and transgene clusters interspersed with genomic DNA [119]. A mixture of DNA fragments interacts with a double-stranded DNA break where a repair complex has already assembled (**a**). The repair complex may stitch together DNA fragments to form concatemers prior to integration, or may integrate single copies. The first integration event stimulates further repair complex activity nearby, resulting in additional nicks and breaks in the genomic DNA that act as further integration sites (**b**). This results in a cluster of transgenes (single copies and concatemers) interspersed with short regions of genomic DNA (**c**)

transgene integration following particle bombardment must take into account some form of three-tier organization, consisting of contiguous transgene arrays, interspersed with short regions of genomic DNA to generate local clusters, and the appearance of widely dispersed signals at metaphase. Two-phase transgene integration mechanisms have been proposed to explain the first two levels of organization, and in such models concatemerization is proposed to occur prior to integration, while interspersion occurs during the integration process [118, 120, 125] (Fig. 4). In each model, penetration of the cell is proposed to elicit a wound response, which would include the induction of DNA repair enzymes, such as nucleases and ligases. The presence of these enzymes and an excess of foreign DNA would result in the linking together of several copies to form concatemers, which would be the substrates for integration. This might be stimulated by homology between individual copies of transforming plasmids, and "backbone" homology might also result in the concatemerization of plasmids carrying different transgenes in cotransformation experiments. However, as stated above, cotransformation and cointegration also occur when two nonhomologous minimal cassettes are used for transformation, so homology might not be as important as the presence of free DNA ends [72]. Kohli et al. [120] suggested that



Crop Plants Transformation Methods. Figure 5

Higher-order transgene locus organization in cereals transformed by particle bombardment [124]. Transformation occurs during interphase, when the chromatin is distributed into specific nuclear zones and territories. If a metal particle causes localized damage, DNA repair complexes will form at these sites and initiate transgene integration (**a**). During metaphase, when FISH analysis is generally carried out, loci that are brought together in interphase may be separated, resulting in multiple signals from the same transformation event (**b**). If the DNA were stretched out, this would reveal large (megabase) interspersed sequences, which have also been observed in fiber-FISH experiments

transgene clusters arise in a second phase where a primary integration event occurring by illegitimate recombination at a chromosome break generates a "hot-spot" for further integration events in the same area. This might be due, for example, to the presence of local repair complexes that can slide along the DNA and introduce nicks which can be exploited by more foreign DNA. Pawlowski and Somers [125] suggested an alternative second phase where a number of discrete transgene concatemers integrate simultaneously at a site containing multiple replication forks. Although there is no direct evidence for either mechanism, it is interesting to note that DNA integration is stimulated in rapidly dividing cells, and is blocked in Arabidopsis mutants lacking essential components of the DNA recombination machinery.

The higher-order organization of transgenic loci observed by FISH is thus far unique to particle bombardment and demands a model which takes into account the three-dimensional structure of the nucleus. It is possible that the transformation event affects a local region of the interphase nucleus, e.g., a metal particle may cause damage to a particular area of chromatin arranged in loops attached to the nuclear matrix, or to a localized transcription factory. If the particle "skims" several loops or several transcription units, there will be regions of DNA damage close together in *trans*, but widely separated in the *cis* configuration were the DNA to be stretched out (Fig. 5). Each of these sites could act as a nucleation point where foreign DNA diffusing from the metal particle is used to patch up double-strand breaks, generating widely separated

arrays and/or clusters [124, 126]. In support of this induced break and repair model, Svitashev et al. [127] have shown that in 6 of 25 transgenic oat plants generated by particle bombardment, transgene integration sites were associated with rearranged chromosomes. This suggests that DNA breaks caused by incoming particles are repaired with foreign DNA and may also result in deletions, inversions, and translocations involving genomic DNA.

Transgene Structure and Integrity

Transgene rearrangements following particle bombardment have been widely reported in the literature and many publications repeat the "lore" that direct DNA transfer is more likely than T-DNA transfer to generate complex rearranged loci. The number of rearrangements that can be detected depends entirely on the resolution of the method being used. Thus, careful analysis of locus structure by Southern blot hybridization, PCR, and DNA sequencing has shown that rearrangements may be more widespread than first envisaged in both transformation methods.

In the case of direct DNA transfer, the analysis of transgenic oat loci by Somers et al. has shown that transgene rearrangements can be extensive and extremely complex, with multiple small insertions, inversions, and deletions within any transgene, plus the presence of filler DNA [127]. In corn, Mehlo et al. [128] noted that every single plant among the population they analyzed showed some form of rearrangement, and they speculated that undetected "minor" rearrangements could be responsible for many instances of transgene silencing otherwise attributed to epigenetic processes. In particular, certain transgene rearrangements were not detectable by Southern blot hybridization because they were too subtle, but they could be picked up by long-range PCR and sequencing. Because Southern blot hybridization is normally the sole method used to determine whether a given locus is intact or rearranged, this suggests caution should be used in relying on such results, since only "major" rearrangements can be detected in this manner.

In the case of *Agrobacterium*-mediated transformation, Afolabi et al. [129] and Zhu et al. [117] found that nonintact T-DNAs were present in >70% of transgenic rice lines, in most cases reflecting loss of the mid to right border portion of the T-DNA. Similarly, Rai et al. [130] found that about 50% of rice plants transformed with a T-DNA containing the phytoene synthase (*psy*) and phytoene desaturase (*crtI*) genes showed evidence of rearrangements, and in the majority of cases the rearrangements occurred in the *crtI* expression cassette, which was adjacent to the right T-DNA border. Rearrangements involving the left border are often characterized by the insertion of variable-size regions of filler DNA, possibly derived from the T-DNA sequence or from plant genomic DNA [116, 131].

Few researchers have characterized transgene rearrangements in detail, but work by Kohli et al. [132] has shown that rearrangements may involve palindromic sequences in the transforming plasmid, which tend to form secondary structures such as hairpins and cruciforms. These investigators characterized 12 transgenic rice lines created by particle bombardment, which had been shown to contain rearranged transgenes. Interestingly, they found that an imperfect palindrome in the CaMV 35S promoter was involved in one third of all rearrangements, i.e., the sequence of this palindrome was adjacent to the rearrangement junction. Similar phenomena have been noted in T-DNA transformants containing the same promoter. This sequence has the ability to adopt a cruciform structure that may stimulate recombination events. Many other promoters contain palindromic sequences of variable length within 100 bp of the transcription start site. The secondary structures formed at these sites enable DNA–protein interactions for transcription under normal circumstances, but may also participate in aberrant recombination events. The fully sequenced papaya genome [123] also revealed a number of previously unidentified transgene rearrangements, i.e., a 1,533-bp fragment comprising a truncated, nonfunctional *tet*A gene and flanking vector backbone sequence, and a 290-bp nonfunctional fragment of the *npt*II gene, in addition to the intact, primary transgene conferring virus resistance.

Transgene Silencing

A common issue raised in association with nuclear transformation in plants is the phenomenon of transgene silencing, where the phenotype corresponding to

the introduced transgene is not expressed. In the absence of a genetic explanation (e.g., an undetected mutation or rearrangement), silencing is an epigenetic phenomenon that can occur at either the transcriptional or posttranscriptional levels. Transcriptional silencing involves the absence of transgene mRNA, and often occurs due to the integration of the transgene at a genomic position that is already repressed (position-dependent silencing). However, transgenes in active regions of the genome may also be silenced if the promoter region is inactivated by hypermethylation, which can occur in response to unusual DNA structures and compositions that attract de novo methylation, or DNA sequences that allow the synthesis of short double-stranded RNA (dsRNA) molecules. In contrast, posttranscriptional silencing actually requires transcription to take place, but the mRNA is rapidly degraded. This is confirmed by nuclear run-on assays, which measure the amount of pre-mRNA in the nucleus. Like transcriptional silencing, posttranscriptional silencing appears to have evolved as a defense against invasive nucleic acids and is also triggered by dsRNA, in this case matching the transcribed region. There is considerable cross talk between the transcriptional and posttranscriptional silencing pathways and if a transgene is homologous to an endogenous gene, the silencing effect can spread to that gene resulting in a phenomenon known as cosuppression.

Transcriptional silencing may be encountered in plants where several copies of the same transgene or part thereof are present in the transgenic locus, or when the same promoter is used to control several transgenes. However, the context is very important. There have been plenty of reports describing transgenic plants carrying multiple transgenes under the control of the same promoter yet showing strong and stable expression. For example, although Zhu et al. [133] used five different endosperm-specific promoters in corn to achieve the high-level expression of carotenogenic genes, Naqvi et al. [134] achieved strong expression of four genes in the same system using the barley D-hordein promoter to control each transgene, with no adverse effects. Transcriptional gene silencing resulting from repetitive promoter regions is correlated with increased promoter methylation [135] and appears to be driven by the production of dsRNA matching the promoter sequence [136]. This has been demonstrated by deliberately expressing dsRNA corresponding to the *nos* promoter in transgenic plants carrying a second transgene driven by the *nos* promoter [137] and by creating transgenic plants with a transgene locus that triggers both transcriptional and posttranscriptional silencing simultaneously, by producing dsRNA corresponding to promoter and transcribed sequences of different target genes [138]. In the absence of deliberately created promoter dsRNA, the transcriptional silencing seen in some transgenic plants carrying multiple copies of the same promoter appears to arise from dsRNA produced either by unfortunate transgene positioning or rearrangements that create hairpin structures, or by transgenes with such high levels of expression that the polyadenylation machinery is saturated. Evidence from many transformation experiments indicates that there is no simple correlation between transgene copy number and expression level, with the exception of certain carefully controlled experiments using boundary elements. In some cases, higher copy numbers have suppressed overall expression levels whereas in others higher copy numbers have enhanced expression. Where suppression effects have occurred, it has been suggested that "runaway expression" resulting in the generation of aberrant RNAs lacking polyadenylate tails has triggered potent silencing through the posttranscriptional silencing pathway [139].

The organization of a transgenic locus is difficult to control, and it is therefore a common occurrence in both *Agrobacterium*-mediated transformation and direct DNA transfer that the juxtaposition of transgenes or fragments thereof can result in the creation of hairpin promoter structures at the DNA level that are transcribed into aberrant dsRNA species. Such arrangements can be obvious and easy to detect, but even where gross rearrangements are absent it is possible that undetected "micro-rearrangements" are present in the transgenic locus, as observed by Mehlo et al. [128] when investigating the structure of a transgenic locus in corn generated by direct DNA transfer. The siRNAs that trigger RNA-dependent DNA methylation are just 24 bp in length, so it is conceivable that inverted repeats of <50 bp could be sufficient for transgene silencing, and such structures would be undetectable using the coarse analysis methods typically employed

to study transgenic plants, such as Southern blot hybridization. The likelihood of dsRNA production depends not only on the presence of damaged or rearranged transgenes, but also on the relative position of intact transgenes, which is itself a reflection of the mechanism of transgene integration. The organization of integrated T-DNA sequences differs among *Agrobacterium* strains, but a common feature of nopaline-type derivatives such as C58 is the preferential integration of T-DNA as dimers with an inverted repeat configuration, linked either at the left or right borders [140]. Where cotransformation is carried out with two T-DNAs containing different genes, the different T-DNAs often integrate as heterodimeric inverted repeats, preferentially around the right border [141]. If the same promoter is used for both genes, this would favor the formation of hairpin structures that could be transcribed from the opposite strand. The structure of loci generated by direct DNA transfer is more variable, but inverted repeat structures involving promoter sequences are not uncommon, allowing the same silencing mechanism to operate [126].

Plastid Transformation Methods

The introduction of DNA directly into the chloroplast genome is considered beneficial for a number of reasons including the high level of gene expression that can be achieved, reflecting the presence of thousands of chloroplasts in photosynthetic cells and the absence of gene silencing. Chloroplast transformation also provides a natural containment method for transgenic plants, since in most crops the transgene cannot be transmitted through pollen (reviewed in [142]).

The first reports of chloroplast transformation were serendipitous, and the integration events were found to be unstable. For example, an early experiment in which tobacco protoplasts were cocultivated with *Agrobacterium* resulted in the recovery of one transgenic plant line in which the transgene was transmitted maternally. Southern blot analysis of chloroplast DNA showed directly that the foreign DNA had become integrated into the chloroplast genome [143]. However, *Agrobacterium* is not an optimal system for chloroplast transformation because the T-DNA complex is targeted to the nucleus. Therefore, direct DNA transfer has been explored as an alternative strategy and efficient chloroplast transformation has been achieved both through particle bombardment and PEG-mediated transformation (reviewed in [144]).

Stable chloroplast transformation was first achieved in the alga *Chlamydomonas reinhardtii*, which has a single large chloroplast occupying most of the volume of the cell [145]. Particle bombardment was used in this experiment and the principles established using this simple organism were extended to tobacco, allowing the recovery of stable transplastomic tobacco plants [146]. These principles included the use of vectors containing chloroplast homology regions, allowing targeted integration into the chloroplast genome, and use of the selectable marker gene *aad*A (encoding aminoglycoside adenyltransferase) which confers resistance to streptomycin and spectinomycin [147]. Basic vectors for plastid transformation include flanking sequences and chloroplast-specific expression cassettes. Species-specific chloroplast flanking sequences are generated by PCR using primers designed from the available chloroplast genomes. The chloroplast expression cassette is composed of a promoter, the selectable marker, and 5′/3′ regulatory sequences to enhance the efficiency of transcription and translation. The most frequently used integration site is the transcriptionally active intergenic region between the *trn*I and *trn*A genes, within the *rrn* operon. The first-generation plastid transformation vectors included the pPRV series and plasmids pRB94/95, in which both the marker gene and the primary transgene have their own 5′ and 3′ regulatory sequences (reviewed in [144]). More recent vectors include modified restriction sites, *lox*P sequences for posttransformation marker excision, and homology regions targeting insertions to the *rbc*L-*acc*D intergenic region [148]. Thus far, chloroplast transformation by particle bombardment has been achieved only in crops that allow direct organogenesis, and this does not include any monocots (reviewed in [142]).

Future Directions

The vast majority of the transgenic plants generated thus far carry a single primary transgene plus a selectable marker. The transgene integrates randomly into the genome, which means it is subject to unpredictable position effects that may result in silencing; the locus structure is also very difficult to control.

In the future, there will be a stronger emphasis on strategies to increase the scope of gene transfer and the predictability and preciseness of DNA integration, and consequently the likelihood of stable and predictable transgene expression.

Transfer of Large DNA Molecules Using Modified Conventional Vectors

A precise upper limit for T-DNA transfer has not been established. It is greater than 50 kbp [2, 3], but using standard vectors it is difficult to transfer inserts larger than 30 kbp routinely due to instability in the bacterial host. However, the analysis of very large genes or the transfer of multiple genes linked in series can now be achieved thanks to the development of high-capacity binary vectors based on the artificial chromosome type vectors used in *E. coli*.

The first to be described was BIBAC2 [149]. This contains an F-plasmid origin of replication and is modeled on the bacterial artificial chromosome. The basic vector transforms tobacco with high efficiency, but the efficiency of transformation drops substantially when large inserts are used. This vector has been used to introduce 150 kbp of human DNA flanked by T-DNA borders into the tobacco genome, although virulence helper plasmids supplying high levels of VirG and VirE in *trans* were critical for successful DNA transfer. An alternative vector carrying a P1 origin of replication and modeled on the P1 artificial chromosome was constructed by Liu et al. [150]. This transformation-competent bacterial artificial chromosome (TAC) vector was used to introduce up to 80 kbp of genomic DNA into Arabidopsis, and while there was some loss of efficiency with the larger inserts, it was still possible to produce many transgenic plants. Both vectors contain a kanamycin resistance marker for selection in bacteria and *hpt* for hygromycin selection in transgenic plants. Both vectors also contain the Ri origin for maintenance in *Agrobacterium*, and within the T-DNA region, the *sac*B marker for negative selection, interrupted by a multiple cloning site for transgene insertion. One of the most attractive uses of high-capacity binary vectors is for the positional cloning of genes identified by mutation. The ability to introduce large segments of DNA into the plant genome effectively bridges the gap between genetic mapping and sequencing, allowing the position of mutant genes to be narrowed down by complementation. Genomic libraries have been established for several plant species in BIBAC2 and TAC vectors [151, 152] and cloning in high-capacity vectors has been simplified by the inclusion of Cre/*lox*P and Gateway site-specific recombination technology [153, 154].

Large (80–150 kbp) DNA molecules have also been transferred to plants by direct DNA transfer [155], and although this is not a routine procedure a novel transformation method has been developed recently, based on bombardment with DNA-coated "bioactive beads" to deliver up to 150 kbp of DNA into rice protoplasts [156].

Plant Minichromosomes

In bacteria, plasmid vectors are maintained as episomal replicons to make cloning and isolating recombinant DNA a simple procedure. When it comes to expressing heterologous genes in eukaryotic cells, episomal vectors are widely used to avoid position effects, hence the development of yeast episomal vectors, yeast artificial chromosomes, mammalian plasmid vectors carrying virus origins of replication (e.g., SV40-based vectors, herpes virus-based vectors), and plant expression vectors based on plant viruses (all of which replicate episomally). The yeast artificial chromosome (YAC) system is the most relevant in this context because it allows genes of any size to be introduced into the yeast genome as an independent replicating unit that is treated by the cell as an additional chromosome. YACs comprise a yeast centromere and telomeres, the origin of replication (autonomous replicating sequence), and selectable markers.

More recently, analogous systems have been developed to maintain genes as episomal minichromosomes in plants. These have many advantages for plant genetic engineering including the ability to express large transgenes or groups of transgenes, and the ability to rapidly introduce new linkage groups into diverse germplasm. Carlson et al. [157] created plant minichromosomes by combining the DsRed and *npt*II marker genes with 7–190 kb of corn genomic DNA fragments containing satellites, retroelements, and other repeat sequences commonly found in centromeres. The circular constructs were introduced into embryogenic corn tissue by particle bombardment

and transformed cells were regenerated and propagated for several generations without selection. The minichromosomes were maintained as extrachromosomal replicons through mitosis and meiosis, and showed roughly Mendelian segregation ratios (93% transmission as a disome with 100% expected, 39% transmission as a monosome crossed to wild type with 50% expected, and 59% transmission in self crosses with 75% expected). The *DsRed* reporter gene was expressed over four generations, and DNA blot analysis indicated the genes were intact.

Gene Targeting (Homologous Recombination)

Gene targeting is the directed modification of an endogenous DNA sequence by homologous recombination, an efficient procedure in bacteria, yeast, certain animal cells, and in the plastid genomes of plants, but typically not in the nuclear genome. Only one plant species has been shown to undergo efficient nuclear homologous recombination and that is the moss *Physcomitrella patens* [158]. Among higher plants, low-level gene targeting has been achieved in certain dicots with frequencies ranging from 10^{-3} to 10^{-6} [159]. However, targeting frequencies of up to 1% have been achieved using a T-DNA-mediated gene targeting strategy involving a long homology region in combination with a strong counterselectable marker in rice [160, 161].

There has also been interest in the use of zinc-finger endonucleases to make targeted double-strand breaks in the plant genome, so that homologous recombination is favored at such sites [162]. The modular nature of zinc-finger transcription factors means that recombinant DNA technology can be used to "mix and match" these DNA-binding domains to create recombinant proteins with unique sequence specificities. Zinc-fingers are motifs approximately 30 amino acids in length which coordinate a Zn^{2+} ion and bind to DNA sequences 3-bp long. Combining different zinc fingers in series allows proteins to be tailor made to bind longer DNA sequences. When a nonspecific DNA endonuclease is incorporated into such a protein, it becomes a targeted DNA cutting tool [163, 164]. The recent achievement of targeted transgene integration and endogenous gene disruption in corn [165] and tobacco [166] using zinc-finger endonucleases provides a tantalizing glimpse of the future of plant biotechnology in which precise changes can be made to the genome of any plant genome that is amenable to DNA transfer.

Site-Specific Recombination

Although site-specific recombination has already been described as a cloning tool, particularly the Cre/*lox*P and Gateway systems for rapid vector assembly, it can also be used in transgenic plants to introduce DNA at a specific, favorable locus, or remove DNA sequences *in vivo*. The Cre/*lox*P system has been most widely used in plants for the controlled excision of selectable marker genes after transformation (e.g., [153]), but also for controlled transgene insertion (e.g., [167]). Controlled integration has been studied in transgenic plants already engineered to contain recipient *lox*P sites [168]. In this study, three different recipient wheat lines were generated by bombarding plants with the *lox*P sequence, and these were subsequently bombarded with a *gusA* construct also containing flanking *lox*P sequences, and a *cre* gene. Following transformation, about 80% of lines contained *gusA* at the recipient site, many with single-copy transgenes and others with concatemers. Both types of locus were stably inherited. There was much less variation in expression among the single-copy lines [168]. Chawla et al. [169] generated 18 different transgenic rice lines containing a precise single copy of *gusA* at a designated site. In seven of these lines, additional copies of the transgene integrated at random sites by illegitimate recombination while 11 showed "clean" integration by site-specific recombination only. The single-copy lines were stable over at least four generations and showed consistent levels of expression, which doubled in homozygous plants. In contrast, the multicopy lines showed variable expression and some fell victim to transgene silencing. Interestingly, where the site-specific and illegitimate integration loci segregated in later generations, transgene expression was reactivated in the plants carrying the site-specific integration site alone, whereas close linkage between the site-specific and random integration prevented segregation in other lines and the silencing persisted.

An exciting recent development is the GENE DELETOR system, which is a hybrid of the Cre-*lox*P

and FLP-*FRT* systems. The GENE DELETOR is based on a fusion recognition site (*lox*P-*FRT*), which is inefficient when both recombinases are expressed but highly efficient when either one of the recombinases is expressed alone, giving up to 100% efficiency in populations of up to 25,000 T1 transgenic tobacco plants [170].

Another use for Cre-*lox*P is the simplification of locus structure by resolving multicopy loci to a single transgene copy [171]. A strategy was developed in which the transformation vector contained a transgene flanked by *lox*P sites in an inverted orientation. Regardless of the number of copies integrated between the outermost transgenes, recombination between the outermost sites resolved the integrated molecules into a single copy. The principle was proven by resolving four multicopy loci successfully into single-copy transgenes.

Bibliography

Primary Literature

1. Bevan M, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187
2. Herrera-Estrella L, DeBlock M, Messens E, Hernalsteens JP, Montagu MV, Schell J (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J 2:987–995
3. Herrera-Estrella L, Depicker A, Van Montagu M, Schell J (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303:209–213
4. Twyman RM, Christou P, Stoger E (2002) Genetic transformation of plants and their cells. In: Oksman-Caldentey KM, Barz W (eds) Plant biotechnology and transgenic plants. Marcel Dekker, New York, pp 111–141
5. Shaw CH, Watson MD, Carter GH, Shaw CH (1984) The right hand copy of the nopaline Ti-plasmid 25 bp repeat is required for tumour formation. Nucleic Acids Res 12: 6031–6041
6. Peralta EG, Hellmiss R, Ream W (1986) Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. EMBO J 5:1137–1142
7. Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318:624–629
8. Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256
9. Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the gene jockeying tool. Microbiol Mol Biol Rev 67:16–37
10. Lai EM, Kado CI (2000) The T-pilus of *Agrobacterium tumefaciens*. Trends Microbiol 8:361–369
11. Tinland B, Koukolikova-Nicola Z, Hall MN, Hohn B (1992) The T-DNA-linked *virD2* protein contains two distinct functional nuclear localization signals. Proc Natl Acad Sci USA 89:7442–7446
12. Tinland B (1996) The integration of T-DNA into plant genomes. Trends Plant Sci 1:179–184
13. Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol 12:121–129
14. Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet 20:375–383
15. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 42:819–832
16. Karimi M, Depicker A, Hilson P (2007) Recombinational cloning with plant gateway vectors. Plant Physiol 145:1144–1154
17. Chen QJ, Zhou HM, Chen J, Wang XC (2006) A Gateway-based platform for multigene plant transformation. Plant Mol Biol 62:927–936
18. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231
19. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282
20. Sheng OJ, Citovsky V (1996) *Agrobacterium* plant cell DNA transport: have virulence proteins will travel. Plant Cell 8:1699–1710
21. Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174
22. Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F (1987) Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol Biol 8:363–373
23. Shillito RD, Saul MW, Pazkowski J, Muller M, Potrykus I (1985) High efficiency direct gene transfer to plants. Biotechnology (N Y) 3:1099–1103
24. Frame BR, Drayton PR, Bagnall SV, Lewnau CJ, Bullock WP, Wilson HM, Dunwell JM, Thompson JA, Wang K (1994) Production of fertile transgenic maize plants by silicon-carbide whisker-mediated transformation. Plant J 6:941–948
25. Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. J Particulate Sci Technol 6:559–563
26. Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity micro-projectiles for delivering nucleic acids into living cells. Nature 327:70–73

27. Christou P, McCabe DE, Swain WF (1988) Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiol 87:671–674
28. Gordon-Kamm WJ, Spencer TM, Mngano ML, Adams TR, Daines RJ, Start WG, O'Brian JV, Chambers SA, Adams WR Jr, Willets NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618
29. Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Biotechnology (N Y) 8:833–839
30. Twyman RM, Christou P (2004) Plant transformation technology – particle bombardment. In: Christou P, Klee H (eds) Handbook of plant biotechnology. Wiley, New York, pp 263–289
31. Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15:305–327
32. Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Nat Biotechnol 9:957–962
33. Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Biotechnology (N Y) 10:667–674
34. Somers DA, Rines HW, Gu W, Kaeppler HF, Bushnell WR (1992) Fertile, transgenic oat plants. Biotechnology (N Y) 10:1589–1594
35. McCabe D, Christou P (1993) Direct DNA transfer using electric discharge particle acceleration (Accell® technology). Plant Cell Tissue Organ Cult 33:227–236
36. Christou P (1996) Particle bombardment for genetic engineering of plants. RG Landes Co, Austin
37. Finer JJ, Finer KR, Ponappa T (1999) Particle bombardment mediated transformation. Curr Top Microbiol Immunol 240:59–80
38. Bechtold N, Ellis J, Pelletier G (1993) In *planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris Life Sci 316:1194–1199
39. Clough SJ, Bent A (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743
40. Qing CM, Fan L, Lei Y, Bouchez D, Tourneur C, Yan L, Robaglia C (2000) Transformation of Pakchoi (*Brassica rapa* L. ssp. *chinensis*) by *Agrobacterium* infiltration. Mol Breed 6:67–72
41. Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, Blaylock LA, Shin H, Chiou TJ, Katagi H, Dewbre GR, Weigel D, Harrison MJ (2000) Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. Plant J 22:531–541
42. Curtis IS, Nam HG (2001) Transgenic radish (*Raphanus sativus* L. var. *longipinnatus* Bailey) by floral-dip method – plant development and surfactant are important in optimizing transformation efficiency. Transgenic Res 10:363–371
43. Feldmann KA, Marks MD (1987) *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana* – a non-tissue culture approach. Mol Gen Genet 208:1–9
44. Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the *in planta* transformation of *Arabidopsis thaliana*. Genetics 155:1875–1887
45. Chang SS, Park SK, Kim BC, Kang BJ, Kim DU, Nam HG (1994) Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation *in planta*. Plant J 5:551–558
46. Dafny-Yelin M, Tzfira T (2007) Delivery of multiple transgenes to plant cells. Plant Physiol 145:1118–1128
47. Komori T, Imayama T, Kato N, Ishida Y, Ueki J, Komari T (2007) Current status of binary vectors and superbinary vectors. Plant Physiol 145:1155–1160
48. Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721
49. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25:989–994
50. van Engelen FA, Molthoff JW, Conner AJ, Nap J-P, Pereira A, Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res 4:288–290
51. Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14:745–750
52. Jin S, Komari T, Gordon MP, Nester EW (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A281. J Bacteriol 169:4417–4425
53. Komari T (1990) Transformation of cultured cells of *Chenopodium quinoa* by binary vectors that carry a fragment of DNA from the virulence region of pTiBo542. Plant Cell Rep 9:303–306
54. Veluthambi K, Gupta AK, Sharma A (2003) The current status of plant transformation technologies. Curr Sci 84:368–378
55. Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol Plant 40:31–45
56. Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SEK, Li B, Nettleton DS, Pei D, Wang K (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol 129:13–22
57. Hanson B, Engler D, Moy Y, Newman B, Ralston E, Gutterson N (1999) A simple method to enrich an *Agrobacterium*-transformed population for plants containing only T-DNA sequences. Plant J 19:727–734

58. Kuraya Y, Ohta S, Fukuda M, Hiei Y, Murai N, Hamada K, Ueki J, Imaseki H, Komari T (2004) Suppression of transfer of non-T-DNA "vector backbone" sequences by multiple left border repeats in vectors for transformation of higher plants mediated by *Agrobacterium tumefaciens*. Mol Breed 14: 309–320
59. Podevin N, De Buck S, De Wilde C, Depicker A (2006) Insights into recognition of the T-DNA border repeats as termination sites for T-strand synthesis by *Agrobacterium tumefaciens*. Transgenic Res 15:557–571
60. Chen S, Songkumarn P, Liu J, Wang G-L (2009) A versatile zero background T-vector system for gene cloning and functional genomics. Plant Physiol 150:1111–1121
61. Naqvi S, Farre G, Sanahuja G, Capell T, Zhu C, Christou P (2009) When more is better: multigene engineering in plants. Trends Plant Sci 15:48–56
62. Zhu C, Naqvi S, Gomez-Galera S, Pelacho AM, Capell T, Christou P (2007) Transgenic strategies for the nutritional enhancement of plants. Trends Plant Sci 12:548–555
63. Halpin C (2005) Gene stacking in transgenic plants – the challenge for 21st century plant biotechnology. Plant Biotechnol J 3:141–155
64. Chen L, Marmey P, Taylor NJ, Brizard JP, Espinoza C, D'Cruz P, Huet H, Zhang S, de Kochko A, Beachy RN, Fauquet CM (1998) Expression and inheritance of multiple transgenes in rice plants. Nat Biotechnol 16:1060–1064
65. Goderis IJWM, De Bolle MFC, François IEJA, Wouters PFJ, Broekaert WF, Cammue BPA (2002) A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. Plant Mol Biol 50:17–27
66. Tzfira T, Tian GW, Lacroix B, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol 57:503–516
67. Thomson JM, Lafayette PR, Schmidt MA, Parrott WA (2002) Artificial gene-clusters engineered into plants using a vector system based on intron-and intein-encoded endonucleases. In Vitro Cell Dev Biol Plant 38:537–542
68. Chung S-M, Frankman EL, Tzfira T (2005) A versatile vector system for multiple gene expression in plants. Trends Plant Sci 10:357–361
69. Lin L, Liu Y-G, Xu X, Li B (2003) Efficient linking and transfer of multiple genes by a multigene assembly and transformation vector system. Proc Natl Acad Sci USA 100:5962–5967
70. Thole V, Worland B, Snape JW, Vain P (2007) The pCLEAN dual binary vector system for *Agrobacterium*-mediated plant transformation. Plant Physiol 145:1211–1219
71. Coutu C, Brandle J, Brown D, Brown K, Miki B, Simmonds J, Hegedus DD (2007) pORE: a modular binary vector series suited for both monocot and dicot plant transformation. Transgenic Res 16:771–781
72. Fu X, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, Twyman RM, Christou P, Kohli A (2000) Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res 9:11–19
73. Breitler JC, Labeyrie A, Meynard D, Legavre T, Guiderdoni E (2002) Efficient microprojectile bombardment-mediated transformation of rice using gene cassettes. Theor Appl Genet 104:709–719
74. Potenza C, Aleman L, Sengupta-Gopalan C (2004) Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. In Vitro Cell Dev Biol Plant 40:1–22
75. Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the Cauliflower mosaic virus 35S promoter. Nature 313:810–812
76. Fang RX, Nagy F, Sivasubramaniam S, Chua NH (1989) Multiple *cis*-regulatory elements for maximal expression of the Cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1:141–150
77. Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299–1302
78. Zheng X, Deng W, Luo K, Duan H, Chen Y, McAvoy R, Song S, Pei Y, Li Y (2007) The Cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters. Plant Cell Rep 26:1195–1203
79. Peremarti A, Twyman RM, Gómez-Galera S, Naqvi S, Farré G, Sabalza M, Miralpeix B, Dashevskaya S, Yuan D, Ramessar K, Christou P, Zhu C, Bassie L, Capell T (2010) Promoter diversity in multigene transformation. Plant Mol Biol 73(4–5):363–378
80. Stavolone L, Ragozzino A, Hohn T (2003) Characterization of Cestrum yellow leaf curling virus: a new member of the caulimoviridae family. J Gen Virol 84:3459–3464
81. Schünmann PHD, Llewellyn DJ, Surin B, Boevink P, Feyter RCD, Waterhouse PM (2003) A suite of novel promoters and terminators for plant biotechnology. Funct Plant Biol 30:443–452
82. Schünmann PHD, Surin B, Waterhouse PM (2003) A suite of novel promoters and terminators for plant biotechnology II. The pPLEX series for use in monocots. Funct Plant Biol 30:453–460
83. McElroy D, Brettell RIS (1994) Foreign gene expression in transgenic cereals. Trends Biotechnol 12:62–68
84. Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218
85. McElroy D, Blowers AD, Jenes B, Wu R (1991) Construction of expression vectors based on the rice actin 1 (Act1) 50 region for use in monocot transformation. Mol Gen Genet 231:150–160
86. He C, Lin Z, McElroy D, Wu R (2009) Identification of a rice *actin2* gene regulatory region for high-level expression of transgenes in monocots. Plant Biotechnol J 7:227–239
87. Wobus U, Borisjuk L, Panitz R, Manteuffel R, Baumlein H, Wohlfart T, Heim U, Weber H, Misera S, Weschke W (1995) Control of seed storage protein gene expression: new aspects on an old story. J Plant Physiol 145:592–599

88. Lu CA, Lim EK, Yu SM (1998) Sugar response sequence in the promoter of a rice alpha-amylase gene serves as a transcriptional enhancer. J Biol Chem 273:10120–10131
89. Martinez A, Sparks C, Hart CA, Thompson J, Jepson I (1999) Ecdysone agonist inducible transcription in transgenic tobacco plants. Plant J 19:97–106
90. Padidam M, Gore M, Lu DL, Smirnova O (2003) Chemical-inducible, ecdysone receptor-based gene expression system for plants. Transgenic Res 12:101–109
91. Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol 107:193–232
92. Angenon G, Dillen W, van Montagu M (1994) Antibiotic resistance markers for plant transformation. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual, 2nd edn. Kluwer, Dordrecht, pp 1–13, Section C1
93. Wilmink A, Dons JJM (1993) Selective agents and marker genes for use in transformation of monocotyledonous plants. Plant Mol Biol Rep 11:165–185
94. Battraw M, Hall TC (1992) Expression of a chimeric neomycin phosphotransferase II gene in first and second generation transgenic rice plants. Plant Sci 86:191–202
95. Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274–276
96. Hille J, Verhweggen F, Roelvink P, Franssen H, van Kammen A, Zabel P (1986) Bleomycin resistance: a new dominant selectable marker for plant cell transformation. Plant Mol Biol 7:171–176
97. Hayford MB, Medford JI, Hoffmann NL, Rogers SG, Klee HJ (1988) Development of a plant transformation selection system based on expression of genes encoding gentamicin acetyltransferases. Plant Physiol 86:1216–1222
98. Eichholtz DA, Rogers SG, Horsch RB, Klee HJ, Hayford M, Hoffmann NL, Braford SB, Fink C, Flick J, O'Connell KM, Fraley RT (1987) Expression of mouse dihydrofolate reductase gene confers methotrexate resistance in transgenic petunia plants. Somat Cell Mol Genet 13:67–76
99. Rathore KS, Chowdhury VK, Hodges TK (1993) Use of *bar* as a selectable marker gene and for the production of herbicide resistant rice plants from protoplasts. Plant Mol Biol 21: 871–884
100. Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG, Fraley RT (1986) Engineering herbicide tolerance in transgenic plants. Science 233:478–481
101. Guerineau F, Mullineaux P (1989) Nucleotide sequence of the sulfonamide resistance plasmid R46. Nucleic Acids Res 14:4370
102. Haughn GW, Smith J, Mazur B, Somerville C (1988) Transformation with mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol Gen Genet 211:266–271
103. Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G (2000) The use of phosphomannose isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. Plant Cell Rep 19:798–803
104. Kunkel T, Niu QW, Chan YS, Chua NH (1999) Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. Nat Biotechnol 17:916–919
105. Stepanenko OV, Verkhusha VV, Kuznetsova IM, Uversky VN, Turoverov KK (2008) Fluorescent proteins as biomarkers and biosensors: throwing color lights on molecular and cellular processes. Curr Protein Pept Sci 9:338–369
106. Calderon-Villalobos LIA, Kuhnle C, Li H, Rosso M, Weisshaar B, Schwechheimer C (2006) LucTrap vectors are tools to generate luciferase fusions for the quantification of transcript and protein abundance *in vivo*. Plant Physiol 141:3–14
107. Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc Natl Acad Sci USA 94:2122–2127
108. Zhong S, Lin Z, Fray RG, Grierson D (2008) Improved plant transformation vectors for fluorescent protein tagging. Transgenic Res 17:985–989
109. Nishizawa K, Kita Y, Kitayama M, Ishimoto M (2006) A red fluorescent protein, DsRed2, as a visual reporter for transient expression and stable transformation in soybean. Plant Cell Rep 25:1355–1361
110. Ochiai-Fukuda T, Takahashi-Ando N, Ohsato S, Igawa T, Kadokura K, Hamamoto H, Nakasako M, Kudo T, Shibata T, Yamaguchi I, Kimura M (2006) A fluorescent antibiotic resistance marker for rapid production of transgenic rice plants. J Biotechnol 122:521–527
111. Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J 17:6086–6095
112. Somers DA, Makarevistch I (2004) Transgene integration in plants: poking or patching holes in promiscuous genomes? Curr Opin Biotechnol 15:126–131
113. Ward ER, Barnes WM (1988) VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to 5′ end of T-strand DNA. Science 242:927–930
114. Ziemienowicz A, Tinland B, Bryant J, Gloeckler V, Hohn B (2000) Plant enzymes but not *Agrobacterium* VirD2 mediate T-DNA ligation *in vitro*. Mol Cell Biol 20:6317–6322
115. Nam J, Mysore KS, Zheng C, Knue MK, Matthysse AG, Gelvin SB (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by Agrobacterium. Mol Gen Genet 261:429–438
116. Kumar S, Fladung M (2002) Transgene integration in aspen: structures of integration sites and mechanism of T-DNA integration. Plant J 31:543–551
117. Zhu QH, Ramm K, Eamens AL, Dennis ES, Upadhyaya NM (2006) Transgene structures suggest that multiple mechanisms are involved in T-DNA integration in plants. Plant Sci 171:308–322
118. Svitashev SK, Pawlowski WF, Makarevitch I, Plank DW, Somers DA (2002) Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. Plant J 32:433–445

119. Kohli A, Miro B, Twyman RM (2009) Transgene integration, expression and stability in plants: strategies for improvements. In: Kole C, Michler CH, Abbott AG, Hall TC (eds) Biotech plants, vol 1, Concepts & Development. Springer-Verlag, Berlin
120. Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci USA 95:7203–7208
121. Gorbunova V, Levy AA (1997) Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res 25:4650–4657
122. Sawasaki T, Takahashi M, Goshima N, Morikawa H (1998) Structures of transgene loci in transgenic *Arabidopsis* plants obtained by particle bombardment: junction regions can bind to nuclear matrices. Gene 218:27–35
123. Ming R, Hou S, Feng Y, Yu Q, Dionne-Laporte A, Saw JH, Senin P, Wang W, Ly BV, Lewis KL, Salzberg SL, Feng L, Jones MR, Skelton RL, Murray JE, Chen C, Qian W, Shen J, Du P, Eustice M, Tong E, Tang H, Lyons E, Paull RE, Michael TP, Wall K, Rice DW, Albert H, Wang ML, Zhu YJ, Schatz M, Nagarajan N, Acob RA, Guan P, Blas A, Wai CM, Ackerman CM, Ren Y, Liu C, Wang J, Wang J, Na JK, Shakirov EV, Haas B, Thimmapuram J, Nelson D, Wang X, Bowers JE, Gschwend AR, Delcher AL, Singh R, Suzuki JY, Tripathi S, Neupane K, Wei H, Irikura B, Paidi M, Jiang N, Zhang W, Presting G, Windsor A, Navajas-Pérez R, Torres MJ, Feltus FA, Porter B, Li Y, Burroughs AM, Luo MC, Liu L, Christopher DA, Mount SM, Moore PH, Sugimura T, Jiang J, Schuler MA, Friedman V, Mitchell-Olds T, Shippen DE, dePamphilis CW, Palmer JD, Freeling M, Paterson AH, Gonsalves D, Wang L, Alam M (2008) The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). Nature 452:991–997
124. Abranches R, Santos AP, Wegel E, Williams S, Castilho A, Christou P, Shaw P, Stoger E (2000) Widely separated multiple transgene integration sites in wheat chromosomes are brought together at interphase. Plant J 24:713–723
125. Pawlowski WP, Somers DA (1998) Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. Proc Natl Acad Sci USA 95:12106–12110
126. Kohli A, Twyman RM, Abranches A, Wegel E, Shaw P, Christou P, Stoger E (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247–258
127. Svitashev SK, Ananiev E, Pawlonski WP, Somers DA (2000) Association of transgene integration sites with chromosome rearrangements in hexaploid oat. Theor Appl Genet 100:872–880
128. Mehlo L, Mazithulela G, Twyman RM, Boulton MI, Davies JW, Christou P (2000) Structural analysis of transgene rearrangements and effects on expression in transgenic maize plants generated by particle bombardment. Maydica 45:277–287
129. Afolabi AS, Worland B, Snape JW, Vain P (2004) A large-scale study of rice plants transformed with different T-DNAs provides new insights into locus composition and T-DNA linkage configurations. Theor Appl Genet 109:815–826
130. Rai M, Datta K, Parkhi V, Tan J, Oliva N, Chawla HS, Datta SK (2007) Variable T-DNA linkage configuration affects inheritance of carotenogenic transgenes and carotenoid accumulation in transgenic indica rice. Plant Cell Rep 26:1221–1231
131. De Buck S, Jacobs A, Van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. Plant J 20:295–304
132. Kohli A, Griffiths S, Palacios N, Twyman RM, Vain P, Laurie DA, Christou P (1999) Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology-mediated recombination. Plant J 17:591–601
133. Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc Natl Acad Sci USA 105:18232–18237
134. Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106:7762–7767
135. Kooter J, Matzke MA, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci 4:340–346
136. Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJM (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 19:5194–5201
137. Mette MF, van der Winden J, Matzke MA, Matzke AJM (1999) Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. EMBO J 18:241–248
138. Mourrain P, van Blokland R, Kooter JM, Vaucheret H (2007) A single transgene locus triggers both transcriptional and post-transcriptional silencing through double-stranded RNA production. Planta 225:365–379
139. Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in *Arabidopsis* T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. Plant Cell 16:2561–2572
140. Jorgensen RA, Snyder C, Jones JDG (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet 207:471–477
141. De Block M, Debrouwer D (1991) Two T-DNAs co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor Appl Genet 82:257–263
142. Verma D, Daniell H (2007) Chloroplast vector systems for biotechnology applications. Plant Physiol 145:1129–1114

143. De Block M, Schell J, Van Montagu M (1985) Chloroplast transformation by *Agrobacterium tumefaciens*. EMBO J 4:1367–1372
144. Maliga P (2005) New vectors and marker excision systems mark progress in engineering the plastid genome of higher plants. Photochem Photobiol Sci 4:971–976
145. Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988) Chloroplast transformation in *Chlamydomonas* with high-velocity microprojectiles. Science 240:1534–1538
146. Svab Z, Hajdukiewcz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA 87:8526–8530
147. Zoubenko OV, Allison LA, Svab Z, Maliga P (1994) Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Res 22:3819–3824
148. Sinagawa-García SR, Tungsuchat-Huang T, Paredes-López O, Maliga P (2009) Next generation synthetic vectors for transformation of the plastid genome of higher plants. Plant Mol Biol 70:487–498
149. Hamilton CM (1997) A binary-BAC system for plant transformation with high molecular weight DNA. Gene 200:107–116
150. Liu YG, Shirano Y, Fukaki H, Yanai Y, Tasaka M, Tabata S, Shibata D (1999) Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc Natl Acad Sci USA 96:6535–6540
151. Hamilton CM, Frary A, Xu YM, Tanksley SD, Zhang HB (1999) Construction of tomato genomic DNA libraries in a binary-BAC (BIBAC) vector. Plant J 18:223–229
152. Shibata D, Lui Y-G (2000) *Agrobacterium*-mediated plant transformation with large DNA fragments. Trends Plant Sci 5:354–357
153. Vega JM, Yu W, Han F, Kato A, Peters EM, Zhang ZJ, Birchler JA (2008) *Agrobacterium*-mediated transformation of maize (*Zea mays*) with Cre-lox site specific recombination cassettes in BIBAC vectors. Plant Mol Biol 66:587–598
154. Schmidt MA, LaFayette PR, Artelt BA, Parrott WA (2008) A comparison of strategies for transformation with multiple genes via microprojectile-mediated bombardment. In Vitro Cell Dev Biol Plant 44:162–168
155. Mullen J, Adam G, Blowers A, Earle E (1998) Biolistic transfer of large DNA fragments to tobacco cells using YACs retrofitted for plant transformation. Mol Breed 4:449–457
156. Wada N, Kajiyama S, Akiyama Y, Kawakami S, No D, Uchiyama S, Otani M, Shimada T, Nose N, Suzuki G, Mukai Y, Fukui K (2009) Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes. Plant Cell Rep 28:759–768
157. Carlson SR, Rudgers GW, Zieler H, Mach JM, Luo S, Grunden E, Krol C, Copenhaver GP, Preuss D (2007) Meiotic transmission of an *in-vitro*-assembled autonomous maize minichromosomes. PLoS Genet 3:1965–1974
158. Schaefer DG (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. Annu Rev Plant Biol 53: 477–501
159. Li J, Hsia AP, Schnable PS (2007) Recent advances in plant recombination. Curr Opin Plant Biol 10:131–135
160. Terada R, Urawa H, Inagaki V, Tsugane K, Lida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20:1030–1034
161. Cotsaftis O, Guiderdoni E (2005) Enhancing gene targeting efficiency in higher plants: rice is on the move. Transgenic Res 14:1–14
162. Kumar S, Allen GC, Thompson WF (2005) Gene targeting in plants: fingers on the move. Trends Plant Sci 11:159–161
163. Cai CQ, Doyon Y, Ainley WM, Miller JC, Dekelver RC, Moehle EA, Rock JM, Lee YL, Garrison R, Schulenberg L, Blue R, Worden A, Baker L, Faraji F, Zhang L, Holmes MC, Rebar EJ, Collingwood TN, Rubin-Wilson B, Gregory PD, Urnov FD, Petolino JF (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol 69:699–709
164. Zeevi V, Tovkach A, Tzfira T (2008) Increasing cloning possibilities using artificial zinc finger nucleases. Proc Natl Acad Sci USA 105:12785–12790
165. Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X, Choi VM, Rock JM, Wu YY, Katibah GE, Zhifang G, McCaskill D, Simpson MA, Blakeslee B, Greenwalt SA, Butler HJ, Hinkley SJ, Zhang L, Rebar EJ, Gregory PD, Urnov FD (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459:437–441
166. Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, Voytas DF (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459:442–445
167. Marjanac G, De Paepe A, Peck I, Jacobs A, De Buck S, Depicker A (2008) Evaluation of Cre-mediated excision approaches in *Arabidopsis thaliana*. Transgenic Res 17:239–250
168. Srivastava V, Ariza-Nieto M, Wilson AJ (2004) Cre-mediated site-specific gene integration for consistent transgene expression in rice. Plant Biotechnol J 2:169–179
169. Chawla R, Ariza-Nieto M, Wilson AJ, Moore SK, Srivastava V (2006) Transgene expression produced by biolistic-mediated, site-specific gene integration is consistently inherited by the subsequent generations. Plant Biotechnol J 4:209–218
170. Luo K, Duan H, Zhao D, Zheng X, Deng W, Chen Y, Stewart CN Jr, McAvoy R, Jiang X, Wu Y, He A, Pei Y, Li Y (2007) "GM-gene-deletor": fused *lox*P-FRT recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants. Plant Biotechnol J 5:263–274
171. Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA 96: 11117–11121

Books and Reviews

Deblaere R, Reynaerts A, Hofte H, Hernalsteens JP, Leemans J, Van Montagu M (1987) Vectors for cloning in plant cells. Meth Enzymol 153:277–292

Kado CI (1998) *Agrobacterium*-mediated horizontal gene transfer. Genet Eng 20:1–24

Komari T, Ishida Y, Hiei Y (2004) Plant transformation technology: *Agrobacterium*-mediated transformation. In: Christou P, Klee H (eds) Handbook of plant biotechnology. Wiley, New York, pp 233–288

Slater A, Scott N, Fowler M (2003) Plant biotechnology. Oxford University Press, Oxford

Twyman RM (2006) Gene transfer to plants. In: Primrose SB, Twyman RM (eds) Principles of gene manipulation and genomics. Wiley-Blackwell, Oxford

Twyman RM, Stoger E, Kohli A, Christou P (2002) Foreign DNA: integration and expression in transgenic plants. In: Setlow JK (ed) Genetic engineering: principles and practice, vol 24. Kluwer Academic/Plenum Publishers, New York, pp 107–136

Valentine L (2003) *Agrobacterium tumefaciens* and the plant: the David and Goliath of modern genetics. Plant Physiol 133:948–955

Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant J 23:11–28

Crop Radiation Capture and Use Efficiency

Erik Murchie[1], Matthew Reynolds[2]
[1]Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington, UK
[2]International Maize and Wheat Improvement Centre (CIMMYT), Mexico DF, Mexico

Article Outline

Glossary

C3 The C3 pathway of photosynthesis, found in most plant species, for example, rice, potato, and wheat.

C4 The C4 pathway of photosynthesis, found in some tropical species, for example, maize, sugarcane, sorghum.

PAR Photosynthetically active radiation. Solar radiation in the wavelength region 400–700 nm.

RUE Radiation use efficiency, the ratio of biomass produced per unit radiation intercepted.

Definition of the Subject and Its Importance

The rate of growth (the rate of the accumulation of dry matter) of all plants is entirely dependent on the interception of energy (electromagnetic radiation) from the sun in the wavelength range 400–700 nm. This energy is utilized by photosynthesis to synthesize carbohydrates and other biological molecules needed for essential plant processes.

The amount of energy intercepted or captured by the whole plant and community system (the canopy) is determined by the organization of leaves into an efficient spatial structure with a large total surface area. The amount of radiation captured will determine the rate of photosynthesis possible and the rate of growth. However, the final growth rate is then determined by *losses* in the system that originate from a number of sources, including the type of photosynthetic mechanism, metabolic and hydraulic constraints, the relationship between photosynthetic source and non-photosynthetic sink organ, variability in environmental conditions, and limitations imposed by management techniques.

The discovery that plant and crop growth is closely linked to the amount of intercepted radiation led to the establishment of methods for measuring radiation use efficiency (RUE). RUE is measured as the amount of dry matter produced per unit intercepted radiation over a given time period and it is often separated into key developmental stages within the life cycle of the crop. It was quickly established that values of RUE tend to be stable for a given species, growth stage, and environment, but there are important differences across crop species and plant types. In the absence of other factors, RUE will set the theoretical limit to biomass production and ultimately crop yield. It is now accepted that RUE is a fundamental measurement which underpins potential crop productivity and yield and it has become embedded into modern methods of crop growth analysis.

In many cases, plants absorb more photosynthetically active radiation (PAR) radiation than they utilize for growth: given the current emphasis on global food security, there is currently much interest in raising the RUE of key crops in important agroecosystems.

Introduction and History

All green plants use sunlight as their sole energy source for assimilation of carbon dioxide into carbohydrates. At its most fundamental level, this process is the formation of energy-rich bonds in a form that is easily stored, transported, and utilized in most essential plant functions. This process is of course photosynthesis and

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

it is the sole supplier of energy for almost all human nutrition and fuel requirements, via current and prehistoric photosynthesis.

The continuing expansion of the human population and the steady increase in consumption per capita is placing pressure on land availability. A variety of factors including urbanization and the erosion of existing land certainly necessitate production of more food and fuel per hectare with fewer inputs (water, fertilizer) and all of this within the uncertainty of changes in climate and increased pressure to use land which may be currently unsuitable for cultivation. For example, 700 million people depend on rice for calorific intake. Each hectare can currently feed around 27 people; by 2050 this will have to rise to 43 people per hectare [1]. This is compounded by the fact that Asian rice yield potential appears to be stagnating [2]. Wheat is grown on over 200 million hectares of land providing approximately one-fifth of the total calorific input of the world's population, and while there have been steady increases in productivity since the green revolution, global demand for wheat is predicted to increase at a faster rate than the annual genetic gains that are currently being realized [3]. Since the highest yields of major crops are usually only achieved within high input systems, it is not surprising that many suggest a reappraisal of the basis of crop productivity.

It seems clear that an increase in the rate of application of fertilizer or water is neither desirable nor even possible. Nitrogen fertilizer is heavily dependent on the continued availability (physically, financially, and politically) of fossil fuels. Water is increasingly scarce not just in equatorial regions but in temperate regions. One way to improve resource use efficiency is to increase the rate of overall biomass production and this is starting to be associated with yield progress of major crops such as rice and wheat [3, 4]. As shall be described, an increase in biomass production by crops, whilst helping to improve upon current rates of breeding progress in yield potential, would also (1) underpin future genetic improvements in adaptive processes, (2) improve the relative efficiency of resource use in terms of biomass production per unit water, light, and fertilizer, and therefore (3) increase the attractiveness of using waste products of arable crops for secondary uses such as fuel, and (4) if applied universally, reduce the competition for land between biofuel crops and food crops, and (5) increase the feasibility of using marginal land for fuel and food production.

A History of Radiation Use Efficiency

Given that it underlies so much of human activity and endeavor it is perhaps curious that the relationship between radiation and plant growth did not become explicitly defined and quantified until relatively recently. It has been suggested that a tendency to retain methods of classical crop growth analysis during the first half of the twentieth century delayed a mechanistic analysis of the processes of biomass accumulation [5]. In these early studies, growth (crop mass) was described as a function of time possibly because of the ease with which mass could be measured through the growing season. This approach was confounded by its inclusion in measurements of relative growth rate where crop biomass increase is closely related to existing biomass. The central role of light interception in growth was recognized by Watson [6] who suggested a measurement of net assimilation rate which worked in some situations but did not adequately account for the complex relationship between leaf area and variation in assimilation rate (photosynthesis) of individual leaves.

Another advancement in understanding came with the consideration that efficiency of light use declined at high levels (light saturation) [7, 8] and that light levels were lower at the base of the canopy. In fact, as pointed out by Hirose [9], the first mathematical model of canopy photosynthesis was first produced by Monsi and Saeki in 1953 but this was not recognized for more than a decade. This resulted in the first real considerations of growth as a function of the amount of light intercepted by the plant canopy. At this time, it was observed that the amount of light intercepted by the canopy was closely related to dry matter accumulation [5]. Despite this there were many studies in the 1970s which used incident light rather than intercepted radiation to make key studies in agronomy and ecology.

The sole use of incident radiation measurements will lead to errors in the calculation of radiation use efficiency especially before canopy closure when not all radiation is absorbed and when crops possess differing rates of canopy development. Over the whole life cycle of the crop, the proportion of incident radiation that is

intercepted and available for assimilation can be quite small. It was not until the mid 1970s that John Monteith established the relationship between accumulated intercepted radiation and accumulated biomass within canopies [10]. This was accompanied by experimental data demonstrating a level of conservation of radiation use efficiency of crop species when grown under optimal growth conditions with high resource availability and a consideration of the role of leaf photosynthetic capacity in crop canopies. This has since proved to be a robust approach and has remained a central feature of crop growth analysis ever since. Many studies attempted to provide values for RUE across a wide range of species and growth conditions across the world [11, 12]. It was quickly claimed (1) that it was possible to attain consistent values for a given species when growing conditions were good, (2) that suboptimal (e.g., nitrogen or water deficient) or stressful conditions caused RUE to decline, and (3) that there were clear differences between crop species: plants possessing the C4 photosynthetic mechanism had the highest RUE followed by most C3 plant species and finally legumes with the lowest RUE values. Early data suggested that RUE was a conservative or even constant value for a given species. However, data published since has demonstrated significant variation and it has been pointed out that close attention in each case must be paid to the methods of analysis, growth conditions, developmental phase, and genotype.

RUE is commonly used within the crop sciences although it is applicable to growth of any autotrophic organism and most ecosystems. Its calculation requires knowledge of the amount of radiation absorbed in a given time period and measurement of the resulting biomass and energy content. These are easier to measure in a uniform system like a monoculture. The historical development of RUE was no doubt dependent on the advancement in other areas of science such as photosynthetic regulation and the technical development of instruments to accurately measure key attributes, that is, radiation, photon flux density, and the rate of photosynthetic carbon assimilation.

The understanding that RUE is fundamental to crop productivity leads naturally to the question of whether it can be improved. The observation that C4 plants possess a higher RUE leads to the notion that such a change would give a higher biomass productivity which could be exploited to increase yield per hectare. The green revolution provided a step change in agricultural productivity that was able to prevent the tragedy of mass starvation in some regions and also to support population increase. As outlined here, a step change of similar magnitude now would probably require an improvement in RUE.

Determination of Radiation Use Efficiency

RUE is the ratio between accumulated plant biomass and the accumulated radiation intercepted by the crop. A note on terminology: some authors use the terms "radiation conversion factor" [13] and "radiation conversion efficiency." Some authors have pointed out that use of the word "conversion" is inappropriate because energy is not being directly converted to matter but rather converted from one form of energy to another (i.e., solar radiation to higher energy state of chlorophyll molecules) [5]. The term radiation use efficiency (RUE) shall be used.

To calculate RUE, it is necessary to know the amount of radiation arriving at the top of the canopy, the amount of radiation intercepted by the canopy, and the biomass accumulated during a given period. In many studies, the energy content of the dry matter is also required. This section will summarize how RUE is measured in a practical sense and tackle some of the diversity in approaches that have been taken.

Radiation arrives at the edge of the earth's atmosphere at a mean rate of 1.4 kJ m^{-2} s^{-1}, the so-called solar constant. By the time photons reach the earth's surface a number of geometrical and atmospheric factors have reduced this value significantly. Cloud cover notwithstanding the greatest influx occurs in tropical regions at low latitudes. The greatest measured flux at the earth's surface itself occurs in low latitude regions where cloud cover is minimal (20–30°). High latitudes can have extremely low solar radiation levels.

Light is absorbed and scattered by molecules, aerosols, and particles in the atmosphere, reducing flux even further. Gas molecules such as CO_2, H_2O, and CH_4 absorb energy at specific infrared wavelengths whilst gas molecules O_2 and O_3 absorb at lower wavelengths. Photosynthesis is restricted to a range of wavelengths that approximate to those visible to the human

eye: 400–700 nm. Higher wavelengths do not contain sufficient energy to drive photosynthesis. At ground level, this range of wavelengths makes up 49% of total solar energy. In other words, the photosynthetic process does not use over half of the energy available. However, longer wavelengths have an important heating effect which raises plant tissue temperature and accelerates metabolic and developmental processes such as leaf and canopy construction.

Variation from day to day means that it is necessary to integrate measurements of radiation over long time periods. This is often done by positioning devices above the canopy and below the canopy. Most commonly these have been fairly inexpensive tube solarimeters connected to a device that continually stores data produced. Reflection can be measured by inverting the device. Instantaneous measurements (spot measurements) of fractional interception (f) are also used but these can be misleading because they are most reliably taken at midday during the same, usually sunny, conditions and therefore do not account for cloud cover or low solar elevation and give lower values of f.

The proportion of PAR is higher in scattered light than direct beam radiation and so it is useful to distinguish between the two and a few commercially available devices are capable of doing so. This also means that the proportion of PAR changes according to solar elevation, although it is around 0.45 at elevations greater than 30° [5]. Growth in predominantly scattered radiation commonly causes an increase in RUE.

Wavelengths used for growth (400–700) are preferentially absorbed by chlorophyll within the canopy resulting in transmitted light that is depleted in red and blue and enriched in far-red. The formula for fractional interception is presented in its simplest form by Eq. 1.

$$f = 1 - \frac{(I + r)}{Io} \quad (1)$$

where f = fractional interception, Io = incident radiation, r = reflected radiation, I = intercepted radiation.

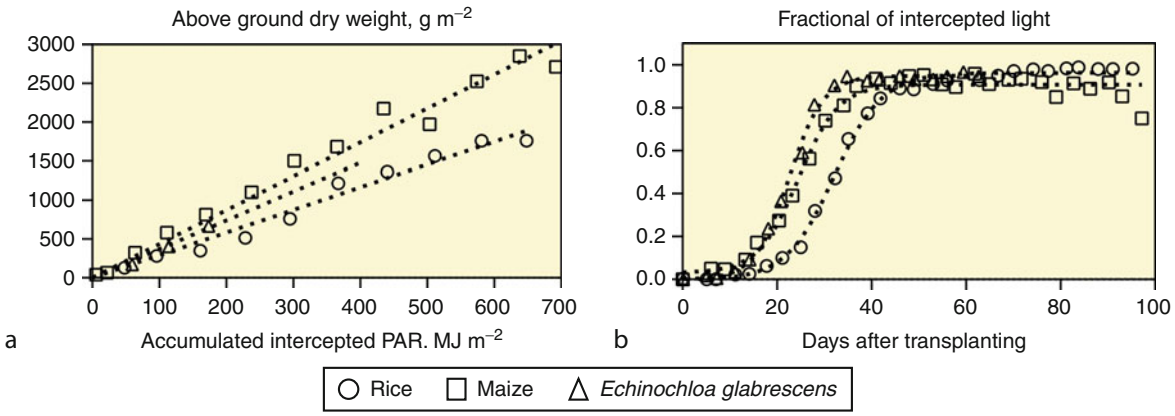
An alternative method for measuring f is spectral reflectance. This compares reflectance from the canopy in the red band and the near IR band to produce a normalized difference vegetation index (NDVI). It has been possible to show the relationship between NDVI and f in many crops [14] and is a technique that is possible to use remotely from satellites and aircraft. Although it is clearly convenient and rapid, one must be aware that it has the drawback of being a spot measurement. Measurements made from satellites suffer from problems of images that deviate from 90° to the earth's surface [15].

Photography is also used to estimate f using a camera fitted with a fish-eye lens positioned below or above the canopy. This has the advantage of encompassing a large area, producing permanent image and being taken rapidly in the field. This method has been shown in many cases to correlate with f measured using solarimeters; however, errors arise due to common bias in positioning the camera. All indirect measurements of f should consider the potential errors that may result.

RUE is usually calculated by measuring the difference in biomass between many consecutive harvests (i.e., accumulated intercepted growth) and plotting this against the measured accumulated intercepted radiation. This is usually a linear relationship and RUE is calculated as the slope of this relationship (Fig. 1). The points at which measurements are made are critical because RUE varies according to developmental state.

Care must be taken when comparing values from different sources [5, 13]. Some of the common sources of variation are as follows:

1. If root mass is not included then RUE values will be lower. However, it is extremely difficult to measure root mass directly and it is often ignored or assumed to be a fixed percentage of total plant mass. Nonuniform growth can therefore be a significant source of error. Energy required to grow and maintain roots is also a function of the biotic and abiotic environment of the soil.
2. Solar radiation flux density is measured using solarimeters, pyranometers, or radiometers which usually cover the wavelengths 300–3,000 nm. The photon flux density is commonly measured using sensors that detect within the photosynthetically active range of 400–700 nm. Therefore the conversion between energy and quanta-based measurements must take into account the energy of each waveband. Data based on different regions of the electromagnetic spectrum can cause inaccuracies, for example, the transmission of shortwave radiation through canopies differs from that of PAR [16].



Crop Radiation Capture and Use Efficiency. Figure 1

An example of the relationship between accumulated intercepted radiation and accumulation of dry matter by three species : the C3 crop rice (*circle*), the C4 crop maize (*square*), and the C4 weed *Echinochloa glabrescens* (*triangle*). Note the higher slope for maize in (**a**), that maize reached full interception before rice in (**b**), and that the weed is the first to form a full canopy but does not achieve the same amount of biomass as the crop species (Redrawn from Sheehy et al. [1])

However, the solar spectrum is fairly constant, and the reliable figure for this is 4.6 µmol quanta. Inaccuracies can arise from badly positioned, poorly leveled or uncalibrated instruments. It is common practice to keep devices free of anything that would attenuate radiation in a way that would bias the results, for example, dead leaves are removed and glass is cleaned.

3. It is important to distinguish *absorbed* from *intercepted radiation*. The latter does not take into account the reflection of PAR from the top surface of the canopy. This is generally assumed to be about 5% of total PAR but variation is likely. Not all of the plant tissues that absorbed energy are necessarily alive or of equal photosynthetic potential.
4. When comparing different species of plant with contrasting harvest organs it is important to consider the *energy content* of the organs involved. Lipid has a higher calorific content than protein, which in turn has a higher calorific content than carbohydrates [17]. The heats of combustion of carbohydrates, proteins, and lipids are 17.3, 22.7, and 37.7 kJ g^{-1}, respectively. Since RUE is comparing the input of energy to the output of dry matter it can vary according to the energy content per unit dry matter and it is essential to adjust RUE accordingly. For example, the RUE of crops that produce particularly oily seeds should decrease during the seed-filling period. However, the nutrient content of plant tissue does not greatly directly influence the measurement of RUE.
5. The stage of growth can have a large influence on the RUE measured (see section Source–Sink Processes and Partitioning of Assimilates). It can be common practice to calculate the RUE over the entire growing season but this does not necessarily represent the maximal RUE value. For example, in many crops RUE appears to be steady during the vegetative period but to decline following the onset of the reproductive phase.

Radiation Capture by Crop Canopies

Canopy structure and therefore the efficiency of light capture was a common feature in the domestication and the later improvement of many crop species. For example, the reduction of branching in maize and sunflower allowed dense planting [18] and there is evidence of further adaptation to higher planting density [19]. The reduction in height of cereals was a key factor in the green revolution, permitting an increase in harvest index and reduction in lodging. Crop canopies

must intercept or capture as much radiation in the 400–700 nm bandwidth as possible. Most of the light interception occurs by leaves; however, stems, petioles, leaf sheaths, and reproductive structures can also absorb significant amounts of radiation. To achieve high interception, they must construct and present a canopy with large leaf area and in many crops the minimum leaf area index (L: the ratio of leaf area per unit ground area) is around three. The L can be expressed by the product of the number of plants per unit ground area, the number of leaves per plant, and the mean area of leaves per plant (plus green stem area). Light can penetrate the canopy and strike the ground below as direct beam radiation (so-called sunflecks), scattered radiation, or radiation that has passed through leaves and other plant organs, that is, transmitted. The amount of penetrated radiation is dependent on the three-dimensional arrangement of leaves in the canopy and for a given L will be a function of a large number of features such as leaf size, leaf density per cubic meter, the angle of individual leaves, heterogeneity of leaves in space (clumping), leaf thickness, and albedo. There is clear variation between species and even between crop varieties in these features. Additionally, it is possible to alter these features through management techniques such as planting density and the application of NPK fertilizer. Therefore there needs to be a way of describing radiation distribution within canopies mathematically and linking this to agronomic characteristics such as leaf area index and nitrogen content.

The relationship between L and fractional interception can be described by Monsi–Saeki equation (Eq. 2), a modification of Beer's law. This assumes that the canopy is a homogeneous medium whose leaves are randomly distributed in space, that is, there is no effect of row structure or clumping and under these conditions Beer's law will apply [9]. The Monsi–Saeki equation can be applied if the canopy is considered to be divided into horizontal layers with each layer possessing a particular L and the irradiance within each layer is measured. The irradiance at each layer will depend on the three-dimensional characteristics of the leaves both within that layer and the layers above. It has been found that this provides an accurate estimate in many crops in which this has been measured: $\ln(I/Io)$ against L provides a linear relationship, the slope of which gives the value k which provides a simple but useful mathematical description of the architecture of the crop canopy in question.

$$I = Io \exp(-kL) \tag{2}$$

I and Io as in Eq. 1, L = leaf area index, and k = extinction coefficient for a given waveband

Crops have been found to vary for the value of k and the major feature is the erectness of leaves. Canopies with erect and narrow leaves such as cereals have a lower value of K than those with flat, broad, and horizontal leaves. This also applies to differences within species, for example, rice varieties vary in erectness [20].

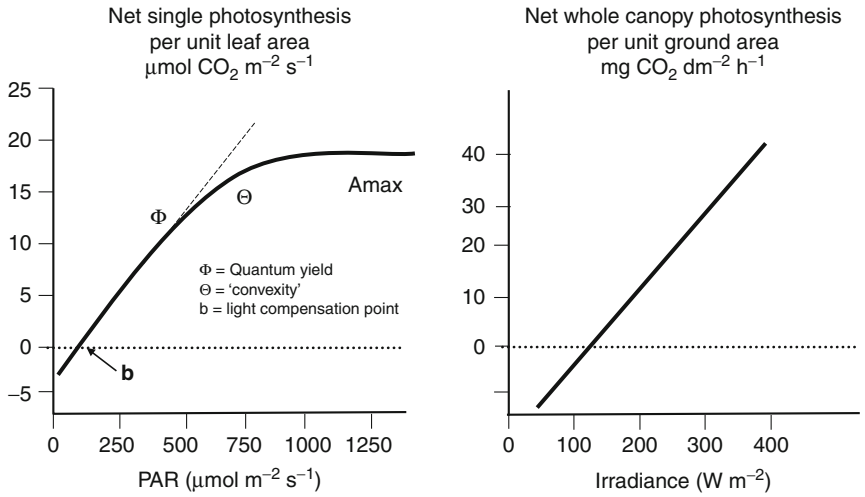
If the Monsi–Saeki equation holds and the k value for a given canopy type is known then it becomes possible to calculate f simply from a knowledge of L (Eq. 3).

$$f = 1 - \exp(-kL) \tag{3}$$

Equation 3, letters as Eqs. 1 and 2

This analysis is useful when canopies are considered to be a three-dimensional "box" of vegetation with radiation penetrating only from above. It can fail where plants are sparsely populated and do not achieve full ground cover or are simply planted in rows with space in between, which is the case for some crops that are tended by hand. The calculation of intercepted radiation in these cases can become quite complex and impractical at high levels of heterogeneity although estimations are often made on the basis of incident radiation and leaf area per plant. Another source of error is angle of solar elevation which can significantly alter the proportion of reflected light and the proportion of scattered light within the canopy. It also does not account for the commonly seen variation in leaf angle that occurs between the top and the bottom of the canopy. Even at a point close to full canopy cover there can be a "bimodal" type of variation in crop canopies. For example, rice plants tend to form "inverted cone" shapes and this causes a complex three-dimensional variation in irradiance distribution especially during canopy development.

The optimal "design" of plant canopies must consider not just maximum interception but also the relationship with photosynthetic rate. In principle, a plant could achieve close to 100% interception with a single, planar chlorophyll-rich leaf and a L of 1.0. However,



Crop Radiation Capture and Use Efficiency. Figure 2
The relationship between photosynthesis and *incident* radiation levels for (**a**) single leaves and (**b**) a full canopy: (**a**) typical features of the leaf response, the linear phase (maximum quantum efficiency), the light compensation point, convexity, and the light saturated rate (Amax, and (**b**) schematic figure: note the linear response of whole canopies in comparison to single leaves

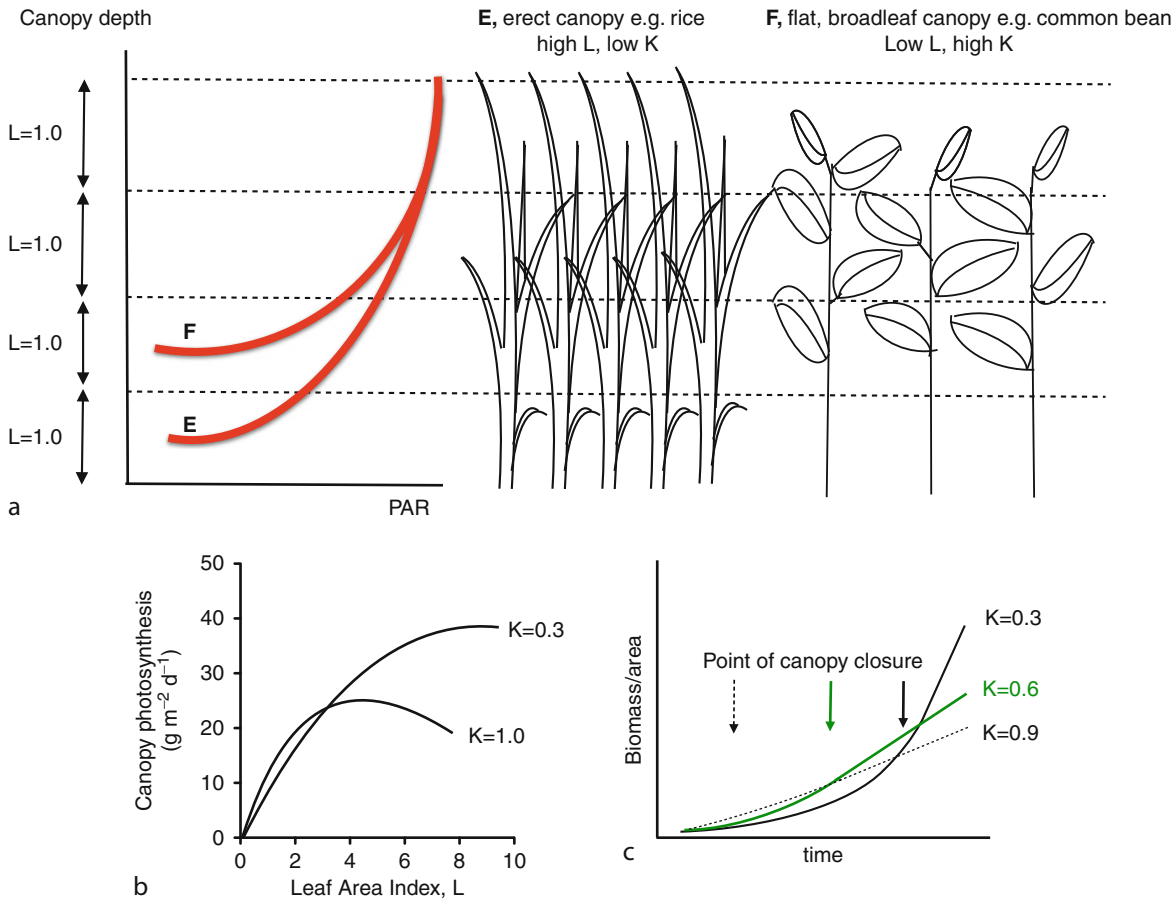
this does not result in optimum productivity largely due to the fact that photosynthesis saturates below full sunlight and this is especially marked in C3 plants. This is a central point: the response of leaf photosynthesis to irradiance is shown in Fig. 2. This is a useful measurement and easy to make with today's equipment. It provides not only a measurement of the maximum rate of photosynthesis (Amax) but also the potential quantum yield of photosynthesis.

A greater canopy carbon gain is achieved by reducing the proportion of leaves in the canopy that exist in the light-saturated state. The leaves lower in the canopy are retained at light limitation while those at the top will be prone to light saturation. If the irradiance increases (e.g., moving from cloud-cover to full sunlight) then those lower in the canopy will be able to respond accordingly. This has lead to the widely observed phenomenon that when irradiance is plotted against carbon-gain (photosynthesis) one commonly observes a linear response for canopies but a saturation in leaves (normally described by a non-rectangular hyperbola) (Fig. 3) [11]. This is important: if canopies demonstrated light saturation then this would severely limit their ability to improve RUE.

Canopy Properties and Photosynthesis

The optimum design for biomass production in terms of carbon gain is predicted to be one that permits a higher proportion of radiation to penetrate to lower layers, whilst also reducing light saturation at the top (Fig. 3). Indeed, this seems to be the trend in species such as rice and wheat although the advantage here is thought to be greatest at lower latitudes where light is overhead for a greater proportion of the year and therefore penetration into the canopy is greater. This also has the advantage that it prevents the unwanted senescence of leaves when light levels drop below or close to the light compensation point [21].

This raises the question of whether there is room for improvement in canopy architecture to improve RUE. Early work found that canopies with a low L benefitted from horizontal posture while those with high L benefitted from upright posture [5, 22]. This work suggested that RUE would not be sensitive to extreme upright posture of leaves. However, it is clear that upright leaves are associated with the highest yielding lines in wheat [23, 24] and it has been suggested that some cereal canopies may benefit from increasing



Crop Radiation Capture and Use Efficiency. Figure 3

Canopy structure and function according to leaf "architecture." (**a**) The schematic figure on the *left* shows canopy depth where the canopy has been divided into layers, each with a leaf area index (*L*) of 1.0. Attenuation of light in the canopy follows the Monsi–Saeki equation. For a broadleaf canopy, the radiation reaches extinction at a higher point and a lower *L* compared to an upright canopy. The canopy extinction coefficient value "*K*" is derived from Eq. 2 and provides a mathematical description of the link between *F* and *L*. (**b**) The calculated relationship between canopy photosynthesis and *L*, measured at irradiance saturating at the top of the canopy for species with two values of *k*. Note the higher maximum value of the species with higher *k* (Redrawn from [9]). (**c**) A highly schematic figure redrawn and adapted from Ong and Monteith [32] and shows the theoretical differences in canopy biomass accumulation over time in species with different values of *k*. Maximum values of carbon gain are achieved after canopy closure which occurs earlier in broadleaf species (high *k*). Species with a lower value of *k* have higher potential rates of total canopy photosynthesis

erectness further [3, 23, 25]. There are relatively few genes controlling erectness and this should be straightforward to test, although work has shown that compensatory effects such as leaf size may be hard to account for.

Modern high-yielding rice lines also have highly erect leaves and it is assumed that this also results in higher potential productivity. In rice, this also permits denser planting which accelerates canopy closure and improves total radiation intercepted over a short tropical growing season.

A further feature is that leaves have different characteristics according to the light intensity to their canopy position. Most notably they have "sun leaf" and

"shade leaf" characteristics, the former having higher light compensation points, higher photosynthetic capacity, and higher nitrogen and enzyme content. This is considered to be an efficient use of resources with nitrogen being located only where it is required for high photosynthetic activity. Additionally, there are a number of acclimation mechanisms to improve light harvesting in shade and diffuse light such as the synthesis of light harvesting complexes enriched in chlorophyll b [20, 26]. The efficiency of use of shade and diffuse light within the canopy is a relatively unexplored area in crops. This raises the question of whether leaf ageing or leaf acclimation gives rise to the nitrogen content of the leaf. There is evidence that both processes are at play: in fact the distribution of nitrogen in plant canopies should be closely related to photosynthesis and irradiance level [27, 28]. There is some indication that this has been achieved although the role of "storage" of assimilated nitrogen in leaves needs further analysis [20, 21].

Canopy development occurs by the successive emergence of leaves from primordia which are localized points of tissue formed around the apical dome of the vegetative shoot. The arrangements of these primordia will largely determine the positions of the leaves in the final canopy. Therefore there is great interest in the genetic manipulation of developmental processes in crops [29, 30]. The thermal time interval between the initiation of successive primordia is critical and determines the time taken for production of successive leaves. The genetic and molecular processes determining leaf development and expansion is an exciting area of research [31].

For annual crops with a limited growing season, it is critical that canopy development is synchronized with periods of high radiation. In temperate regions, low temperatures in Spring can increase the time taken for canopy closure. Since maximum rates of production are not attained until full canopy cover is achieved, this can be deleterious and is referred to as "lost time" [32]. Faster rates of canopy closure can be achieved by different strategies such as the application of nitrogen increasing planting density, irrigation, and the planting of crops in autumn so that the time taken for establishment is reduced. For example, in the UK, maximum radiation receipts occur in June, and for Spring-sown crops such as potato and sugar beet, productivity can depend on the establishment of a high L before this period. In rice, the remarkable erectness of leaves in some modern cultivars has allowed for extremely dense planting and a shift toward a reduction in tillering. This has the advantage that canopy closure can be achieved quickly and the so-called lost time is reduced.

There is no doubt that canopy structure is a central feature of crops with high RUE although the precise three-dimensional characteristics are difficult to quantify by direct measurement and this has meant that the techniques to describe crop canopies mathematically have remained relatively simple and continued to assume that they exist as randomly distributed photosynthetic elements in space. As shall be described in the next section, it will be essential to have a more sophisticated approach which links leaf arrangement and light distribution at higher resolutions and accounts for the dynamics of both light distribution and localized photosynthetic responses. For this various photographic and laser-based methods are available for "digitization" of plant and crop canopies although the high density of many crop canopies makes such measurements difficult in a practical sense. Mathematical modeling of plant canopy structure is progressing rapidly along with increased computing power and more sophisticated programming [33]. It would seem likely that methods that can use empirical measurements to model canopy architecture at high resolution and accurately predict photosynthetic light responses are not far away.

"Conversion" of Captured Radiation: Photosynthetic Mechanisms

The efficiency of radiation use by the whole canopy is the sum of photosynthesis occurring by each leaf and each leaf portion within the canopy. In turn, the photosynthetic rate of each leaf is determined by the sum of photosynthesis in the chlorophyll-containing organelles (chloroplasts). Each leaf and organelle will exist in a dynamic microenvironment and the photosynthetic productivity will depend on the resources available to it at a given moment in time (light, water, nutrients). Due to limitations placed on the photosynthetic process such as light saturation and CO_2 diffusion, the rate of photosynthesis at the leaf level is a strong determinant of canopy carbon gain. This section will describe the photosynthetic process, the different types of photosynthesis, and limitations to leaf level photosynthesis.

The vast majority of chloroplasts are located in leaves and they are numerous, with each mesophyll cell containing between 50 and 200 chloroplasts [34]. Chloroplasts are responsible for absorption of the 400–700 waveband in crop canopies and are the reason that plants appear green. They are sac-like subcellular organelles that contain membranous structures that possess the chlorophyll used for light harvesting. Most of the chlorophyll is contained within pigment protein complexes called "light harvesting complexes" and these are extremely efficient at absorbing visible light, and through a series of resonance transfer mechanisms, they pass the excitation energy to a reaction center where a special pair of chlorophyll molecules use this energy to generate a redox potential capable of oxidizing water via the oxygen evolving complex. The resulting electrons are passed through an electron transport chain and used to generate a proton gradient that synthesizes ATP. Ultimately, the electrons produce a reductant, NADPH. The NAPDPH and ATP are utilized within the Calvin–Benson or photosynthetic carbon reduction cycle to reduce CO_2 to triose phosphates that are used in hexose and starch synthesis or exported from the chloroplast for sucrose synthesis.

How Efficient Is Crop Photosynthesis?

It is useful to consider the amount of light energy arriving at a canopy top or leaf surface and calculate energy losses at each stage based on current knowledge of the photosynthetic process in order to estimate the potential system productivity [35, 36]. As mentioned, only 49% of solar energy is available for photosynthesis. Within the PAR range chlorophyll does not absorb strongly in the green band and the reasons for this adaptation have been well explored [37]. Green photons make up around 10% in the PAR range. Red and blue photons drive photosynthesis with equal efficiency despite the fact that blue light contains more energy per photon than red light. Chlorophyll is excited to higher energy states by blue photons but the extra absorbed energy is not used to drive photochemistry. This extra energy which is calculated to make up 6.6% of incident solar energy is effectively lost [36].

C3 photosynthesis is the dominant form among plant life which uses the enzyme ribulose bis phosphate carboxylase (Rubisco) to fix CO_2 where the initial product of photosynthesis contains three carbon atoms. Rubisco is part of the Calvin–Benson cycle. Examples of C3 crop species are rice, wheat, potato, soybean, cotton, and chickpea. In C3 photosynthesis, three ATP molecules and two NADPH molecules are used to assimilate one molecule of CO_2 and regenerate the acceptor, RuBP. Zhu et al. [36] provide a calculation of the energy required to fix one carbon atom and compare this to the energy contained within that one-sixth of a mole of glucose. A calculation of the quantum requirement of each ATP and NADPH molecule reveals that 8 mol of photons are needed for the fixation of CO_2 molecule and this represents 1,388 kJ of energy [36] whilst the energy content of this carbon atom within the glucose molecule is 477 kJ.

C3 plants operate the process of photorespiration. This is a consequence of the dual reaction of Rubisco: in addition to the fixation of CO_2 into carbohydrates, Rubisco also reacts with O_2 to produce glycolate. This molecule must be metabolized through the photorespiratory pathway, a process that consumes ATP, reducing power and releases CO_2. The product of this is phosphoglycerate which reenters the Calvin–Benson cycle. The losses caused by photorespiration are significant and are greatly dependent on temperature: the specificity of Rubisco for CO_2 and the solubility of CO_2 relative to O_2 in water declines with increasing temperature. In tropical C3 plants the rate of photorespiratory flux can be significant especially when stomata limit the internal leaf CO_2 concentration. In tropical rice in field temperatures of 35°C under otherwise optimal conditions, saturating leaves with CO_2 resulted in an increase in leaf photosynthesis of over 40% [38].

A subset of plants called C4 plants have evolved a fascinating mechanism which minimizes the oxygenase reaction of Rubisco to often insignificant levels. It does this by restricting Rubisco to a bundle sheath cell, which is oxygen free and non-leaky. Initial fixation of CO_2 takes place in mesophyll cells using an alternative enzyme, phosphoenol pyruvate carboxylase which generates C4 acids. These acids are pumped into the bundle sheath cell where they are de-carboxylated, and the CO_2 released is concentrated around Rubisco in the bundle sheath chloroplasts. This effectively reduces the extent of the oxygenase reaction and the photorespiratory pathway to insignificant levels. It is

thought to have evolved in a climate where high temperatures, low CO_2 levels, and low humidity could all have been strong selective pressures. The C4 mechanism is largely found in warm regions. Measurements show that in many plants the advantage of the C4 mechanism over C3 starts to become particularly significant at growth temperatures above 25°C. C4 requires additional ATP synthesis which increases the quantum requirement of the photosynthetic process to 12 photons per CO_2 molecule, in comparison with 8 for C3 photosynthesis. However, this is more than overcome by the elimination of photorespiration and low activity of Rubisco resulting in a greatly enhanced capacity for carbon assimilation at most light intensities in C4 plants. Other advantages become apparent, for example, the higher efficiency of Rubisco means that lower amounts of this protein are needed. Since Rubisco can make up to 35% of total leaf protein in some C3 plants [20], this increases the photosynthetic nitrogen use efficiency of C4 plants. The CO_2 concentrating mechanism of C4 plants means that stomatal conductance does not need to be high and water loss can be reduced.

A substantial "loss" of biomass is mitochondrial respiration which has often been divided into growth respiration and maintenance respiration in accordance with the associated physiological process. Respiration is unsurprisingly difficult to measure and to define in practice [39]. The relative rates can vary substantially according to growth stage. For example, maintenance respiration increases substantially as a proportion of total carbon flux following ear emergence in barley [35]. During vegetative growth, growth respiration is substantially higher than maintenance respiration. Respiration as a whole can vary from 30% to 60% of total carbon exchange and therefore it has a significant impact on RUE. Studies have shown that it undergoes optimization according to the environment and developmental state: for example, it is well known that the relationship between L and respiration is not linear because this would result in a negative net carbon balance in the lower, shaded regions of the canopy. Attempts have been made to optimize respiratory rates to improve crop productivity but this is a difficult task because of the problems with measurement (especially with roots) on different scales from tissue to whole canopy, and of the separation between maintenance and growth respiration. It is often considered that there is greater scope for the improvement of maintenance respiration because this is more sensitive to prevailing conditions. Recent work suggests that the possibilities should be revisited [39, 40].

At any given moment in time photosynthesis can be limited by light level (e.g., under cloud cover or self-shading), stomatal conductance (this becomes severely limiting under conditions of low soil water), and Rubisco (under saturating irradiance and high stomatal conductance Rubisco limits photosynthetic rate in C3 plants). Interactions between growth condition and genotype will determine the final rate of photosynthesis. Growth under optimal conditions is considered: the imposition of resource limitation on RUE will be discussed in the final section.

Taking into account the minimum essential losses that occur, the percentage of "biomass energy" produced per unit solar radiation (total solar spectrum) for C3 and C4 plants is generally agreed to be 4.6% and 6%, respectively (calculated on a kJ/kJ basis) [36]. The highest recorded values are 2.4% and 3.7% [36] with common measured values being much lower than this, for example, [41]. The large difference between measured and attained photosynthetic efficiency of crop canopies is the cause of some debate and some mechanisms focusing on metabolic constraints are discussed here. It is possible that canopy architecture remains a limitation (discussed above). It is however noted that much of the observed reduction in RUE and photosynthetic efficiency in agricultural systems is caused by growth under suboptimal conditions [36].

The above has considered photosynthetic efficiency in the context of a leaf canopy. However there is current interest in exploiting photosynthesis in non-leaf organs such as the green ear in wheat [42, 43] and the spikelets in rice. Despite the relatively small surface area (compared to LAI) it seems clear that the high exposure to radiation and close proximity to the grain sink may mean that their photosynthetic contribution has been underestimated [42]. In the case of oil seed rape, the photosynthesis in seed pods is the dominant supply due to a diminished leaf area [44]. This is a source of photosynthate that deserves closer investigation.

Metabolic and Regulatory Constraints to RUE

Although photosynthesis underpins growth and yield it has often been considered that it is a feature of primary metabolism which has undergone optimization through natural selection and empirical breeding. This view has partly arisen from the conservative nature of the basic mechanism and composition of the photosynthetic apparatus. However, firstly, it is clear that canopy photosynthesis has undergone improvement via total leaf area and nitrogen per unit leaf area (fertilizer) and, secondly, there is considerable genetic variation in leaf photosynthetic capacity and in the response of leaf photosynthesis to environmental factors and abiotic stress. Nevertheless, the role of any improvement in leaf photosynthesis in crop yield progress has been hard to quantify due to the difficulty of routinely measuring net leaf and canopy carbon gain and of eliminating compensating processes, although some success has been achieved [24, 45, 46]. and photosynthesis has been successfully linked to yield progress in some cases, for example, [24]. There is some recent evidence that leaf photosynthesis can exert an effect when biomass production is the dominant limiting factor [47–49]. Nevertheless leaf photosynthesis is considered to be the dominant factor determining RUE [5] and its improvement is increasingly viewed as an important target [49, 50].

Many of the suggested routes to the leaf CO_2 assimilation rate of crops have focused on Rubisco. Increasing the amount of Rubisco in the leaf is problematic: it is already at extremely high levels and to accumulate more would require an increase in nitrogen fertilizer application. There are indications that Rubisco may be accumulated to excess capacity in some leaves [51]. There may be opportunities to improve the properties of the Rubisco enzyme [52] and there is some natural variation among plants and algae in the properties of Rubisco. For example, forms of Rubisco present in the genus *Limonium* have a higher specificity factor than that in all crop species [53]. However, there is a well-cited inverse relationship between specificity for CO_2 and maximum catalytic activity [54]. It has been suggested that different forms of Rubisco could be assigned different roles within the plant according to environmental condition, tailored, for example, to high light or low light conditions. Other enzymes in the Calvin–Benson cycle have been shown to have promise for improvement such as sedoheptulose-1,7-bisphosphatase [55].

Elimination or reduction in photorespiration has been of interest for a long time even though the seemingly wasteful process has been assigned metabolic and photoprotective roles. The increased growth rates, biomass, and yield of plants grown under high CO_2 where photorespiratory flux is reduced indicates that the process is largely wasteful. Methods for blocking the pathway have proved ineffective [56] probably due to the accumulation of intermediates. A recent and novel approach has avoided this problem by using bacterial enzymes to "shortcut" the pathway [57]. In *Arabidopsis thaliana* plants, this has had the effect of improving biomass production and shows great promise although the precise mechanism of improved growth has not been described yet.

The greatest improvements in yield of crops native to warmer climates would come from the introduction of the C4 mechanism into C3 crop species. In the case of rice, for example, it has been calculated that this is the only way to bring about an increase in biomass production sufficient to meet a 50% improvement in yield by 2050 [1, 58]. Early attempts to introduce elements of the C4 pathway into rice by transformation with C4 genes from maize or other C4 species [59] are considered ambiguous or partially successful at best and introduction of the full "Kranz" anatomy seems to be the most likely way to achieve the required goal. Some natural C4 mechanisms exist in a single cell [60] but it is unclear whether this would provide sufficient rates of assimilation.

The evolution of the C4 syndrome independently on more than 60 occasions in angiosperms would suggest there is no intrinsic reason why the C4 pathway could not be introduced into a major crop such as rice or wheat. A combination of advanced molecular techniques, transformation of key genes and smart screening of germplasm may achieve this [61]. Indeed there is now a funded international consortium of scientists formed to address this task in rice (www.irri.org).

Recent modeling work suggests that photosynthesis may not be optimized in many plant species. For example, Zhu et al. [62] used an evolutionary algorithm to partition nitrogen between enzymes associated with different processes within a plant cell. The combination of enzyme amounts and activities that produced

a higher photosynthetic rate was allowed to proceed to the next generation, and after 1,500 generations it was found that photosynthesis was substantially increased. It seems that an over investment in photorespiratory metabolism and an underinvestment in enzymes of the Calvin–Benson cycle may be critical. It is possible that this represents a lack of adaptation to contemporary higher CO_2 levels.

Photosynthesis is a dynamic process and constantly responds to changing environmental conditions. It is still debated as to whether the responses observed in situ are optimized. For example, when light is absorbed in excess of that required for photosynthesis, a series of regulatory mechanisms are activated which dissipate the excess excitation energy within the thylakoid membrane [63, 64]. This is considered a photoprotective process which reduces the likelihood of photooxidative stress. This process often has no impact on the light-saturated rate of CO_2 assimilation but it does reduce the quantum yield at low irradiance levels. Therefore, following a transfer to low light (caused, e.g., by cloud cover or leaf and solar movement), the slow relaxation of photoprotection causes a potential reduction in the rate of CO_2 assimilation. Given the large variation in irradiance in canopies in space and time, this has long been considered a factor in canopy photosynthesis. Indeed the manipulation of photoprotection has been shown to influence fitness in *A. thaliana* [65]. Recently, photoprotection was modeled in a tall three-dimensional canopy using ray tracing algorithms [66]. The reduction in canopy carbon gain was predicted to be as large as 30% under low temperature conditions. This would seem to indicate that there is room for improvement in terms of optimization of photoprotection.

Acclimation of photosynthetic capacity to environmental conditions such as irradiance can occur over longer timescales such as days and weeks. It has been hypothesized that acclimation conferred an advantage in terms of carbon gain [49]. Recent experiments using *A. thaliana* indicate that over long growth periods under naturally variable light levels this is indeed the case [67].

The question of whether plant and crop responses are appropriate for any switch in environmental conditions has been expanded to include those of growth and storage of carbon. For example, there is evidence that transfer of carbon to storage organs can occur at the expense of allocation to new organs [68]. Cross et al. [69] observed that accessions that allocated less carbon to storage at night had a higher growth rate. This concept has yet to be tested in crop species. Genes that are involved in the regulation are being identified, for example, DELLA proteins are negative growth regulators of central importance which are thought to integrate the effects of various growth-promoting hormones such as gibberellins and have been shown to have an effect on rates of tissue growth [70].

Source–Sink Processes and Partitioning of Assimilates

This section discusses the different assimilate sinks that have either direct or indirect effects on light interception (LI) and RUE in crops. As discussed, radiation capture is a highly dynamic process affected not only by sun angle and fluxes in radiation intensity, but also by gross morphological above ground structures that evolve over a plant's life cycle and include leaf area development, stem dynamics, and the emergence of floral structures. Within crop species, there is considerable interest in genetic effects on morpho-physiological traits that affect light interception and distribution. Genetic effects include early vigor; stem density (m^{-2}) and dynamics; leaf anatomy and geometry; the composition, distribution, and duration of light harvesting and photosynthetic proteins in the canopy; the architecture of floral structures; and the continual interaction of these with crop development. Crop management is an additional factor which will affect radiation interception principally through row spacing, N fertilization, and irrigation, and by controlling biotic stresses that may reduce LI.

Direct Effects of Sinks on RI and RUE

It is axiomatic that all photosynthetic structures begin as carbon sinks while at some point becoming net exporters (i.e., assimilate sources). The exact investment strategy in these photosynthetic structures will determine the RUE of the "photosynthetic canopy" as a whole. In this context, leaf area index (L) is a useful parameter to consider. Typically L values above 3 are considered optimal for maximizing RUE in annual crops. However, theoretical considerations suggest

that the relative distribution of leaf area and light harvesting and photosynthetic proteins among different layers of the canopy can also modulate RUE and are currently targets for genetic improvement [25].

In the context of canopy photosynthesis, two other important sinks with direct impact on RUE are the stems and the reproductive structures. Stems, as well as being covered with a green leaf sheath, provide the skeleton for leaf display and, therefore, to a large extent, determine the height and geometry of the leaf canopy. Stems are highly dynamic and may appear and disappear within the main part of the crop cycle. In small grain cereals, full light interception is typically accelerated through tiller development, a strategy which results in the subsequent shedding of nonproductive tillers after optimum *L* is achieved; the investment of assimilates in tillers apparently being offset by the increased *L* [71]. The strategy also has benefit under favorable years when a larger proportion of tillers achieve reproductive success. Reproduction requires a further investment of assimilates in floral structures, which, though often photosynthetic themselves, may also intercept incident light and, therefore, shade other photosynthetic tissue. Reducing panicle height for this reason has proved beneficial in rice [72]. Genetic modification of tiller dynamics and spike photosynthesis are both promising areas in crop breeding; neither has been systematically addressed yet as outlined above, and both traits interact with LI and potentially with RUE. There is considerable genetic diversity for tillering capacity in small grain cereals and a tiller inhibitor gene (*Tin*) has also been identified in wheat [73]. Considerable morphological diversity is also apparent in the reproductive structures of many crop species. However, those which are photosynthetic show a complex physiology, the measurement of LI and RUE is extremely challenging, and studies to establish genetic diversity are scant [43]. Nonetheless, shading studies in wheat have suggested genetic diversity for the contribution of spike photosynthesis to grain filling under drought, and given that reproductive structures intercept a significant proportion of light in many crops, it may be expedient to incorporate them into models of canopy photosynthesis.

Finally, non-photosynthetic sinks such as roots and structural components of the plant (stem wall, rachis, etc.) may impact on LI and RUE by competing with photosynthetic tissue for assimilates, however, these effects are not well documented [74].

Indirect Effects of Sinks on RI and RUE

There are other non-photosynthetic sinks which may also compete directly with photosynthetic tissue for assimilates but whose indirect effects may be much more significant in terms of overall RUE of the crop. These are (1) the accumulation and remobilization of carbohydrates (such as water-soluble carbohydrates in wheat and starch in rice) to and from stems and (2) the partitioning of assimilates to the reproductive spike.

While it seems clear that stem storage carbohydrates accumulate to provide a buffer against post-anthesis stress conditions when current photo-assimilates may be insufficient for grain filling [75–77], it is unclear what trade-offs may be involved in terms of assimilate partitioning. Given that genetic variation for storage carbohydrates is large and may constitute up to 50% of the stem dry weight shortly after anthesis, it could affect not only competition for assimilates from other sinks (e.g., roots, spike) but also RUE if, for example, the demand for stem storage carbohydrates is great enough to solicit feedback responses of the photosynthetic apparatus. While the latter has not been studied, feedback effects that influence RUE have been shown in response to partitioning of assimilates to the reproductive spikes.

A large body of evidence has shown that the number of reproductive sinks that are set in a crop is the main factor determining yield potential [78, 79]. Indeed the post anthesis sink size in wheat has been associated with RUE [80]. Sink strength associated with grain number is the most likely explanation for the relationships demonstrated between yield and photosynthetic rate, for example, in a historic series of wheat cultivars [24]. Furthermore, in other studies in spring wheat it was shown using both genetic and physiological treatments that RUE responded to increased spike fertility, resulting in increased yield and aboveground biomass [3]. It has been suggested that one way to enhance the sink capacity in wheat is to lengthen the stem elongation phase. The stem elongation phase encompasses the spike growth period and this would therefore result in a heavier spike during this period [3]. Another strategy would be to alter the sinks that

compete with spike index, such as roots, stems, leaf sheaths, and infertile shoots. It must be ensured that any reduction in leaf lamina does not have an effect on LI and RUE. Reducing the allocation of biomass to roots may improve RUE by permitting increased partitioning to spikes (discussed in Reynolds et al. [3]). Some caution is urged since future yields may be dependent on increasing the ability to access soil water and nutrients. However, there is a possibility that the efficiency of uptake of water and nutrients could be improved with no change in root mass, for example, partitioning root length density at greater soil depths [81]. Structural stem carbohydrates (not the reserve carbohydrates discussed above) could be reduced by classical methods such as reducing plant height although wheat plant heights of below 70–90 cm are associated with lower biomass [3, 82]. Such approaches must be offset against increased lodging susceptibility. Other possible targets such as infertile tillers and awns are discussed in Reynolds et al. [3].

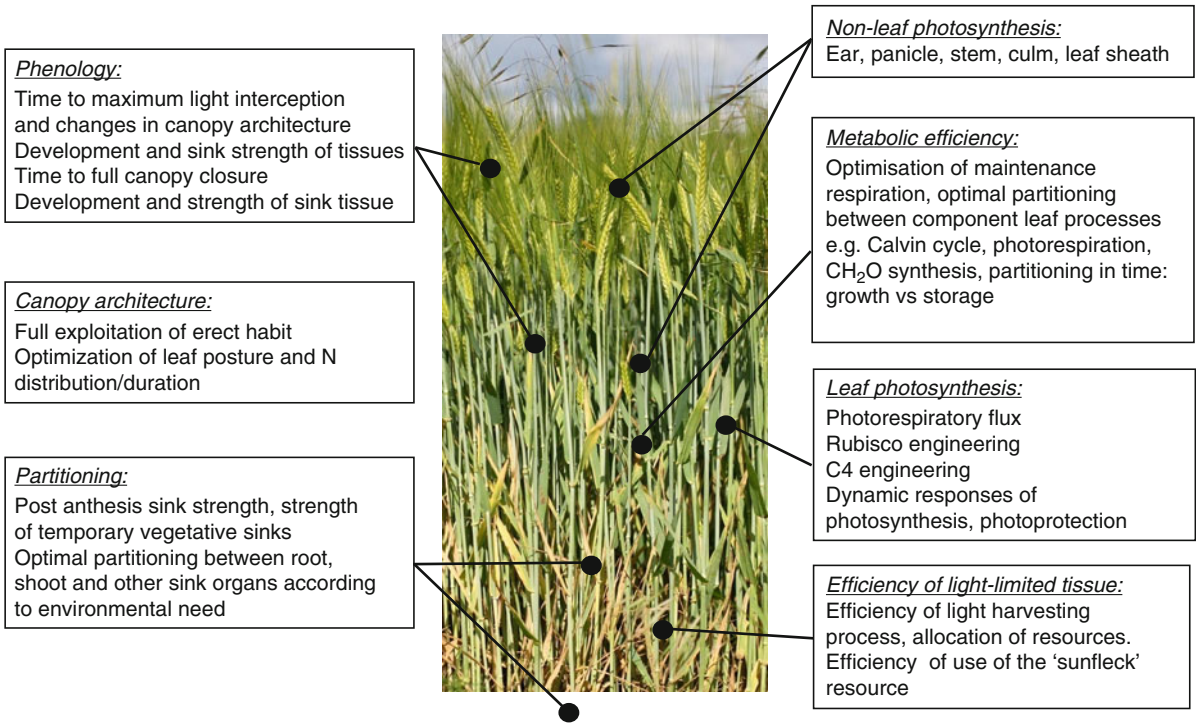
In particular, the maintenance of fertility under unpredictable environments is highlighted: in wheat, kernel set can be very sensitive to a number of environmental conditions such as moisture stress and irradiance. This is part of a set of evidence suggesting that plant signaling is involved in reducing grain number. The signaling (local or long distance within the plant) is a well-established phenomenon: the transport of molecules regulates growth, partitioning, and metabolism and is a fundamental feature of plant biology, likened in some cases to neural networks that sense, quantify, and memorize the environment around them [49, 68, 83]. The most well-known example is that drying soils induce the synthesis of abscisic acid in roots, and transport of this hormone to shoots induces a reduction in stomatal aperture to increase water use efficiency of remaining soil water [84]. It is possible that plant responses to environmental events are preemptive certainly but may also be simply too "conservative" predicting unfavorable conditions and setting seed size accordingly, while a higher yield can be attained by maintaining larger seed number under well-managed conditions. Floral abortion in maize in response to drought appears to be controlled by the up- or downregulation of a few enzymes [85]. In wheat, day length can alter sugar supply to fertile florets leading to cell death [86], and ethylene is a high-temperature signal leading to kernel abortion. Cytokinin appears to regulate the number of spikelets in rice [87]. Therefore, better targeted regulation of grain abortion before the onset of seed filling is a potential target for improvement of RUE and yield potential (Fig. 4).

Theoretical Considerations Related to the Improvement of RUE

The concept of RUE has received some criticism as being one that contains circular reasoning: one cannot have growth without biomass accumulation and vice versa. Additionally it is the product of almost every process in plant canopy growth and development, which makes a mechanistic description quite complex. However, it is established as a unifying concept in crop physiology, for example, [5, 10, 88]. Moreover the understanding of the component parts of the system and their integration is continually improving, and increasingly these parts are not viewed as a "black box," giving more options in the future with regard to RUE improvement.

For a given crop genotype, RUE is largely sensitive to leaf photosynthesis (and therefore nitrogen content) and the proportion of radiation that is diffuse or direct [89]. The latter is a much under-studied area in plant and crop physiology. The stability of RUE has been used to question whether it is possible to improve upon crops with existing C3 or C4 types.

Theoretical figures were outlined above for the maximum efficiency with which photosynthesis can operate and it was concluded that crops operate below this. However, what is the realistic "spare capacity" for RUE improvement in a major crop plant? Reynolds et al. [23] estimate potential productivity for the entire growth cycle of irrigated wheat in a specific and well-characterized location, NW Mexico. Radiation fluxes were measured throughout the season. Due to the time it takes for canopy establishment, large losses can occur before canopy closure, and this was taken into account using a model that used measured intercepted radiation and the time before canopy closure. A value of 1,748 MJ m^{-2} was obtained for radiation absorbed by photosynthetically active tissue. Estimates of field quantum requirement can vary between 10 and 30 mol quanta mol^{-1} CO_2, taking



Crop Radiation Capture and Use Efficiency. Figure 4
Summary of the potential target areas which would result in an improvement of crop radiation use efficiency

into account photorespiration and photochemical inefficiency. For a wheat crop, best estimates would seem to be in the range 15–24 mol quanta mol^{-1} CO_2 [90] which results in a range of RUE between 1.5 and 2.6 g carbohydrate MJ^{-1}. The calculated value of biomass ranges from 2,620 to 4,545 g m^{-2} whilst the measured value for wheat in this environment is up to 2,100 g m^{-2}. This suggests that improvements in field RUE are conceivable.

Sinclair and Horie [88] claimed that the observed stability of RUE arose from consistently high light-saturated leaf photosynthetic capacity (Amax). They plotted the leaf photosynthetic capacity against RUE for C3, C4, and leguminous crops types. The saturation of this response indicated that further modest increase in leaf photosynthetic capacity will only have a limited impact on the RUE, hence the stability of the RUE response [5]. However under conditions of restricted nitrogen, water, or under stress much lower values of photosynthetic capacity caused a larger reduction in RUE. This has been used as an argument that seeking modest improvements in leaf photosynthetic capacity may not be worthwhile. As pointed out here it is possible to trace improvements in biomass production that are linked to Amax. Biomass, yield, and Amax were associated in irrigated wheat cultivars in warm conditions [23] and in temperate conditions [24]. In the latter case, higher Amax may reflect a feedback response caused by greater partitioning to reproductive structures – the differences in Amax were greatest during grain filling and not well associated with above-ground biomass.

There has been a lot of attention paid to the use of Amax in the selection of varieties with a higher photosynthetic potential, largely because it is convenient to measure and shows genetic variability. However, it should be clear from this entry that Amax will mostly be expressed at the top of the canopy (most leaves will not be at light saturation) and may not be a good proxy for canopy photosynthetic rate especially when integrated over long time periods. Care should be taken when extrapolating from spot measurements to whole

canopy photosynthesis. It is not surprising then that attempts to improve yield by simply selecting for Amax have had largely unsuccessful results.

Perhaps more importantly, many of the improvements that show great promise are not solely associated with capacity at light saturation but rather to the dynamic responses of photosynthesis over time and therefore to total canopy carbon gain. These processes are much harder to measure experimentally although advances in techniques such as continual chlorophyll fluorescence monitoring may provide breakthroughs. As the knowledge of photosynthetic regulation improves, these should be installed into agricultural photosynthesis. It would seem necessary to tailor photosynthetic responses to improvements in canopy architecture.

Sources of Variation in Agricultural RUE

In any comparative analysis of RUE, it is necessary to ensure that the methods of analysis are directly comparable and provide an accurate estimate that do not contain any of the potential errors in measurement outlined above. Additionally, consideration must be taken when comparing different environments because varieties may not be well adapted to their locality and therefore able to "express" their maximum RUE. Other reviews, for example, Sinclair and Muchow [5] provide a comprehensive survey of the literature for many key crop species. A summary is provided here of the species and of the environmental factors affecting RUE in agriculture, and available information for a few key species has been provided.

It is in fact not common to find studies that specifically link RUE with yield progress; however, it has been shown, for example, that yield progress in wheat associates with both harvest index and total biomass production [74]. Some evidence also suggests that older wheat cultivars had a lower RUE and this was associated with a lower post-anthesis sink capacity [4, 80].

Species Variation in RUE

As pointed out by many workers, there is no "constant" for RUE and there is considerable variation between species. It is important to pay attention to the developmental stage of each crop in question and maximum attained RUE is referred to here. The highest recorded values for season-long RUE across C4 species are 3.4 g MJ^{-1} and those of C3 species 2.8 g MJ^{-1} [5, 35]. Mitchell et al. [13] reported that average values for RUE in rice, wheat, maize, and soybean in the vegetative stage were 2.2, 2.7, 3.3, and 1.9 g MJ^{-1}, respectively.

Therefore the tendency for a distinction between C3 and C4 crops seems to be consistent. However, care must be taken. For example, sorghum, a C4 species, possesses a relatively low RUE and this has been attributed to low leaf N content. Conversely, sugarcane, also a C4 species, seems to have an exceptionally high RUE. Potato, a C3 species, has shown values for RUE higher than all other C3 crops and even some C4 species (sorghum). It must once again be emphasized that the energy content of dry matter should be taken into account. It has been suggested that the high values for sugarcane and potato reported are due to the exceptionally low energy content of the products (sucrose and starch). It is often claimed that sugarcane has the highest radiation use efficiency among the plant kingdom, although there are relatively few studies.

A few studies have indicated the relatively low RUE of rice among C3 crops [13, 91], although there are surprisingly few studies available for this species and recent suggestions that RUE may not be currently closely linked to high yield potential [92].

Leguminous species such as soya bean have lower RUEs and a high percentage of PAR utilization. This is due to the higher lipid and protein content (see earlier section). However, even when the vegetative stage alone is considered, soybean has shown lower RUE values than wheat, rice, and maize [13, 93]. This has also been observed in other grain legumes [5]. RUE of legumes may be an important future target. For detailed information on RUE values for each species, the reader is referred to detailed reviews such as [5].

In recent years, there has been much interest in "energy crops." These are crops that are grown for the sole purpose of fuel production or combustion for energy generation. This has been stimulated by concerns over emissions of greenhouse gasses and the growth of crops represents a CO_2-neutral strategy. Drawbacks include the clear competition with food supply and the possible threat to natural vegetation. Nevertheless it has been shown that certain species have the potential to be extremely productive in this regard, especially C4 grasses such as *Miscanthus* [94]. *Miscanthus* has the

highest annual primary production of any crop species producing 50% more biomass than corn due to a high leaf area and longer duration, although the energy conversion rate is about the same [95]. Other examples of energy crops under study are willow, poplar, and oil crops such as *Jatropha*, although radiation use efficiency of this category of crop species is not well studied. These crops may find an application on land unsuitable for food crops and low input environments.

Developmental Stage

The stage of crop development has a clear effect on the RUE attained. Firstly the photosynthetic potential may be dependent on growth stage. Secondly the appearance and disappearance of vegetative and reproductive sinks can influence photosynthesis via the presence of feedback signaling mechanisms and carbohydrate accumulation; this has been discussed in detail above. In many species, a lower RUE was observed during earlier crop establishment stages in comparison with later stages and this was attributed to a lowered photosynthetic capacity [5]. The effect of senescence during the post flowering has the effect of reducing photosynthesis, and therefore RUE is usually observed to be higher during the vegetative stages than post flowering. Recent studies have indicated that post-anthesis RUE in wheat is strongly linked to sink size: Older cultivars with smaller sinks had significantly reduced RUE during this phase [4].

There is an indication in some studies that in rice this post-anthesis effect is not as pronounced [13]. In a recent study, Takai et al. [96] suggested that a maintenance of high growth rate and RUE in the late reproductive phase of rice is a key to higher grain yield potential in this species and may be linked to the short tropical life cycle and requirement for rapid photosynthesis during grain filling.

Environmental Factors

There are many practical difficulties involved with linking leaf-level photosynthesis with canopy-level RUE, even though it is technically straightforward to measure leaf photosynthesis. However, increasing the photosynthetic rate per unit leaf area of leaves has direct relationship with increasing RUE. It is frequently observed that the response between light-saturated photosynthetic *capacity* and RUE is curvilinear rather than linear. However, as discussed above it is critical to consider photosynthetic efficiency at all light levels. As a generalization it is fair to say that any factor, biotic or abiotic, that reduces the photosynthetic potential of a canopy is also likely to reduce the RUE. This applies to many of the common factors that reduce growing conditions below optimum such as water availability, nutrients, and extreme temperature.

It is not surprising that nitrogen has a significant impact upon RUE due to the close and well-established relationship with leaf photosynthesis. There is a strong nitrogen-dependent effect on leaf area and consequently light capture in plants, therefore, nitrogen content per unit leaf mass or per unit leaf area is considered. This has been the subject of a number of studies in several species: Usually a curvilinear response with leaf nitrogen is obtained, with RUE increasing up to a saturating value beyond which response is limited such as sunflower (average canopy leaf nitrogen [97]), soybean (specific leaf nitrogen [98]), and maize and sorghum (leaf nitrogen per unit leaf area [99]). However, this relationship is not always observed (see [5]). In examining species differences, the plant matter energy content and the nitrogen–photosynthesis relationship is important. For example, C4 plants have a higher potential photosynthetic nitrogen use efficiency [88]. Naturally site-variation in soil nitrogen is common and potentially a large source of variation in RUE.

Soil water and atmospheric humidity have the potential to reduce photosynthetic rate and therefore RUE. In experiments that imposed soil water deficit, reductions in RUE have been observed in some cases [100] but not others [100]. It has been suggested that the variability in response is a result of variation in the extent of the soil water deficit: When this was measured in a quantitative manner, it could be established that RUE would decline when the level of extractable soil water declined below a certain limit (in this case 30%) [101].

An increase in vapor pressure deficit has been associated with lower RUE in some studies. It is noted that the impact in RUE was greater than that would have been predicted from leaf photosynthesis alone and it was suggested that environmental factors associated with vapor pressure deficit are involved.

Other studies indicated only a small impact of vapor pressure deficit on RUE [5, 102].

Temperature has been associated with RUE when it has an influence on leaf CO_2 assimilation rate and this has also been shown to be related to the physiological effects of nighttime temperature [5].

Future Directions

Due to the effects of climate change caused by anthropogenic emissions, an altered environment for crop growth is likely to be required [103]. Some of these changes can be predicted with a high probability, for example, CO_2 is rising and is likely to continue at a similar rate to the current one with a slowing of rate according to internationally agreed emission cuts. Since 1750, atmospheric CO_2 has risen by around 100 ppm and continues to rise at the rate of around 2 ppm per year. By the end of the twenty-first century, temperature is predicted to rise by between 0.5°C and 4°C in response to levels of the greenhouse gasses CO_2, methane (CH_4), and nitrous oxide (N_2O). Agriculture itself is a major contributor to greenhouses gasses. Studies have already indicated that climate change is having an effect on productivity of crop systems [103–105].

These changes must be considered according to geographical region and in the context of deleterious effects such as a likely reduction in water availability, a rise in pollutants such as ozone, and the increasing frequency of extreme damaging events such as flooding, drought, and storms. They must also be weighed against possible beneficial effects such as higher photosynthetic rates induced by elevated CO_2 and a longer growing season at higher latitudes leading to higher biomass and higher yield. Accordingly, a reduction in yield in the tropics and a rise in temperate regions may be experienced. The effect of most of these factors on RUE has been discussed above and a detailed discussion of the wider impact of climate change on agriculture is beyond the scope of this article. Clearly suboptimal growing conditions will potentially reduce RUE and yield. However, it is important to ensure that the crops are adapted appropriately to future climates.

Experiments using completely enclosed or open topped chambers in the field in which CO_2 levels are elevated above ambient have shown significant increases in growth and yield. The reasons are clear and related to the suppression of photorespiration and consequently an increase in RUE. Additional beneficial effects related to a lowered stomatal conductance [25, 106]. These data have been used in models that predict an increase in future crop production in many northern regions. Experiments with more realistic field conditions (Free Air CO_2 Enrichment, FACE) have also demonstrated an increase crop yields but these were lower than those predicted by chamber experiments. This is lowered further when additional effects such as ozone and disease are accounted for and the net result may be negative [107, 108]. This suggests that the predicted beneficial effects of an increased atmospheric CO_2 on plant growth have been overestimated. However, as pointed out by Long et al. [107] there is still a paucity of data for CO_2 enrichment effects in many agroecosystems so a level of uncertainty remains.

There is also a need for further work into the adaptation of crops to high CO_2 levels: Theoretical work by Zhu et al. [62] using evolutionary algorithms to predict optimal levels of CO_2 for photosynthesis have shown that the relative levels of enzymes involved in primary metabolism are not optimized for carbon assimilation. It is possible that there has been insufficient adaptation during crop improvement since the start of the industrial revolution, raising the intriguing possibility that plants should be bred or engineered for adaptation to higher CO_2 levels of the future in order to maximize yield and RUE. Presumably this also applies to other environmental changes.

Therefore it is clear that there is a demand for greater crop yield per unit area of land with fewer resources available. This step change in yield will arise from an integrated set of targets, most of which have been outlined in this article. Assuming that management of the crop is optimal and improvement of partitioning and sink capacity reach their limits, it will be increasingly necessary to underpin any substantial yield improvements with increased biomass production and this means that RUE will need to increase.

RUE represents a complex system and as pointed out by Reynolds et al. [23], it is a less frequent outcome of the genetic recombination events required for breeding, in comparison to manipulation of sink size. It will be necessary to focus on methods for improving RUE

of crops and to introduce this into breeding techniques. In addition, there have been significant advances in the plant molecular and genomic sciences in the past 2 decades and these should be exploited to raise RUE. The discovery of new genes and combinations of genes requires routine screening of large amounts of leaf material for photosynthetic potential. It is difficult to do this manually using traditional gas exchange techniques; however, there are a number of techniques developing rapidly that could be exploited to act as surrogates or proxies of CO_2 assimilation rate. For example, chlorophyll fluorescence is a rapid method that can be used to image leaf material for photosynthetic efficiency extremely rapidly. It is routine to image chlorophyll fluorescence; however, it is technically difficult to do this on a three-dimensional structure such as a canopy. Another method is canopy temperature depression which measures the difference between leaf and air temperature caused by evaporation. It therefore provides an indication of stomatal conductance and has been correlated with plant performance [109]. Considerable recent interest has been placed on the imaging plant growth leading to the emergence of the field of "plant phenomics." Advances in methods for rapid and high-resolution screening of plant material for photosynthetic performance can be anticipated. Additionally, the complexity of RUE means that a mathematical understanding of the integration and functioning of component processes will be critical. Given the difficulty of direct measurement, modeling of canopy photosynthesis is already used routinely and will be needed for the continued testing of new traits.

The Role in Wheat and Rice Research: Current Research at CIMMYT and IRRI

The challenge of improving photosynthesis and therefore RUE in crops during both optimal and suboptimal conditions has been recognized by the research organizations that were central to the last great agricultural revolution (the green revolution), the International Rice Research Institute (IRRI) in the Philippines and the International Maize and Wheat Improvement Centre (CIMMYT) in Mexico. IRRI has a number of research programs that are centered toward the sustainable improvement of rice yield in changing environments. Perhaps the most relevant here is the C4 rice consortium whose goal is a step change in rice photosynthesis through the introduction of the C4 pathway of photosynthesis (http://beta.irri.org/projects15/en/c4rice). The impact this would have on yield and resource use efficiency of the poorer rice growing regions of the world should not be underestimated.

Given the urgency of the current food crisis coupled with the likely negative impact of climate change on productivity, especially in less developed countries [110], both IRRI and CIMMYT have strategic initiatives to raise the yield potential of rice and wheat, respectively, by around 50%. The IRRI C4 rice initiative is already underway as mentioned. CIMMYT is currently facilitating a Wheat Yield Consortium (WYC) among a group of leading scientists with the view to raising the genetic yield threshold of wheat. A central issue is that the fundamental bottleneck to raising productivity, namely, photosynthetic capacity, has hardly changed since wheat breeding started. Nonetheless, basic research in photosynthesis suggests that substantial improvements in yield are theoretically possible. While increasing photosynthetic potential will require considerable research focused at cellular and subcellular processes (such as the genetic modification of Rubisco and its regulation), intervention at this level must go hand in hand with modification of structural and reproductive aspects of growth, since these will determine the net agronomic benefit of increased RUE .For example, even at current levels of yield potential, a significant portion of yield is lost to lodging damage each year so improved structural integrity will be prerequisite to realizing genetic gains in RUE and biomass. The other major factor determining yield potential is adaptation of the reproductive processes which affect harvest index [3, 111] and whose physiological and genetic basis is relatively poorly understood [112]. The aim of the WYC is to develop or identify sources and genes for the combination of traits necessary to realize improved expression of wheat yield potential and combine them through strategic crossing using the most expedient combination of conventional, physiological, and molecular breeding approaches; the consortium approach is expected to realize impacts in farmers fields in a shorter time frame than if bottlenecks to yield potential are identified successively and investigated in relative isolation.

Bibliography

Primary Literature

1. Sheehy JE, Mitchell PL, Hardy B (2007) Charting pathways to C4 rice. International Rice Research Institute, Los Banos
2. Cassman K (1994) Breaking the yield barrier. In: Proceedings of a workshop on rice yield potential in favourable environments, International Rice Research Institute, Los Banos
3. Reynolds M, Foulkes MJ, Slafer GA, Berry P, Parry MAJ, Snape JW, Angus WJ (2009) Raising yield potential in wheat. J Exp Bot 60:1899–1918
4. Acreche MM, Briceno-Felix G, Sanchez JAM, Slafer GA (2009) Radiation interception and use efficiency as affected by breeding in Mediterranean wheat. Field Crops Res 110:91–97
5. Sinclair TR, Muchow RC (1999) Radiation use efficiency. Adv Agron 65:215–265
6. Watson DJ (1952) The physiological basis of variation in yield. In: Norman AG (ed) Advances in agronomy. Academic, New York
7. DeWit CT (1959) Potential photosynthesis of crop surfaces. Neth J Agric Sci 7:141–149
8. Loomis RS, Williams WA (1963) Maximum crop productivity: an estimate. Crop Sci 3:67–72
9. Hirose T (2005) Development of the Monsi-Saeki theory on canopy structure and function. Ann Bot 95:483–494
10. Monteith JL (1977) Climate and efficiency of crop production in Britain. Philos Trans R Soc Lond B Biol Sci 281:277–294
11. Gallagher JN, Biscoe PV (1978) Radiation absorption, growth and yield of cereals. J Agric Sci 91:47–60
12. Evans LT (1993) Crop evolution, adaptation and yield. Cambridge University Press, Cambridge
13. Mitchell PL, Sheehy JE, Woodward FI (1998) Potential yields and the efficiency of radiation use in rice. In: IRRI discussion paper series No. 32, International Rice Research Institute, Manila
14. Garcia R, Kanemasu ET, Blad BL, Bauer A, Hatfield JL, Major DJ, Reginato RJ, Hubbard KG (1988) Interception and use efficiency of light in winter-wheat under different nitrogen regimes. Agric For Meteorol 44:175–186
15. Hanan NP, Prince SD, Begue A (1995) Estimation of absorbed photosynthetically active radiation and vegetation net production efficiency using satellite data. Agric For Meteorol 76:259–276
16. Marshall B, Willey RW (1983) Radiation interception and growth in an intercrop of pearl-millet groundnut. Field Crops Res 7:141–160
17. Penningdevries FWT, Brunstin A, Vanlaar HH (1974) Products, requirements and efficiency of biosynthesis - a quantitative approach. J Theor Biol 45:339–377
18. Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. Cell 127:1309–1321
19. Duvick DN (2005) Genetic progress in yield of united states maize (*Zea mays* L.). Maydica 50:193–202
20. Murchie EH, Hubbart S, Chen YZ, Peng SB, Horton P (2002) Acclimation of rice photosynthesis to irradiance under field conditions. Plant Physiol 130:1999–2010
21. Sinclair TR, Sheehy JE (1999) Erect leaves and photosynthesis in rice. Science 283:1456–1457
22. Duncan WG (1971) Leaf angles, leaf area, and canopy photosynthesis. Crop Sci 11:482–485
23. Reynolds MP, van Ginkel M, Ribaut JM (2000) Avenues for genetic modification of radiation use efficiency in wheat. J Exp Bot 51:459–473
24. Fischer RA, Rees D, Sayre KD, Lu ZM, Condon AG, Saavedra AL (1998) Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. Crop Sci 38:1467–1475
25. Long SP, Zhu XG, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? Plant Cell Environ 29:315–330
26. Murchie EH, Horton P (1997) Acclimation of photosynthesis to irradiance and spectral quality in British plant species: Chlorophyll content, photosynthetic capacity and habitat preference. Plant Cell Environ 20:438–448
27. Murchie EH, Yang JC, Hubbart S, Horton P, Peng SB (2002) Are there associations between grain-filling rate and photosynthesis in the flag leaves of field-grown rice? J Exp Bot 53:2217–2224
28. Ackerly DD (1992) Light, leaf age, and leaf nitrogen concentration in a tropical vine. Oecologia 89:596–600
29. Morinaka Y, Sakamoto T, Inukai Y, Agetsuma M, Kitano H, Ashikari M, Matsuoka M (2006) Morphological alteration caused by brassinosteroid insensitivity increases the biomass and grain production of rice. Plant Physiol 141:924–931
30. Boccalandro HE, Ploschuk EL, Yanovsky MJ, Sanchez RA, Gatz C, Casal JJ (2003) Increased phytochrome B alleviates density effects on tuber yield of field potato crops. Plant Physiol 133:1539–1546
31. Fleming AJ (2005) The control of leaf development. New Phytol 166:9–20
32. Ong CK, Monteith JL (1992) Canopy establishment: light capture and loss by crop canopies. In: Baker NR, Thomas H (eds) Crop photosynthesis: spatial and temporal determinants. Elsevier, Amsterdam, pp 1–9
33. Watanabe T, Hanan JS, Room PM, Hasegawa T, Nakagawa H, Takahashi W (2005) Rice morphogenesis and plant architecture: Measurement, specification and the reconstruction of structural development by 3D architectural modelling. Ann Bot 95:1131–1143
34. Pyke KA, Leech RM (1987) The control of chloroplast number in wheat mesophyll-cells. Planta 170:416–420
35. Hay R, Porter J (2006) The physiology of crop yield. Blackwell, Oxford
36. Zhu X-G, Long SP, Ort DR (2008) What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? Curr Opin Biotechnol 19:153–159
37. Nishio JN (2000) Why are higher plants green? Evolution of the higher plant photosynthetic pigment complement. Plant Cell Environ 23:539–548
38. Murchie EH, Chen YZ, Hubbart S, Peng SB, Horton P (1999) Interactions between senescence and leaf orientation determine in situ patterns of photosynthesis and photoinhibition in field-grown rice. Plant Physiol 119:553–563

39. Amthor JS (2000) The McCree-de Wit-Penning de Vries-Thornley respiration paradigms: 30 years later. Ann Bot 86:1–20
40. Nunes-Nesi A, Carrari F, Lytovchenko A, Smith AMO, Ehlers Loureiro M, Ratcliffe RG, Sweetlove LJ, Fernie AR (2005) Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol 137:611–622
41. Beadle CL, Long SP (1985) Photosynthesis: is it limiting to biomass production. Biomass 8:119–168
42. Araus JL, Brown HR, Febrero A, Bort J, Serret MD (1993) Ear photosynthesis, carbon isotope discrimination and the contribution of respiratory CO_2 to differences in grain mass in durum-wheat. Plant Cell Environ 16:383–392
43. Tambussi EA, Bort J, Araus JL (2007) Water use efficiency in C-3 cereals under Mediterranean conditions: a review of physiological aspects. Ann Appl Biol 150:307–321
44. Diepenbrock W (2000) Yield analysis of winter oilseed rape (*Brassica napus* L.): a review. Field Crops Res 67:35–49
45. Watanabe N, Evans JR, Chow WS (1994) Changes in the photosynthetic properties of Australian wheat cultivars over the last century. Aust J Plant Physiol 21:169–183
46. Gutierrez-Rodriguez M, Reynolds MP, Larque-Saavedra A (2000) Photosynthesis of wheat in a warm, irrigated environment - II. Traits associated with genetic gains in yield. Field Crops Res 66:51–62
47. Peng S, Laza RC, Visperas RM, Sanico AL, Cassman KG, Khush GS (2000) Grain yield of rice cultivars and lines developed in the Philippines since 1966. Crop Sci 40:307–314
48. Hubbart S, Peng S, Horton P, Chen Y, Murchie EH (2007) Trends in leaf photosynthesis in historical rice varieties developed in the Philippines since 1966. J Exp Bot 58:3429–3438
49. Murchie EH, Pinto M, Horton P (2009) Agriculture and the new challenges for photosynthesis research. New Phytol 181: 532–552
50. Horton P (2000) Prospects for crop improvement through the genetic manipulation of photosynthesis: morphological and biochemical aspects of light capture. J Exp Bot 51:475–485
51. Quick WP, Fichtner K, Schulze ED, Wendler R, Leegood RC, Mooney H, Rodermel SR, Bogorad L, Stitt M (1992) Decreased ribulose-1, 5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with antisense rbcS. 4. Impact on photosynthesis in conditions of altered nitrogen supply. Planta 188:522–531
52. Parry MAJ, Andralojc PJ, Mitchell RAC, Madgwick PJ, Keys AJ (2003) Manipulation of Rubisco: the amount, activity, function and regulation. J Exp Bot 54:1321–1333
53. Galmes J, Flexas J, Keys AJ, Cifre J, Mitchell RAC, Madgwick PJ, Haslam RP, Medrano H, Parry MAJ (2005) Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. Plant Cell Environ 28:571–579
54. Zhu G, Spreitzer RJ (1996) Directed mutagenesis of chloroplast ribulose-1, 5-bisphosphate carboxylase/oxygenase. Loop 6 substitutions complement for structural stability but decrease catalytic efficiency. J Biol Chem 271:18494–18498
55. Raines CA (2006) Transgenic approaches to manipulate the environmental responses of the C-3 carbon fixation cycle. Plant Cell Environ 29:331–339
56. Medrano H, Keys AJ, Lawlor DW, Parry MAJ, Azconbieto J, Delgado E (1995) Improving plant-production by selection for survival at low CO_2 concentrations. J Exp Bot 46:1389–1396
57. Kebeish R, Niessen M, Thiruveedhi K, Bari R, Hirsch HJ, Rosenkranz R, Stabler N, Schonfeld B, Kreuzaler F, Peterhansel C (2007) Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in *Arabidopsis thaliana*. Nat Biotechnol 25:593–599
58. Hibberd JM, Sheehy JE, Langdale JA (2008) Using C-4 photosynthesis to increase the yield of rice - rationale and feasibility. Curr Opin Plant Biol 11:228–231
59. Matsuoka M, Furbank RT, Fukayama H, Miyao M (2001) Molecular engineering of C4 photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 52:297–314
60. Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C4 plant photosynthesis. Nature 414:543–546
61. Sage R, Sage T (2007) Learning from nature to develop strategies for the directed evolution of C4 rice. In: Sheehy JE, Mitchell PL, Hardy B (eds) Charting new pathways to C4 rice. International Rice Research Institute, Los Banos
62. Zhu X-G, de Sturler E, Long SP (2007) Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. Plant Physiol 145:513–526
63. Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol 47:655–684
64. Ruban AV, Berera R, Ilioaia C, van Stokkum IHM, Kennis JTM, Pascal AA, van Amerongen H, Robert B, Horton P, van Grondelle R (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. Nature 450:575–U522
65. Frenkel M, Bellafiore S, Rochaix JD, Jansson S (2007) Hierarchy amongst photosynthetic acclimation responses for plant fitness. Physiol Plant 129:455–459
66. Zhu XG, Ort DR, Whitmarsh J, Long SP (2004) The slow reversibility of photosystem II thermal energy dissipation on transfer from high to low light may cause large losses in carbon gain by crop canopies: a theoretical analysis. J Exp Bot 55:1167–1175
67. Athanasiou K, Dyson BC, Webster RE, Johnson GN (2010) Dynamic acclimation of photosynthesis increases plant fitness in changing environments. Plant Physiol 152:366–373
68. Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell Environ 30:1126–1149
69. Cross JM, von Korff M, Altmann T, Bartzetko L, Sulpice R, Gibon Y, Palacios N, Stitt M (2006) Variation of enzyme activities and metabolite levels in 24 arabidopsis accessions growing in carbon-limited conditions. Plant Physiol 142:1574–1588
70. Achard P, Renou JP, Berthome R, Harberd NP, Genschik P (2008) Plant DELLAs restrain growth and promote survival of

adversity by reducing the levels of reactive oxygen species. Curr Biol 18:656–660
71. Berry PM, Spink JH, Foulkes MJ, Wade A (2003) Quantifying the contributions and losses of dry matter from non-surviving shoots in four cultivars of winter wheat. Field Crops Res 80:111–121
72. Setter TL, Conocono EA, Egdane JA, Kropff MJ (1995) Possibility of increasing yield potential of rice by reducing panicle height in the canopy. 1. Effects of panicles on light interception and canopy photosynthesis. Aust J Plant Physiol 22:441–451
73. Spielmeyer W, Richards RA (2004) Comparative mapping of wheat chromosome 1AS which contains the tiller inhibition gene (tin) with rice chromosome 5S. Theor Appl Genet 109:1303–1310
74. Shearman VJ, Sylvester-Bradley R, Scott RK, Foulkes MJ (2005) Physiological processes associated with wheat yield progress in the UK. Crop Sci 45:175–185
75. Blum A (1998) Improving wheat grain filling under stress by stem reserve mobilisation (Reprinted from Wheat: Prospects for global improvement, 1998). Euphytica 100:77–83
76. Foulkes MJ, Sylvester-Bradley R, Weightman R, Snape JW (2007) Identifying physiological traits associated with improved drought resistance in winter wheat. Field Crops Res 103:11–24
77. Yang J, Zhang J, Liu L, Wang Z, Zhu Q (2002) Carbon remobilization and grain filling in Japonica/Indica hybrid rice subjected to postanthesis water deficits. Agron J 94:102–109
78. Miralles DJ, Slafer GA (2007) Sink limitations to yield in wheat: how could it be reduced? J Agric Sci 145:139–149
79. Slafer GA, Calderini DF, Miralles DJ, Dreccer MF (1994) Preanthesis shading effects on the number of grains of 3 bread wheat cultivars of different potential number of grains. Field Crops Res 36:31–39
80. Acreche MM, Slafer GA (2009) Grain weight, radiation interception and use efficiency as affected by sink-strength in Mediterranean wheats released from 1940 to 2005. Field Crops Res 110:98–105
81. Reynolds M, Dreccer F, Trethowan R (2007) Drought-adaptive traits derived from wheat wild relatives and landraces. J Exp Bot 58:177–186
82. Miralles DJ, Slafer GA (1995) Yield, biomass and yield components in dwarf, semi-dwarf and tall isogenic lines of spring wheat under recommended and late sowing dates. Plant Breed 114:392–396
83. Brenner ED, Stahlberg R, Mancuso S, Vivanco J, Baluska F, Van Volkenburgh E (2006) Plant neurobiology: an integrated view of plant signaling. Trends Plant Sci 11:413–419
84. Davies WJ, Wilkinson S, Loveys B (2002) Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. New Phytol 153:449–460
85. Boyer JS, McLaughlin JE (2007) Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. J Exp Bot 58:267–277
86. Ghiglione HO, Gonzalez FG, Serrago R, Maldonado SB, Chilcott C, Cura JA, Miralles DJ, Zhu T, Casal JJ (2008) Autophagy regulated by day length determines the number of fertile florets in wheat. Plant J 55:1010–1024
87. Ashikari M, Sakakibara H, Lin SY, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. Science 309:741–745
88. Sinclair TR, Horie T (1989) Leaf nitrogen, photosynthesis, and crop radiation use efficiency - a review. Crop Sci 29:90–98
89. Hammer GL, Wright GC (1994) A theoretical-analysis of nitrogen and radiation effects on radiation use efficiency in peanut. Aust J Agric Res 45:575–589
90. Loomis RS, Amthor JS (1996) LImits to yield revisited. In: Reynolds M, Rajaram S, McNab A (eds) Increasing yield potential in wheat: breaking the barriers. CIMMYT, Mexico, pp 76–89
91. Inthapan P, Fukai S (1988) Growth and yield of rice cultivars under sprinkler irrigation in southeastern Queensland. 2. Comparison with maize and grain-sorghum under wet and dry conditions. Aust J Exp Agric 28:243–248
92. Zhang YB, Tang QY, Zou YB, Li DQ, Qin JQ, Yang SH, Chen LJ, Xia B, Peng SB (2009) Yield potential and radiation use efficiency of "super" hybrid rice grown under subtropical conditions. Field Crops Res 114:91–98
93. Rochette P, Desjardins RL, Pattey E, Lessard R (1995) Crop net carbon-dioxide exchange-rate and radiation use efficiency in soybean. Agron J 87:22–28
94. Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of *Miscanthus*. Glob Change Biol 14:2000–2014
95. Dohleman FG, Heaton EA, Leakey ADB, Long SP (2009) Does greater leaf-level photosynthesis explain the larger solar energy conversion efficiency of *Miscanthus* relative to switchgrass? Plant Cell Environ 32:1525–1537
96. Takai T, Fukuta Y, Shiraiwa T, Horie T (2005) Time-related mapping of quantitative trait loci controlling grain-filling in rice (*Oryza sativa* L.). J Exp Bot 56:2107–2118
97. Hall AJ, Connor DJ, Sadras VO (1995) Radiation-use efficiency of sunflower crops - effects of specific leaf nitrogen and ontogeny. Field Crops Res 41:65–77
98. Sinclair TR, Shiraiwa T (1993) soybean radiation-use efficiency as influenced by nonuniform specific leaf nitrogen distribution and diffuse-radiation. Crop Sci 33:808–812
99. Muchow RC, Sinclair TR (1994) Nitrogen response of leaf photosynthesis and canopy radiation use efficiency in field-grown maize and sorghum. Crop Sci 34:721–727
100. Muchow RC (1989) Comparative productivity of maize, sorghum and pearl-millet in a semi-arid tropical environment. 2. Effect of water deficits. Field Crops Res 20:207–219
101. Singh P, Rama YV (1989) Influence of water deficit on transpiration and radiation use efficiency of chickpea (*Cicer-arietinum*-l). Agric For Meteorol 48:317–330
102. Sinclair TR, Muchow RC (1999) Occam's Razor, radiation-use efficiency, and vapor pressure deficit. Field Crops Res 62: 239–243
103. IPCC (2007) Summary for policymakers. In: Climate change 2007: the physical science basis. A report of working group

I of the 4th assessment report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge/New York
104. Parry M, Rosenzweig C, Livermore M (2005) Climate change, and risk global food supply of hunger. Philos Trans R Soc B Biol Sci 360:2125–2138
105. Peng SB, Huang JL, Sheehy JE, Laza RC, Visperas RM, Zhong XH, Centeno GS, Khush GS, Cassman KG (2004) Rice yields decline with higher night temperature from global warming. Proc Natl Acad Sci USA 101:9971–9975
106. Drake BG, GonzalezMeler MA, Long SP (1997) More efficient plants: A consequence of rising atmospheric CO2? Annu Rev Plant Physiol Plant Mol Biol 48:609–639
107. Long SP, Ainsworth EA, Leakey ADB, Morgan PB (2005) Global food insecurity. Treatment of major food crops with elevated carbon dioxide or ozone under large-scale fully open-air conditions suggests recent models may have overestimated future yields. Philos Trans R Soc B Biol Sci 360:2011–2020
108. Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytol 165:351–371
109. Amani I, Fischer RA, Reynolds MP (1996) Canopy temperature depression association with yield of irrigated spring wheat cultivars in a hot climate. J Agron Crop Sci 176:119–129
110. Lobell DB, Burke MB, Tebaldi C, Mastrandrea MD, Falcon WP, Naylor RL (2008) Prioritizing climate change adaptation needs for food security in 2030. Science 319:607–610
111. Reynolds MP, Delgado MI, Gutierrez-Rodriguez M, Larque-Saavedra A (2000) Photosynthesis of wheat in a warm, irrigated environment - I: Genetic diversity and crop productivity. Field Crops Res 66:37–50
112. Barnabas B, Jager K, Feher A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31:11–38

Books and Reviews

Bolhar-Nordenkampf HR, Leegood RC, Long SP (eds) (1993) Photosynthesis and productivity in a changing environment: a field and laboratory manual. Chapman & Hall, London

Laisk A, Nedbal L, Govindjee (eds) (2009) Photosynthesis in silico: understanding complexity from molecules to ecosystems. Springer, Dordrecht

Lawlor DW (2000) Photosynthesis. Taylor & Francis, Boca Raton

Reynolds MP, Rajaram S, McNab A (eds) (1996) Increasing yield potential in wheat: breaking the barriers. CIMMYT, Mexico

Russell G, Marshal B, Jarvis PG (eds) (1990) Plant canopies, their structure growth and function. Cambridge University Press, Cambridge

Sage RF, Monson RK (eds) (1999) C4 Plant biology. Academic, London

Crop Responses to Available Soil Water

Manuela M. Chaves[1,2], O. Zarrouk[1]
[1]Plant Molecular Ecophysiology Laboratory (LEM), Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal
[2]Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Lisbon, Portugal

Article Outline

Glossary

Dehydration avoidance Dehydration avoidance is the strategy of the plants that are able to maintain tissue water potential as long (and as high) as possible under drought conditions.

Dehydration tolerance Dehydration tolerance is the strategy of the plants that are able to cope with severe tissue dehydration.

Harvest index Harvest index is the biomass of the harvested product expressed as a percentage of the total crop biomass.

Photoassimilates Photoassimilates is the energy-storing carbohydrates produced by photosynthesis in the green tissues of the plants.

Water-use efficiency (WUE) Water-use efficiency (WUE) is the carbon gain (or biomass formed) per unit of water transpired or the ratio between photosynthesis (A) and stomatal conductance (g_s), termed as intrinsic WUE.

Definition of the Subject

Sustainable intensification of global agriculture is a major purpose (and challenge) for twenty-first century scientific, social, and political communities, in order to guarantee food security, while preserving natural resources. Fast growing population and climate change could lead to a global crisis if efforts from different disciplines and countries are not congregated. Among limiting factors is water scarcity, which may dramatically decrease crop production worldwide.

Mitigation measures are therefore a major goal for sustaining crop production and they are based either on management practices that will enable water savings or on breeding efforts for more adequate crops. Improved physiological and molecular knowledge on plant's response to water deficits is essential to get improvements in crop yield under adverse environments. Because of the complexity of these responses, it is imperative to integrate disciplines as functional genomics, transcriptomics, proteomics, and metabolomics with plant physiology to improve breeding strategies.

According to the present knowledge, the key factors responsible for high yield under drought in annual plants include an appropriate phenology that will enable escaping drought and getting the timing of flowering right, as well as high water-use efficiency (WUE) and harvest index. On the other hand, the basic knowledge for fruit tree crop breeding under water scarcity is much more fragmented than for annuals because of the highest complexity of fruit trees. Therefore more efforts are needed in this area of research.

Introduction

Scarcity of water resources is an increasingly important issue since it will dictate global production of food and feed for the next generations, as dramatically described by The Economist (May 2010): "Water is the new oil: a resource long squandered; now growing expensive and soon to be overwhelmed by insatiable demand. Aquifers are falling, glaciers vanishing, reservoirs drying up and rivers no longer flowing to the sea. Climate change threatens to make the problems worse. Everyone must use less water if famine, pestilence and mass migration are not to sweep the globe."

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

The International Panel for Climate Change [1] predicts that water scarcity, together with incidence of high temperature, will increase in the near future in many regions of the globe with dramatic effects on agriculture. Of the world's water resources, c. 80% is currently consumed by irrigated agriculture. In this context, the investment in research for improving water use by the crops ("more crop per drop" as in the CGR program Challenge Program on Food and Water) [2], either through improved management technologies [3, 4] or more efficient genotypes [5, 6], is crucial. Increased water use by the crop can be obtained by agronomic practices that decrease water losses by soil evaporation, runoff, through-flow, deep drainage, and competing weeds, thereby making more water available for the crops, and in irrigated agriculture by using deficit irrigation practices.

Crop resistance to drought has been the subject of extensive research in the last decades, ranging from the physiological traits underlying resistance to water deficits [7–12] to breeding efforts [13, 14] including classical and genetic engineering approaches [15–17]. The world's most important crops for human and animal consumption in terms of total production – wheat, maize, rice, soybean, barley, and sorghum – have endured large yield increases during the twentieth century [18–22] and recently became the target of investigation aiming at improved performance under harsh environments, namely water scarcity [5, 6, 15, 23]. To articulate the knowledge obtained in different scientific disciplines (agronomy, breeding, physiology, and molecular biology) still remains a challenge, with the ultimate goal of providing farmers with better performing crops under water scarcity [7, 14]. Designing robust field trials is also essential in order to test improved crops under the multi-stress conditions they generally face under natural conditions. Indeed, there have been several reports of apparently promising biotechnological manipulations that have failed to deliver drought-tolerant crops in yield terms, when the novel material is transferred from the growth room to the field [4].

The key factors responsible for high yield under drought in annual plants are an appropriate phenology that will enable escaping drought and getting the timing of flowering right, high water-use efficiency (WUE) and a high harvest index [14, 24]. To get high WUE would involve the improvement in crop transpiration efficiency but the increased partitioning of biomass into the harvested product will also lead by itself to higher WUE. The relative importance of each of these processes will depend on how water is available during the crop cycle [13]. Indeed, it is noticeable that under most dryland situations where seasonal rainfall is unpredictable, maximizing soil moisture use is a crucial component of drought resistance (avoidance), which is generally expressed in lower WUE [25].

It is also important to recognize that many of the traits that explain plant adaptation to drought (e.g., phenology, the size and depth of the root system, xylem properties or the storage of reserves) are mostly constitutive [21, 26] and therefore can be found within the species population when it exhibits a large intraspecific variability. Moreover, there is the general recognition that the efficacy of high throughput screening of plant genetic material will speed-up crop improvement via breeding programs (see, e.g., [19]).

In fruit crop production, improvement under conditions of water scarcity has been achieved mostly by optimized management technologies such as deficit irrigation [3, 27], with breeding efforts being much more modest. Indeed the basic knowledge for tree crop breeding under water scarcity is much more fragmented than for annuals because of the highest complexity of fruit trees. In tree crops economic return is highly dependent on fruit quality, generally not related with total plant biomass produced and therefore water availability [3]. Moreover, yield-determining processes in fruit trees may not be sensitive to water deprivation at some developmental stages [28] and fruit quality may even beneficiate from a mild to moderate water deficits, as is well documented in grapevines [29]. High-density fruit orchards use composite plants – the scion cultivar grafted into a rootstock. The rootstock can alter the behavior of the scion, including its vegetative growth, flower numbers and flowering time, crop yield [30], and also drought tolerance [31]. The mechanisms underlying the regulation of scion growth and development by the rootstock are not fully understood [32].

Research in the so-called new climate proof crops such as *Chenopodium quinoa* Willd (quinoa) and *Amaranthus* spp is also developing. Quinoa is an

Andean seed crop, very nutritious [33], showing high tolerance to drought, frost, salinity, and biotic factors [34–36]. Quinoa has been selected as one of the crops to secure food production in the twenty-first century [37]. The protein quantity and the quality of quinoa seed is superior to those of cereals, because of the high content of lysine, methionine, and threonine, in addition to a range of vitamins (B2) and minerals (iron, calcium). Although amaranth is less tolerant to a range of stresses as compared to quinoa, it tolerates higher temperatures because it is a C4 plant [38]. The protein content of amaranth grain ranges from 12% to 17%, with a high level of lysine, similar to quinoa [39].

In this review the focus is on the key factors responsible for sustained plant growth and production under water scarcity, referring to both annual and perennial (fruit) crops. During evolution, plants developed different strategies to successfully cope with water stress; they comprised either acclimation to a slowly developing water deficits or the response to a sudden drought. These issues will be discussed in the context of the agriculture needs and related to production (fruit) quality, a major target of modern agriculture nowadays.

Revisiting Plant Strategies to Cope with Water Scarcity: The Crop Perspective

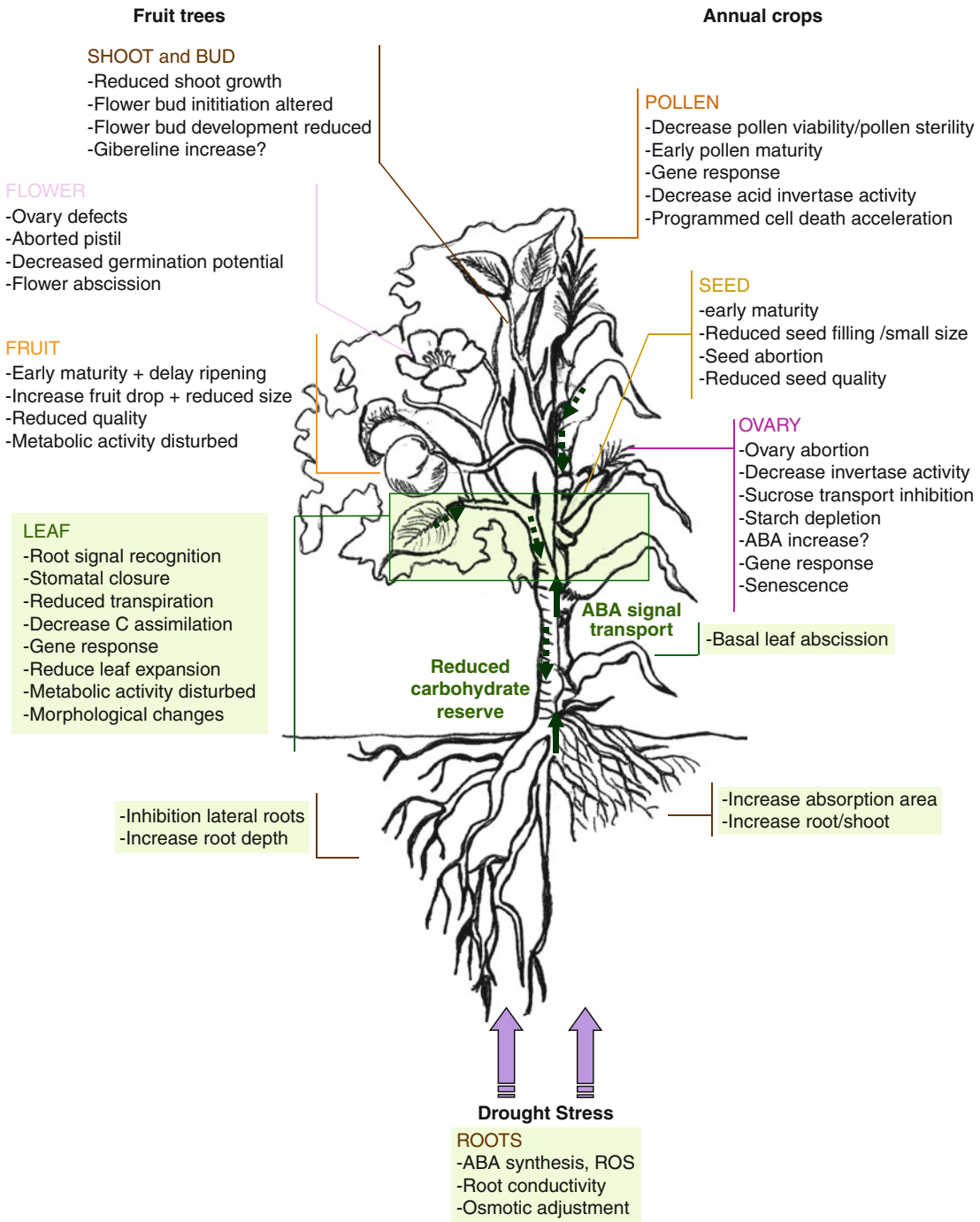
Plants respond and adapt to stress at whole plant, root, reproductive structures, and leaves, by using mechanisms that are being unraveled at the cellular and molecular levels [7, 40].

Classically, plant resistance to drought has been divided into escape, avoidance, and tolerance strategies [7, 41, 42]. However, in practice, most plants combine a range of these different strategies [43].

Plants that escape drought are able to adjust their phenology to the environment, being able to complete their life cycle before drought occurs. Escape strategies rely on successful flowering and therefore reproduction before the onset of severe stress. Plant ability to store reserves in stems and roots and to remobilize them for grain filling under water scarcity is extremely relevant for grain crops under water scarcity [44, 45], as it has been described in cereals such as wheat, maize, and rice [46–48], and legumes [49]. When stem reserve storage/utilization is insufficient to support fruit growth under stress, delayed-senescence genotypes may be desirable in crops where yield is source-limited [50]. High nitrogen availability can further increase the reuse of stored carbohydrates in cereals under moderate soil drying, leading to increased grain yield; under non-limiting water conditions, however, abundant nitrogen was shown to reduce grain yield in stay green genotypes [47].

Dehydration avoidance, which is common to both annuals and perennials, implies the maintenance of tissue water potential as long (and as high) as possible under drought conditions. This can be achieved either by (1) minimizing water loss or by (2) maximizing water uptake. Decreased water loss may derive from stomata closure and/or reduction of absorbed light, including leaf rolling [51], increased reflectance (with, e.g., trichomes), or steep leaf angles [52]. Shedding of older leaves is also relevant to reduce water loss, while allowing the reallocation of nutrients to younger leaves, when water deficit is relieved. Maximizing water uptake may be accomplished by increased investment in the roots at the expense of shoot growth (Fig. 1), which is generally inhibited very early on in response to decreasing water availability [8, 45]. Deep roots have been reported as important drought resistance traits for both annuals and perennials in semi-arid regions [26]. However, in arid environments, where rainfall is sporadic and of short duration, plants may take advantage of shallow roots that proliferate quickly near the soil surface allowing water uptake by the plant before it evaporates [53]. For example, *Zoysia japonica* was reported as having a relatively shallow root system, moderate WUE, while exhibiting a high capacity for osmotic adjustment [53]. High root plasticity is a key factor in this strategy because it will determine how fast roots grow in response to sporadic rain, following a period of drought stress.

Under slowly developing water deficits some plants show osmotic adjustment (OA), a cellular stress adaptive response that may improve tissue resistance to desiccation, by maintaining cell turgor, likely supporting crop yield under stress [25]. Because this mechanism allows plant water uptake at lower soil water potentials it is considered a dehydration avoidance strategy. The alterations observed under OA comprise increases in soluble sugars (like fructans and sucrose) [54], amino acids (e.g., proline, aspartic acid,



Crop Responses to Available Soil Water. Figure 1

Whole plant response to drought stress in fruit trees (*left*) and in annuals crops (*right*). Green boxes and letters correspond to shared responses between the species

and glutamic acid), methylated quartenary ammonium compounds (e.g., glycine betaine and alanine betaine), and some proteins, such as dehydrins [55] and cyclitols (e.g., D-pinitol, mannitol) ([56]; see also review [7]). In addition to the role played via the decrease in osmotic potential, these solutes may protect cell membrane and metabolic machinery under dehydration. According to Bohnert et al. [57] sequestration of H_2O molecules, reducing the solvent-protein interaction, may explain stabilization of protein complexes and membranes.

The positive role of osmotic adjustment on yield has been subject to much discussion since benefits were often not observed (see review by [58, 59]). A possible explanation is that turgor maintenance in cells is often associated with slow growth. It is also likely that osmoprotection mechanisms are not functional until severe dehydration occurs, with the implication that OA may be critical to survival rather than to promote plant growth and crop yield under drought [7].

Mechanisms of protection against oxidative stress are also fundamental to cope with drought and co-occurring stresses under arid semi-arid environments, as it will be discussed below.

Photoassimilates, Water Use, and Crop Yield

As proposed by Monteith [60], yield potential (Yp) of a crop at a given location can be defined as: $Yp = HI.Pn$ with $Pn = \Sigma(PAR^{*}ABSc^{*}\varepsilon c)$ and PAR being the incident solar radiation in the specific location, ABSc the fraction of the radiation intercepted by the crop, εc the efficiency of the conversion of intercepted radiation into biomass and Pn the primary productivity (total biomass produced over the growing season) and HI the harvest index (see also [61]). Under water scarcity a decrease in Yp may occur as a result of less intercepted radiation (due to lower total leaf area and smaller leaf angles) and a lower εc, as a result of a decrease in photosynthesis.

On the other hand, as judiciously proposed by Passioura ([62]; see also [14, 22, 63]), crop yield under water-limited conditions can be estimated by the product of transpiration efficiency (biomass/water transpired) × water transpired (WU) × harvest index (HI). Therefore, optimizing yield under such conditions has to be performed by increasing either water-use (amount and pattern), transpiration efficiency/WUE or partitioning of more biomass to grain. This can be achieved through better water management, the adjustment of crop phenology to the environment, or genetic improvement. The latter implies getting varieties that can give the so-called more crop per drop, either by improved carbon fixation under water deficits or having deeper roots to capture more water or converting more of the biomass into grain (increasing HI). Increasing WUE at the expenses o higher assimilated carbon rather than decreased WU is a desirable way to improve YP under water scarcity, but will generally require biotechnological interventions [17].

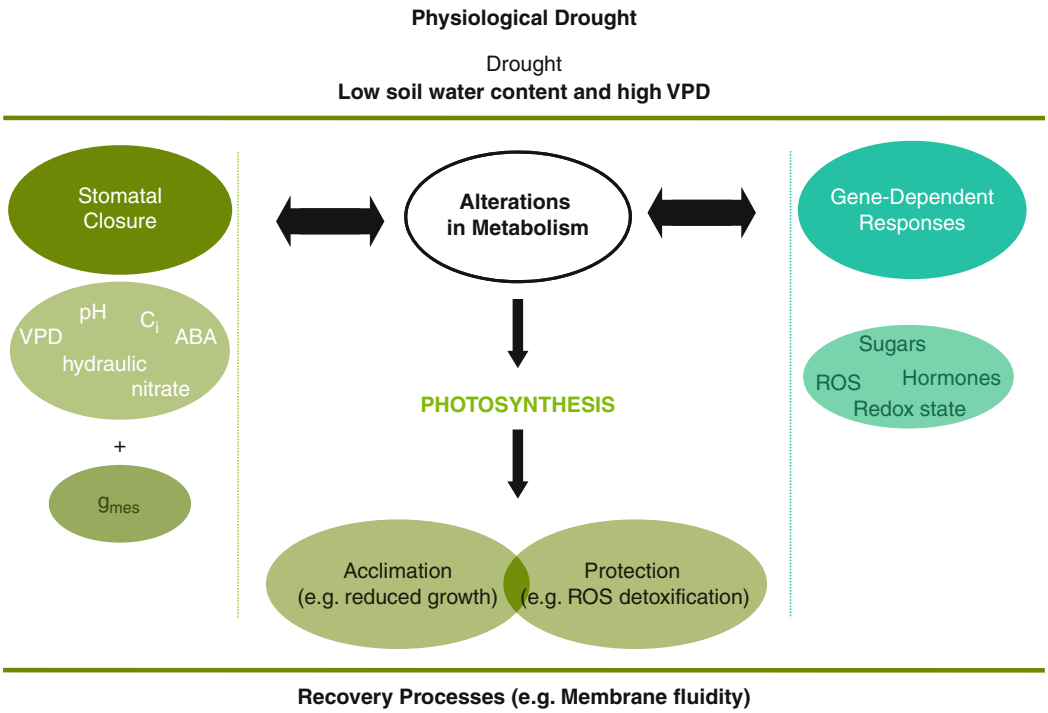
In the last decades, increases in Yp under non-limiting water conditions have been achieved with genotypes of cotton, wheat, and rice that exhibit high stomatal conductance and transpiration. This trait allows a decrease in leaf temperature and a greater CO_2 fixation per unit of leaf and land area [25, 64]. Under water-limited environments crop production has relied mostly in dehydration avoidance traits as described above, which maximize soil moisture use (when it becomes available for example, under sudden rainfall), but is generally associated with lower WUE. On the contrary, drought resistance traits that reduce WU, and are therefore associated with high WUE, will unavoidably reduce YP.

Few successful cases of breeding for water scarcity environments have been reported, one being the dryland wheat grain yield improvement, with selection for high WUE in Australia [65]. This success has been explained by the fact that wheat is grown there mainly on stored soil moisture. Furthermore, as suggested by Blum [2], a major opportunity for yield improvement under water scarcity is the control of WU during the earlier part of the growing season in order to avoid lack of soil moisture later on, during the reproduction phase. An attempt to achieve this was done by Richards and Passioura [66] by selecting for reduced root xylem diameter.

How Metabolic Processes Respond to Drought: Photosynthesis, Respiration, Photorespiration

Photosynthesis

Under water scarcity, restrictions in leaf carbon uptake take place as a result of increased resistance to CO_2 diffusion induced both by decreased stomatal



Crop Responses to Available Soil Water. Figure 2

Direct effects of drought on stomata and mesophyll (g_m) conductance as well as on gene expression, resulting in alterations of photosynthetic metabolism and ultimately on plant acclimation (Adapted from [9])

aperture (Fig. 2) [7, 9] and decreased mesophyll conductance [67]. At the whole plant level, total carbon uptake is further reduced due to the concomitant or even earlier inhibition of shoot growth [8]. Metabolic inhibition of photosynthesis usually occurs under more severe stress conditions or when various stresses co-occur, such as high light and temperature [8, 10], although claims for earlier alterations in photosynthetic metabolism are reported by Lawlor and coworkers ([12]; see a review by [68]). Interestingly, alterations in gene expression are also observed very early on, although these alterations are generally not turned into differential expression of proteins or enzymes in the short term (see revision [9]).

Stomata are able to detect a decrease in water availability either in the soil or in the atmosphere by feedback and feed-forward mechanisms. Feedback mechanisms include the response to dehydration in the leaf itself that is transmitted to the guard cells, either by hydraulic or by chemical signals. Feed-forward responses are generally mediated by hormonal signals and may take place before any alteration in leaf tissue water status occurred. They comprise responses of guard cells to high vapor pressure deficit, whose mechanisms area still not fully resolved [69–71] and to dehydration taking place elsewhere in the plant, namely in the roots [72]. Hormones, with particular relevance to ABA, but also cytokinins and ethylene, have been implicated in the root–shoot signaling, either acting in isolation or concomitantly. This long distance signaling by hormones may be mediated by reactive oxygen species [73].

Primary events of photosynthesis including electron transport capacity are very resilient to moderate drought [74], with the decline in PSII photochemistry being explained by a decrease in substrate availability. In fact, PSII activity often declines concomitantly with carbon uptake under water deficits, suggesting that photosynthetic electron chain is finely tuned by available CO_2 [75]. However, under field conditions, when high light co-occur with water deficits, CO_2 deprivation at the chloroplast (driven by stomatal closure) may

result in the production of excess reducing power and therefore in the decline of the quantum yield of PSII. These stresses also appear to inhibit the repair of PSII through suppression of the synthesis of PSII proteins, in particular, the production of D1 protein [76]. Protection mechanisms against reactive oxygen species (ROS) that are formed under such conditions are an essential component of plant survival [77]. Such protection may be achieved by the regulated thermal dissipation occurring in the light-harvesting complexes, involving the xanthophyll cycle [78, 79] and the lutein cycle [80]. Photoprotective mechanisms compete with photochemistry for the absorbed energy, thus leading to the downregulation of photosynthesis. If the limitation of the rate of CO_2 assimilation is accompanied by an increase in the activity of another sink for the absorbed energy, for example, photorespiration [81] or Mehler-peroxidase reaction [82], the decline in non-cyclic electron transport will be proportionally lower than the decrease observed in the rate of CO_2 assimilation. It was estimated that, in the absence of the repair mechanisms, photodamage would lower the yield of photosynthesis to less than 5% of the yield achieved now [83]. Although these responses have mainly been documented in plants native to semi-arid regions they may also occur in crop plants, even irrigated, when they are subjected to intense heat and irradiance, during the summer period. This is likely to increase its frequency in the near future.

As for Rubisco activity it was shown to be very resistant to water deficits [84, 85], being generally affected only after severe stress [86, 87]. The same authors also found evidence that low CO_2 concentration in the chloroplast (Cc) attained under severe water deficits could induce deactivation of Rubisco sites. It is further suggested that these effects are species-dependent, with species adapted to low Cc being able to maintain active Rubisco longer in response to prolonged drought [87]. These findings are compatible with earlier data suggesting that decreased sink capacity (limited capacity to use photoassimilates), as induced, e.g., by shoot growth inhibition under drought, might feedback to decrease photosynthesis, namely by downregulating the enzymes of the photosynthetic carbon cycle [88].

When studying the effects of drought on photosynthesis it is also important to recognize that crop productivity is dependent on photoassimilates produced at the whole plant level, which partly explains why often there is no correlation between crop yield and leaf photosynthetic rate [61]. Therefore, the impact of drought on shoot and canopy growth may be as important to crop yield as the effects produced at the single leaf level. Indeed, water scarcity by inducing a significant decline in total plant leaf area via the inhibition of new leaf growth or the earlier senescence of older leaves will decrease total carbon uptake by the plant. Moreover, lower leaf angles induced by decreased leaf turgor will reduce total intercepted irradiance, with significant impact on crop yield (Pinheiro and Chaves, unpublished results). However, it must be emphasized that, at least in cereals (wheat, maize and soybean), yield is generally more limited by the sink than by the source [61, 89]. This explains why these crops have the potential to cope with moderate drought and still fill their grains. Of course, this is also possible due to the capacity of remobilizing photoassimilates, previously stored in the shoot, as was discussed above.

Respiration and Photorespiration

The effects of water deficits on *dark respiration* are still unclear, with reports of either decreases, maintenance, or increases in the rates of this process (see the review [90]). Inhibition of respiration under drought has been observed in mature leaves of crops and herbaceous species as well as in roots, and is presumably related with a decreased availability of substrate to mitochondria under conditions of low photosynthesis and growth (e.g., [86, 91, 92]). This may be explained by the need of herbaceous species to quickly respond to water scarcity, thereby lowering their respiration rates in order to optimize their carbon gain over shorter periods of time.

On the contrary, trees and shrubs seem to show slower responses to drought than short-lived species. As suggested by Flexas et al. [86] and Atkin and Macherel [93], a higher demand for respiratory ATP (higher respiration rates) may be required under severe water stress to compensate for the lowered ATP production in the chloroplasts. The increased maintenance respiration will support repair mechanisms needed under acclimation to drought that will ensure a better performance under extended periods of water scarcity.

In general, the changes observed in respiration in response to drought are smaller than those observed in photosynthesis, thereby implying that respiration increases proportionally in relation to photosynthesis, with likely impact on leaf intercellular CO_2 and on plant carbon balance [68].

The role of *photorespiration* during drought stress has been scarcely studied [81, 94], partly due to difficulties in quantifying the rate of photorespiration [95]. Since photorespiration and photosynthetic metabolism are strictly linked and photorespiration depends on the recycling of RuBP in the Benson–Calvin cycle, it may be hypothesized that severe drought stress should result in lower photorespiration. As suggested by Osmond et al. [96], photorespiration and the Mehler-peroxidase pathway could protect the photosynthetic apparatus against photoinhibition in drought-stressed leaves, by sustaining photon utilization in non-assimilatory electron flow, when electron consumption by CO_2 assimilation is reduced due to low internal CO_2 concentrations. However, other studies, such as the one by Brestic et al. [97] concluded that photorespiration was not important for photoprotection in drought-stressed French bean.

In a recent work with glycine decarboxylase-deficient plants, Igamberdiev et al. [98] showed that photorespiration contributes to stomatal regulation. The data obtained with these mutants revealed that the photorespiratory mutants were able to decrease the rate of photorespiration, but only at the expense of increased water loss. Indeed, the necessity to maintain a high CO_2 concentration near the site of carboxylation in the chloroplasts of plants deficient in photorespiratory enzymes required an increased opening of the stomata, with a corresponding increase in water loss and decrease in water-use efficiency.

Yield and Quality Under Water Scarcity

Agriculture depends to a large extent on the success of plant reproduction [99]. Drought affects crop productivity as much as all other environmental factors combined and its impact on crops differs according to the attained developmental stage. The effects of water scarcity are quite different in annuals and perennial species [100]. Reproductive development of cereals, from meiosis to seed set, is highly vulnerable to water deficit [101] and the meiosis stage appears to be the most stress sensitive period of reproduction in all studied species [102]. However, anthesis, pollen fertility, pollination, female fertility, and early zygote development were also reported to be susceptible and finally their failure altered the number and the final quality of grain [102]. Later in the development, water stress tends to reduce grain size [103]. In fruit trees, severe water stress during flowering was reflected in the final fruit number per tree, whereas water stress during the fruit-growth and maturity phases was reflected mainly in fruit size [104]. In addition, the timing and intensity of the drought period dictate the extent of alterations occurring in the final fruit quality [29].

Flower Initiation and Induction

Grain crops are very sensitive to drought during floral initiation and floral pre-meiotic differentiation [105]. Water deficit at this stage causes pollen sterility, but when stress is severe, it usually affects also female fertility. In cereals, water stress during flower induction and inflorescence development leads to a delay in flowering or even to its complete inhibition [105]. The increase of abscisic acid (ABA) in response to water stress has been suggested to play a role in this delay [106].

In woody plants, flower bud initiation is an important feature in fruit tree cultivation. However, the effect of drought on floral meristems is among the least understood aspects of crop reproductive development under water deficit [107]. In species where flowering takes place prior to leaf emergence as *Prunus* trees, flower development occurs in the absence of new photoassimilates and at the expense of pre-stored reserves (starch), either in the flower itself or other plant organs [108]. This explains why in *Prunus* water stress effects are only detected in the subsequent reproduction event and not in the same year. This is the case of peach, apricot, and sweet cherry trees, where drought stress during flower bud initiation markedly slowed the progression of floral differentiation by delaying the differentiation of pistil primordia [109, 110].

In opposition, water stress has been demonstrated experimentally to induce flowering by a direct stimuli

on stem and bud in citrus [111], apple [112], and pear [113]. In these species, water stress showed to stimulate polyamine accumulation, which is linked to floral initiation, thus enhancing the production of floral primordia and re-flowering. However, severe water stress induces little flower production. Indeed, under severe stress during flower bud induction periods, gibberellins level increased, inducing a low level of flower production per shoot [114].

Flowering and Pollen Development

Loss of pollen fertility, spikelet death, and abortion of newly formed seed are associated with a decline in the water status of the reproductive structures and decline of carbohydrate availability [105]. By reducing photosynthesis, drought directly interferes with inflorescence and flower number, flowers life span, flowers opening, and maintenance of floral organs in crops [115] as well as maintenance of nectar production in floral organs [116]. In addition, water stress reduces flower size and sucrose content in nectar [117] leading to an extensive loss in yield [118].

Drought also affects seed yield of plants through an effect on availability and viability of pollen grains [119]. The effect of drought on pollen fertility/availability also depends on the species and it is considered a common symptom in angiosperms [120]. The hypothesis that ABA is a primary controlling agent in water stress induction of pollen viability still remains unanswered [102]. Recently in chickpea [119], water stress was shown to affect pollen growth in the pistil rather than affecting pollen viability. In fact, pistils from well watered plants pollinated with pollen of stressed ones showed fewer germinated pollen grains and fewer pollen tubes that reached the ovary, which suggested that drought has an effect on pollen tube growth, which is inhibited in the pistil [119]. Pollen viability is highly dependent on sugar unloading and pre-anthesis stem reserve accumulation is considered a significant factor affecting flower development in water stressed plants (see Fig. 1) [121]. The decrease of acid invertase activity under low water potential impedes pollen to metabolize incoming sucrose in hexose in the developing pollen, which might lead to pollen sterility [122, 123]. This hypothesis is confirmed by the study of Koonjul et al. [124] who observed a downregulation of the soluble invertase gene *Ivr1* and *Irv5* in wheat microspores, which correlates with accumulation of reducing (fructose and glucose) and non reducing (sucrose) sugars in the ear [125]. Nevertheless, recent studies by Liu and Bennett [126] showed that stress induced sterility in rice is not only caused by disruption in sugar metabolism or by desiccation of reproductive tissue, but also by disturbance of anther pollen development and cell function. In wheat, microspores lost contact with the tapetum at first pollen grain mitosis and the filament degenerated in response to water stress, which resulted in total sterility [127]. The expression of various proteins related to anther wall degradation and cell wall modifications are modified in stressed rice anther [126]. Furthermore, pollen from stressed plants might have a shorter life span and reduced vigor, which explain that pollen tube growth fails to reach the ovule [128]. The depletion of the adenosine triphosphate pool, increased concentration of hydrogen peroxide, and downregulated transcripts in anthers of drought-stressed rice lead to a programmed cell death and may cause pollen sterility [129].

In woody species, as observed in apricot trees, postharvest drought may induce an increase of aborted pistils in the subsequent year, decrease germination potential of pollen (see Fig. 1), and decrease the percentage of fruit set due to an increase of fruit drop [130]. In almond, the number of fruiting positions per tree is negatively associated with water stress [131]. However, drought stress during flower initiation does not influence the percentage of spurs that flowered or set fruit during subsequent years. Although water stress had no apparent effect on spur mortality in the first year, more than a half of spurs died within three subsequent years. In addition, water stress reduces flower bud development in grapevine [132] and peach [133] and increases flower abscission [134] and young fruit drop [135].

Ovary Development

Consequences of inhibited photosynthesis under drought and therefore insufficient assimilates are particularly striking around the time of pollination when reproductive events occur rapidly, namely ovary growth [136, 137]. In maize the pollen does not loose

availability even at severe water stresses (water potential below −12.5 MPa) and low kernel number is explained by a poor receptivity of silks and/or by an ovary abortion. Maize ovaries are normally loaded with glucose and starch on the day of pollination [138, 139]. Under drought, sucrose, the main translocated product from the carbon fixed in photosynthesis, declines (see Fig. 1). The enzymes that convert incoming sucrose to glucose in ovary lose activity and starch starts to be hydrolyzed [137, 138]. Cell wall acid invertase hydrolyses sucrose in the apoplast of the upper pedicel tissues and its activity creates a steep gradient in glucose concentration between the upper pedicel and the nucellus of young ovary. Ovary abortion under drought was attributed to the decrease of glucose and invertase activity in upper pedicel, inhibiting sucrose transport [138]. Sucrose delivery decreased first in ovary and probably triggers an early downregulation of genes coding for sucrose processing enzymes (*INCW2* and *IVR2*). Glucose depletion occurs few days after and triggers an up-regulation of putative senescence genes as *ribosome inactivation protein 2* [139], which suggests the beginning of failed ribosome function and later induces an up-regulation of *phospholipase D1* gene leading to loss of plasma membrane integrity, indicating the onset of senescence [137, 140]. This probably causes the irreversible loss in viability found during abortion [139]. Feeding sucrose during water stress largely prevents these changes, which confirm that senescence genes are "monitoring" the sugar status of the ovary cells and when sugars content decreases these genes turn on in sequence and orchestrate cell death [137].

A transcriptional study of placenta and endosperm of maize under drought showed that both tissues responded differentially to water shortage [141]. While most of the responding genes in placenta involved up-regulation, in endosperm these were downregulated. Downregulated genes relate to cell division and to endosperm growth, which may explain arrested growth and thus decreased demand of photosynthates [141].

These described events are common across crop species [137]. Although wheat requires water potentials to be much lower than maize to lose the same amount of photosynthetic activity [137], starch is also depleted in floral structures after drought (see Fig. 1) [142], inducing an impairment of ovule function [143]. Also in grapevine, drought induces lower availability of sugar due to decreased photosynthesis, which provokes ovary abnormalities and flower abscission [144].

Setter and coauthors [145] suggest an interaction between ABA and sugar that might induce a signal cascade that leads to the abortion process to initiate. The recent finding by Setter and Parra [146] supported the model of the interacting influence of carbohydrates and ABA at least at the pedicel-placenta tissues of basal kernels. Several studies in maize reported that water stress increases ABA in florets, suggesting it might trigger the abortion process [145, 147]. Young and Gallie [148] observed that regulation of programmed cell death during maize endosperm development involves the interaction of ABA and ethylene signaling pathways. However, Boyer and Westgate [99] explained that hormone effects are difficult to interpret because their expression on dry matter or water content basis can cause concentration to increase simply because the ovary dry mass or water content decreased, as it was shown in maize [149].

Yield

All described abnormalities of flowering, fertilization, and zygote development that may occur under drought stress induce yield losses, and the timing of the occurrence of drought stress determines the degree of disturbance. Water stress during ear formation and milk stages in maize was shown to induce early loss of lower leaves and a decrease in plant dry matter and in grain yield, as a result of reduced intercepted radiation [150]. Moreover, all the yield parameters were significantly affected. When water stress occurred at pre-anthesis a reduction in seed numbers was observed due to pollen sterility and ovary abortion. Post-anthesis water stress generally enhances whole-plant senescence and lead to a faster remobilization of carbon from vegetative tissues to seeds, thereby inducing earlier maturity and small seeds [119, 151–155]. This is a typical strategy of Mediterranean annuals, which exhibit a phenological drought-avoidance producing seed before water supplies are exhausted [45]. Remobilization of pre-stored carbon to the seed and acceleration of seed filling rate are associated to an alteration in the hormonal balance of grains, namely

the decrease in gibberellins and the increase in ABA [48, 156]. The reduced duration of grain filling may partly result from reduced number and size of endosperm cells and therefore reduced capacity to accumulate starch following drought stress [157]. Additionally, seed cell expansion is driven by water uptake [158] and when cell expansion in the seed stops, the end of seed growth and seed maturation are predetermined. Indeed, Yang et al. [152] suggested the enhancing of sink strength with water stress is promoted by alterations in sucrose synthase and starch branching enzyme activities taking place in grain during stress. Severe drought stress during seed filling generally resulted in seeds that are shriveled and deformed and with a reduced weight [152, 159]. The shortening in the duration of seed fill limits seed size because grain fill process fails to finish in a short maturation period [155, 160], consequently reducing seed yield [161]. Seed size reduction was highest in late maturing wheat genotypes, suggesting that early maturing genotypes partially escaped late-season water stress [162]. However if water stress occurs after cell division is completed it does not affect yield since sink size per kernel is already predetermined [152]. In sesame, most drought-tolerant cultivars abort a higher proportion of their seeds, and in that case more efficiently shunt available nutrient resources to the healthy growth of remaining seeds [163].

In woody plants and especially in fruit trees, it is generally accepted that post-harvest water stress may negatively influence fruit set and crop load in the next season, as it is the case in peach [164–167], in almond [168] and in sweet cherry [169]. This reduction in fruit set is due to the limitation placed by reduction in the accumulation of carbohydrate reserves. In fact, in cultivated *Prunus* species, floral bud-break and fruit set significantly precede net carbon export from leaves [167]. In citrus, severe water stress taking place during flowering led to decreased fruit number per tree, whereas in water restrictions occurring during the fruit-growth and maturity phases, the effects were reflected mainly in fruit size [104]. Olive tree has a reputation of being a drought resistant crop. However, drought incidence during winter, leading to a low level of soil moisture, may reduce vegetative growth [170] and have an important impact on flowering and fruit set, resulting in a drastic decrease on olive fruit number and size [171, 172]. In fact, severe water stress in phase I of fruit growth was shown to reduce mesocarp cell division and number [170, 173] suggesting a high sensitivity of olive mesocarp cell size to water stress. Grapevines are also well adapted to semi-arid climates, nonetheless severe water stress during the summer largely limit grapevine yield and cluster weight [27, 29]. This reduction is mainly observed in drought-avoiding genotypes, which optimize survival at expense of yield and reduced sugar allocation to reproductive tissues and thereby reducing fertility [174].

The effect of drought in crop plants is enhanced by high temperature and effects are usually difficult to separate [118]. Differences among genotypes are observed; for example barley is much less sensitive to short periods of very high temperature than wheat, and combined drought and high temperatures are more likely than high temperature alone to explain the reductions in grain weight observed in the field [175].

Fruit and Seed Quality

Drought influences end-use quality of major food crops in the world, as it is the case of wheat [176], barley, and maize [177]. Indeed, water stress in wheat changes the patterns of proteins [178]; in barley, grain β-glucan content and malt fine extract decreased with drought stress [179]; in soybean drought during seed development has a large effect on isoflavone concentration in the seeds [180]; in maize, water stress increases flavonoid content in seeds but reduces carotenoids and phenol content inducing a decrease in the antioxidant capacity of kernel oil [181]. In lupin seeds, raffinose quantity and accumulation pattern are reduced by water stress, suggesting an increase in nutritive values of seeds since raffinose is considered to be a major cause of flatulence in animals and humans [161].

Starch granule shape, volume and structure are important factors contributing to starch quality in wheat [182]. Post-anthesis water stress affects the proportions of different types of starch granules and increases the percentage of small granules per seed [183].

Protein content has an important implication in the processing qualities of cereals since it affects the functional properties of processed wheat products [184].

In wheat, grain protein content decreases linearly with the severity of drought stress during grain development [158]. Recently Zhao et al. [184] showed that the intensity of drought dictates the intensity of the effect on protein accumulation and reported a differential accumulation of proteins (especially gliadins and glutenins) in mature grains. The decrease in the accumulation of oil due to water stress was reported in wheat [184], soybean [185, 186], lupin [187] and chickpea [188].

In fruit trees, drought influences fruit development, metabolism, and final composition, and its timing and intensity dictate the extent of alterations occurring in final quality [29]. With regard to the effect of water stress on organoleptic properties, fruit quality is significantly affected by severe water stress during the fruit-growth and maturity periods [104]. Under water restriction, fruit maturity tends to be hastened, supporting the hypothesis that increasing water restriction might encourage early fruit maturity as observed in peach [189, 190]. Post-harvest water deficit was shown to increase fruit soluble solid concentration in the following season in peach [191, 192]. The opposite effect, reducing firmness and soluble solid concentration, was reported in sweet cherry [169].

Water stress was shown to decrease oil content and yield in young olive trees and induce lower level of phenolics in oil [172]. Olive fruits import assimilates from the canopy, but also produce them in situ by photosynthesis in the mesocarp; the fruit is capable of retaining chlorophyll even when its color change [193] and this makes a significant contribution to oil production [194]. This "autonomy" of olive fruits may explain the maintenance of oil quality in trees under water deficits [172]. In fact, although irrigation led to decreased contents of some undesirable sensory qualities as phenols, some favorable intense green notes were also reduced, which suggests complex effects of water stress on olive quality [195]. In grapevine, severe water stress influences final composition of the berries by delaying their ripening but also through an indirect effect on berry size [196, 197] that affects sugar and flavonoids metabolism in the berry (review [29]). However, moderate water deficit was shown to have beneficial effects on grape berries, for example by enhancing photoprotection mechanisms [198] and likely having a positive effect on wine quality [29, 196]. Indeed, moderate water deficit promotes sugar accumulation by inhibition of shoot growth with a subsequent reallocation of carbohydrates to fruits [199] and by the activation of ABA-mediated uptake of hexoses [198]. Under such conditions, sugar accumulation in berries accelerates anthocyanin accumulation or/and biosynthesis [200, 201]. The enhancement of carotenoids content and the up-regulation of genes encoding enzymes involved in biosynthesis of berry aroma were also observed after mild stress imposition [198, 202]. Similar benefits of mild water deficit in fruit quality were also observed in apricot [203], citrus [104] and olive [204].

The Breeding Achievements

Conventional Breeding

To face drought, farm-management practices and plant breeding are used for the improvement of crop yield [18], being the second approach the most promising in the long term [64]. Drought is the most recalcitrant to breeders' efforts due to the complexity of plant drought-tolerance mechanisms [205] and breeders have no reliable method of distinguishing sensitive from tolerant germoplasm other than by measuring yield [19]. Therefore, the main objective of a drought-tolerance breeding program is to select the variety presenting the better yield under stress conditions [206]. However, yield is a trait which is characterized by low heritability, polygenic control, and a high genotype $\times$ environment interaction (G $\times$ E) [21, 207].

During the last 50 years, most of the progress has been derived from empirical (conventional) breeding [208, 209] by selecting desired traits recognized at the phenotypic level. Generally, there is a minimum of 4 years extensive yield testing to further selection at multiple locations before a new variety is released to farmers. Empirical selection for grain yield was effective during the last decades. However, when plants are crossed some undesirable traits may be transferred along with those of interest – including some with negative effects on yield potential. In addition, breeding can only be done between species inter-compatible, which limits the new traits that can be added to those that already exist in a particular species [206]. Furthermore, the variability of rainfall from year to year increases G $\times$ E and reduces heritability for yield,

thereby limiting yield progress [19]. The indirect or analytical approach based on an understanding of the crop physiology, may help to target the key traits that are limiting yield [64]. Such an approach may therefore complement conventional breeding programs and hasten yield improvement [210–212].

A number of physiological studies have identified some traits which presence/expression are associated with plant adaptability to drought prone environment [205] and have been already successfully exploited in crop improvement [213]. Increasing water use by increasing early shoot vigor to escape dryer periods is a common strategy in Mediterranean region [214], but in very dry years this strategy may result in reduced yield. Early maturity by adjusting crop development with seasonal rainfall pattern in environments where they experiment terminal drought, or late flowering with short grain filling period in environments with early season drought, are examples of traits that are being used in breeding. Leaf cuticle waxes appear to play a key role in day and night transpiration and selecting for waxy leaves may reduce water loss and prevent leaf senescence during grain filling [215]. Enhancement of water extraction, by optimizing root architecture that resulted in greater water capture, has also the potential to significantly increase grain yield [63]. In addition, enhancement of root osmolytes accumulation was used in cereals, but a clear evidence of their beneficial role in crop yield was not observed [58].

Carbon isotope discrimination (Δ) is an attractive feature for C_3 plants breeders since it may provide an indirect sensing of transpiration efficiency (TE) [13]. In fact, genotypes showing higher TE (lower Δ) may be more productive under certain environmental conditions [216]. However, in the Mediterranean region the opposite was found and positive correlation between Δ and yield are reported [217], which is probably due to the fact that genotypes with lower TE have maintained a higher CO_2 conductance due to a better water status or by a faster plant development or better access to water [216]. Aerial infrared (IR) methods on vegetative material offer the possibility to detect variation on TE and present the advantage to reduce the cost of measuring carbon isotope discrimination [19, 216].

Fruit tree species propagation is possible only by vegetative methods. However, with the spreading of different species in new areas, the use of rootstock has become the first alternative to adapt scion cultivars to soils or climatic conditions that are otherwise not fully suited to their cultivation. Climatic areas or soils subjected to transient drought conditions require rootstock capable of inducing efficient water use by the scion cultivar. Unfortunately, there is only limited understanding of how rootstock provides tolerance to drought [218] and the majority of rootstock breeding programs addresses fruit quality. Traditionally, rootstocks have been produced using conventional hybridization breeding techniques, which are time and space consuming. Initial selection of main rootstocks is made from open pollinated fruit tree germplasm, generating thereby intra- and interspecific hybrids. The extended use of interspecific hybrids is due to the lower susceptibility of this type of rootstocks to biotic and abiotic stresses and may also overcome soil problems as drought [219], which might derive from heterosis (hybrid vigor) or from the elevation of ploidy levels of these rootstocks [220]. However, the use of known drought-tolerant/resistant rootstocks may not fulfill grower's intents in terms of fruit commercial traits, mainly because they may provide excess vigor. In addition, the problem of graft compatibility of hybrid rootstocks with commercial scion complicated the use of a wide range of cultivars [221, 222].

New Breeding Approaches

Compared to conventional approaches, genomics offer unprecedented opportunities for dissecting quantitative traits into their single genetic determinants, the quantitative trait loci (QTLs), thus paving the way to marker-assisted selection (MAS) and, eventually, cloning of QTLs and their direct manipulation via genetic engineering. Several studies have reported QTLs for roots architecture and investigated their effect on yield under water stress in rice [223] and also in maize, where QTL reported for leaf ABA concentration showed an effect on root size and grain yield [224, 225]. In sorghum QTLs related to 'stay green' trait and yield were identified [226].

Some QTLs of one character were shown to overlap with QTLs of other characters. This is the case in maize, where QTLs of leaf growth sensitivity to water deficit

overlapped with QTLs for leaf responses to evaporative demands, thus suggesting that hydraulic mechanisms are involved in that response [227]. Similarly, overlap between QTLs of leaf growth sensitivity with silk growth was observed [228]. QTLs were also reported for anthesis-silking interval in maize [15], for seed weight and yield under different water conditions in rice [229] and durum wheat [230]. It was also possible to identify WUE QTLs in brassica [231], rice [232], and wheat [233]. However, despite all the recent technological breakthroughs, the overall contribution of genomics-assisted breeding to the release of drought-tolerant cultivars has so far been marginal [234], in part due to the fact that a given QTL can have positive, null or negative additive effects depending on the drought scenario [235]. In addition, cloning QTLs for yield maintenance under drought conditions and drought physiological traits is limited because the unavailable information about the biochemistry of some of these traits [236].

In recent years, proteomic and transcriptomic studies have advanced the basic understanding of protein/gene regulatory networks that are active during the exposure of plants to water stress [237, 238] and there have been several reports of apparently promising biotechnological manipulations emerging from these tools [239]. Remarkable dehydration tolerance has been obtained under laboratory conditions using bacterial RNA chaperones overexpression [16], NFYB2 class transcriptionals regulators [240], as well as modulating the expression of dehydration response element binding (DBF/DREB1) transcription factor [241], using of detoxification of reactive oxygen species that accumulate under stress [242, 243] and of hormone intervening in drought signaling [244]. Introducing RNA silencing in some crops to downregulate poly ADP ribose polymerase and thus inducing tolerance to wide range of stresses [245] and overexpression of cyanobacterial flavodoxin [246] were also reported. However, these reports and others have failed to deliver drought tolerance in yield terms when the novel material is transferred from the growth room to the field [4].

Fruit trees also have benefited from new technologies and it is now possible to reduce the breeding time by MAS. Very recently fruit breeders have begun to examine the possibilities of using such techniques in the production of new rootstocks. The aim is to improve an already good rootstock for yield by modifying its gene expression or introducing new genes to improve resistance to abiotic factors. Unfortunately, the mechanisms by which rootstocks bring about their beneficial effects on the vigor and cropping of scions are still very poorly understood. Until more research is conducted in this field and genes that control such processes are identified, progress in this area may be slow. Recent studies showed that ectopic expression of the transcription factor Osmyb4 gene in transgenic apple trees, improved water stress tolerance and might result in long term ameliorated productivity. Further experiments are required to assess the agronomic value of the plants produced and to verify to what extent the expression of *Osmyb4* may contribute to enhanced drought tolerance under field conditions [247].

Summarizing, to date increased drought resistance of the major crop plants (sustained yield under water scarcity) has been dependent upon the screening of a wide range of germoplasm in order to identify genetic variation in major traits involved in stress resistance [19, 248, 249]. In addition, the effective use by the crop of a limiting water supply has been achieved by adjusting crop phenology to its environment [2, 14] or by using agronomic practices aiming at an improved water use, such as deficit irrigation [4, 29]. A variety of approaches have been successful, particularly in the irrigation of top quality fruit and vineyards [3, 27, 29, 250, 251] but also in annual crops [252–255]. In addition to minimizing changes in shoot water status, deficit irrigation is able to control the balance between fruit and vegetative growth, with likely positive impact on fruit quality [8, 256].

Future Directions

Drought has a great impact on the vegetative and reproductive development of annual and fruit tree crops and thereby impacts on final yield and seed and fruit quality. The key factors responsible for high yield under drought in annual plants are an appropriate phenology that will enable escaping drought and getting the timing of flowering right, high water-use efficiency and a high harvest index. The impact of drought is highly complex and involves diverse processes as photosynthesis, respiration, reserve accumulation, fertilization, gamete and embryo development, and fruit

and seed development. Due to the complexity of crop reproduction and the still incomplete knowledge of mechanisms underlying the response of reproductive events under water stress, it has been until now difficult to construct a model system to guarantee successful reproductive development and high yield under water scarcity. How resource availability (namely carbohydrate reserve) influence the development of reproductive organs under water stress is among the least understood aspects. On fruit trees, it is also urgent to understand (1) how rootstock confers resistance and/or tolerance to drought to the scion cultivar to better adjust breeding strategies; (2) the key events of fruit ripening. In addition, differences between species imply different strategies to improve/maintain yield and quality under such conditions. Spatial strategies also differ: a particular trait may be associated with higher yield in one environment and may have no/or negative effect in other environment [13].

Conventional breeding still appears to be the most effective for a significant drought-tolerance improvement of new cultivars. The application of modern molecular tools to understand responses of crops to water scarcity has only recently begun. In addition, to integrate them in breeding programs it is imperative to incorporate an accurate measurement of the phenotype under water stress. However, the speed of molecular technologies is not matched by the speed on phenotyping, being the latter one of major limitations to improve selection methods for water-limited environments [19]. Moreover, it is imperative to integrate disciplines, as functional genomics, transcriptomics, proteomics and metabolomics with plant physiology to improve breeding strategies. This would allow researchers to address drought tolerance/resistance through an integrated approach based on the knowledge of expression of genes and their products at a particular phenological and developmental stage of the crop.

Bibliography

1. IPCC (2007) Climate change 2007: the physical basis summary for policy makers. Cambridge University Press, Cambridge
2. Blum A (2009) Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. Field Crops Res 112:119–123
3. Fereres E, Soriano MA (2007) Deficit irrigation for reducing agricultural water use. J Exp Bot 58:147–159
4. Chaves MM, Davies B (2010) Drought effects and water use efficiency: improving crop production in dry environments. Funct Plant Biol 37(2):iii–vi
5. Reynolds MP, Pierre CS, Saad ASI, Vargas M, Condon AG (2007) Evaluating potential grains in wheat associated with stress-adaptive trait expression in elite genetic resources under drought and heat stress. Crop Sci 47:172–189
6. Reynolds M, Foulkes MJ, Slafer G, Berry P, Parry MAJ, Snape JW, Angus WJ (2009) Raising yield potential in wheat. J Exp Bot 60:1899–1918
7. Chaves MM, Pereira JS, Maroco J (2003) Understanding plant response to drought – from genes to the whole plant. Funct Plant Biol 30:239–264
8. Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits – Prospects for water-saving agriculture. J Exp Bot 55:2365–2384
9. Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103:551–560
10. Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol 6:269–279
11. Neumann PM (2008) Coping mechanisms for crop plants in drought-prone environments. Ann Bot 101(7):901–907
12. Lawlor DW (2009) Musings about the effects of environment on photosynthesis. Ann Bot 103:543–549
13. Condon AG, Richards RA, Rebetzke GJ, Farquhar GD (2004) Breeding for high water use efficiency. J Exp Bot 55(407):2447–2460
14. Passioura J (2007) The drought environment: physical, biological and agricultural perspectives. J Exp Bot 58:113–117
15. Ribaut JM, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations and alternatives. J Exp Bot 58(2):351–360
16. Castiglioni P, Warner D, Bensen RJ, Anstrom DC, Harrison J, Stoecker M, Abad M, Kumar G, Salvador S, D'Ordine R, Navarro S, Back S, Fernandes M, Targolli J, Dasgupta S, Bonin C, Luethy MH, Heard JE (2008) Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol 147:446–455
17. Parry MAJ, Madgwick PJ, Bayon C, Tearall K, Hernandez-Lopez A, Baudo M, Rakszegi M, Hamada W, Al-Yassin A, Ouabbou H, Labhilili M, Phillips AL (2009) Mutation discovery for crop improvement. J Exp Bot 60:2817–2825
18. Richards RA, Rebetzke GJ, Condon A, van Herwaarden AF (2002) Breeding opportunities for increasing the efficiency of water use and crop yield in temperate cereals. Crop Sci 42:111–121
19. Richards RA, Rebetzke GJ, Watt M, Condon AG, Spielmeye W, Dolferus R (2010) Breeding for improved water productivity in temperate cereals: phenotyping, quantitative trait loci, markers and the selection environment. Funct Plant Biol 37:85–97

20. Araus JL, Ferrio JP, Buxo R, Voltas J (2007) The historical perspective of dryland agriculture: lessons learned from 10 000 years of wheat cultivation. J Exp Bot 58(2):131–145
21. Passioura JB (2002) Environmental biology and crop improvement. Funct Plant Biol 29:537–546
22. Passioura J, Angus JF (2010) Improving productivity of crops in water-limited environments. Adv Agron 106:37–75
23. Lafitte HR, Yongsheng G, Yan Y, Li ZK (2007) Whole plant responses, key processes, and adaptation to drought stress: the case of rice. J Exp Bot 58(2):169–175
24. Richards RA (1991) Crop improvement for temperate Australia: future opportunities. Field Crops Res 26(2):141–169
25. Blum A (2005) Drought resistance, water-use efficiency, and yield potential- are they compatible, dissonant, or mutually exclusive? Aust J Agric Res 56:1159–1168
26. Blum A, Arkin GF (1984) Sorghum root growth and water-use as affected by water supply and growth duration. Field Crops Res 9:131–142
27. Chaves MM, Santos TP, Souza CR, Ortuno MF, Rodrigues ML, Lopes CM, Maroco JP, Pereira JS (2007) Deficit irrigation in grapevine improves water-use efficiency while controlling vigour and production quality. Ann Appl Biol 150:237–252
28. Johnson RS, Handley DF (2000) Using water stress to control vegetative growth and productivity of temperate fruit trees. HortScience 35:1048–1050
29. Chaves MM, Zarrouk O, Francisco R, Costa JM, Santos T, Regalado AP, Rodrigues ML, Lopes CM (2010) Grapevine under deficit irrigation: hints from physiological and molecular data. Ann Bot 105:661–676
30. Lockard RG, Schneider GW (1981) Stock and scion growth relationships and the dwarfing mechanism in apple. Hortic Rev 3:315–375
31. Landsberg JJ, Jones HG (1981) Apple orchards. In: Kozlowski TT (ed) Water deficits and plant growth, vol 6. Academic, London, pp 419–469
32. Atkinson CJ, Policarpo M, Webster AD, Kingswel G (2000) Drought tolerance of clonal Malus determined from measurements of stomatal conductance and leaf water potential. Tree Physiol 20:557–563
33. Repo-Carrasco R, Espinoza C, Jacobsen CE (2003) Nutritional value and use of the Andean crops quinoa (*Chenopodium quinoa*) and kañiwa (*Chenopodium pallidicaule*). Food Reviews Int 19:179–189
34. Jacobsen SE (2003) The worldwide potential for quinoa (*Chenopodium quinoa* Willd.). Food Reviews Int 19:167–177
35. Jacobsen SE, Monteros C, Corcuera LG, Bravo LA, Christiansen JL, Mujica A (2007) Frost resistance mechanisms in quinoa (*Chenopodium quinoa* Willd.). Eur J Agron 26:471–475
36. Bertero HD, de la Vega AJ, Correa G, Jacobsen SE, Mujica A (2004) Genotype and genotype-by-environment interaction effects for grain yield and grain size of quinoa (*Chenopodium quinoa* Willd.) as revealed by pattern analysis of multi-environment trials. Field Crops Res 89:299–318
37. FAO (2006) FAOSTAT data, FAO Statistical databases FAOSTAT. www.fao.org
38. Jacobsen SE, Mujica A (2003) The genetic resources of Andean grain amaranths (*Amaranthus caudatus* L., *A. cruentus* and *A. hipochondriacus* L.) in America. Plant Genet Resour Newsl 133:41–44
39. Bressani R, Gonzales JM, Zuniga J, Brauner M, Elias LG (1987) Yield, selected chemical composition and nutritive value of 14 selections of amaranth grain representing four species. J Sci Food Agric 38:347–356
40. Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol 16(2):123–132
41. Levitt J (1972) Responses of plants to environmental stresses. Academic, New York
42. Turner NC (1986) Crop water deficits: a decade of progress. Adv Agron 39:1–51
43. Ludlow MM (1989) Strategies of response to water stress. In: Kreeb KH, Richter H, Hinckley TM (eds) Structural and functional responses to environmental stresses. SPB Academic, The Hague, pp 269–281
44. Blum A, Sinmena B, Mayer J, Golan G, Shpiler L (1994) Stem reserve mobilization supports wheat-grain filling under heat stress. Aust J Plant Physiol 21:771–781
45. Chaves MM, Pereira JS, Rodrigues ML, Ricardo CPP, Osório ML, Carvalho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field: photosynthesis and growth. Ann Bot 89:907–916
46. Bruce WB, Edmeades GO, Barker TC (2002) Molecular and physiological approaches to maize improvement for drought tolerance. J Exp Bot 53:13–25
47. Yang J, Zhang J, Huang Z, Zhu Q, Wang L (2000) Remobilization of carbon reserves is improved by controlled soil-drying during grain filling of wheat. Crop Sci 40: 1645–1655
48. Yang JC, Zhang JH, Wang ZQ, Zhu QS, Wang W (2001) Remobilization of carbon reserves in response to water deficit during grain filling of rice. Field Crops Res 71:47–55
49. Rodrigues ML, Pacheco CMA, Chaves MM (1995) Soil–plant relations, root distribution and biomass partitioning in *Lupinus albus* L. under drought conditions. J Exp Bot 46:947–956
50. Levy PE, Moncrieff JB, Massheder JM, Jarvis PG, Scott SL, Brouwer J (1997) CO_2 fluxes at leaf and canopy scale in millet, fallow and tiger bush vegetation at the HAPEX-Sahel southern super-site. J Hydrol 188–189:612–632
51. Ehleringer JR, Cooper TA (1992) On the role of orientation in reducing photoinhibitory damage in photosynthetic-twig desert shrubs. Plant Cell Environ 15:301–306
52. Larcher W (2000) Temperature stress and survival ability of Mediterranean sclerophyllous plants. Plant Biosyst 134: 279–295
53. Huang B (1997) Roots and drought resistance. Turfgrass performance during drought depends heavily on root characteristics. Golf Course Management June:55–58

54. Vijn I, Smeekens S (1999) Fructan: more than a reserve carbohydrate? Plant Physiol 120:351–359
55. Pinheiro C, Cruz de Carvalho MH, Bartels D, Ricardo CP, Chaves MM (2008) Dehydrins in *Lupinus albus*: pattern of protein accumulation and gene expression in response to drought. Funct Plant Biol 35:85–91
56. Anderson CM, Kohorn BD (2001) Inactivation of Arabidopsis SIP1 leads to reduced levels of sugars and drought tolerance. J Plant Physiol 158:1215–1219
57. Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7:1099–1111
58. Serraj R, Sinclair TR (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant Cell Environ 25:333–341
59. Turner NC, Abbo S, Berger JD, Chaturvedi SK, French RJ, Ludwig C, Mannur DM, Singh SJ, Yadava HS (2007) Osmotic adjustment in chickpea (Cicer arietinum L.) results in no yield benefit under terminal drought. J Exp Bot 58(2):187–194
60. Monteith JL (1977) Climate and the efficiency of crop production in Britain. Phil Trans R Soc Lond B 281:277–294
61. Long SP, Zhu X-G, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? Plant Cell Environ 29: 315–330
62. Passioura JB (1977) Grain yield, harvest index, and water use of wheat. J Aust Inst Agric Sci 43:117–121
63. Passioura JB (2006) Increasing crop productivity when water is scarce – from breeding to field management. Agr Water Manage 80:176–196
64. Araus JL, Slafer GA, Reynolds MP, Royo C (2002) Plant breeding and water relations in C3 cereals: what should we breed for? Ann Bot 89:925–940
65. Condon AG, Richards RA, Rebetzke GJ, Farquhar GD (2002) Improving intrinsic water-use efficiency and crop yield. Crop Sci 42:122–131
66. Richards RA, Passioura JB (1989) A breeding program to reduce the diameter of the major xylem vessel in the seminal roots of wheat and its effect on grain yield in rain-fed environments. Aust J Agric Res 40:943–950
67. Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H (2008) Mesophyll conductance to CO2: current knowledge and future prospects. Plant Cell Environ 31:602–621
68. Lawlor DW, Tezara W (2009) Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. Ann Bot 103:561–579
69. Bunce JA (1997) Does transpiration control stomatal responses to water vapour pressure deficit? Plant Cell Environ 20:131–135
70. Franks PJ, Cowan IR, Farquhar GD (1997) The apparent feed forward response of stomata to air vapour pressure deficit: information revealed by different experimental procedures with two rainforest trees. Plant Cell Environ 20:142–145
71. Buckley TN, Mott KA, Farquhar GD (2003) A hydromechanical and biochemical model of stomatal conductance. Plant Cell Environ 26:1767–1785
72. Wilkinson S, Davies WJ (2002) ABA-based chemical signalling: the coordination of responses to stress in plants. Plant Cell Environ 25:195–210
73. Lake JA, Woodward FI, Quick WP (2002) Long-distance CO_2 signalling in plants. J Exp Bot 53:183–193
74. Cornic G, Gouvallec JL, Briantais JM, Hodges M (1989) Effect of dehydration and high light on photosynthesis of two C3 plants (*Phaseolus vulgaris* L. and *Elatastema repens* [Cour.] Hall f.). Planta 177:84–90
75. Loreto F, Tricoli D, Di Marco G (1995) On the relationship between electron transport rate and photosynthesis in leaves of the C4 plant Sorghum bicolor exposed to water stress, temperature changes and carbon metabolism inhibition. Aust J Plant Physiol 22:885–892
76. Takahashi S, Murata N (2008) How do environmental stresses accelerate photoinhibition? Trends Plant Sci 13:179–182
77. Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical Implications. Antioxid Redox Signal 11:861–905
78. Demmig-Adams B, Adams WW III (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci 1:21–26
79. Ort DR (2001) When there is too much light. Plant Physiol 125:29–32
80. Matsubara S, Gilmore AM, Osmond CB (2001) Diurnal and acclimatory responses of violaxanthin and *lutein epoxide* in the Australianmistletoe *Amyema miquelii*. Aust J Plant Physiol 28:793–800
81. Wingler A, Lea PJ, Quick WP, Leegood RC (2000) Photorespiration, metabolic pathways and their role in stress protection. Phil Trans R Soc Lond B 355:1517–1529
82. Biehler K, Fock H (1996) Evidence for the contribution of the Mehler peroxidase reaction in dissipating excess electrons in drought-stressed wheat. Plant Physiol 112:265–272
83. Melis A (1999) Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage in vivo? Trends Plant Sci 4:130–135
84. Bota J, Medrano H, Flexas J (2004) Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? New Phytol 162:671–681
85. Grassi G, Magnani F (2005) Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. Plant Cell Environ 28:834–849
86. Flexas J, Ribas-Carbó M, Bota J, Galmés J, Henkle M, Martínez-Cañellas S, Medrano H (2006) Decreased Rubisco activity during water stress is induced by stomatal closure, not by decreased relative water content. New Phytol 172:73–82
87. Galmés J, Ribas-Carbó M, Medrano H, Flexas J (2010) Rubisco activity in Mediterranean species is regulated by the chloroplastic CO_2 concentration under water stress. J Exp Bot (in press)
88. Peet MM, Kramer PJ (1980) Effects of decreasing source-sink ratio in soybeans on photosynthesis, photorespiration, transpiration and yield. Plant Cell Environ 3:201–206

89. Borrás L, Slafer GA, Otegui ME (2004) Seed dry weight response to source-sink manipulations in wheat, maize and soybean: a quantitative reappraisal. Field Crops Res 86: 131–146
90. Gimeno TE, Sommerville KE, Valladares F, Atkin OK (2010) Homeostasis of respiration under drought and its important consequences for foliar carbon balance in a drier climate: insights from two contrasting Acacia species. Funct Plant Biol 37:323–333
91. Ribas-Carbo M, Taylor NL, Giles L, Busquets S, Finnegan PM, Day DA, Lambers H, Medrano H, Berry JA, Flexas J (2005) Effects of water stress on respiration in soybean (*Glycine max*. L.) leaves. Plant Physiol 139:466–473
92. Galmés J, Ribas-Carbo M, Medrano H, Flexas J (2007) Response of leaf respiration to water stress in Mediterranean species with different growth forms. J Arid Environ 68:206–222
93. Atkin OK, Macherel D (2009) Crucial role plant mitochondria orchestrating drought tolerance. Ann Bot 103:581–597
94. Wingler A, QuickWP BRA, Bailey KJ, Lea PJ, Leegood RC (1999) The role of photorespiration during drought stress, an analysis utilizing barley mutants with reduced activities of photorespiratory enzymes. Plant Cell Environ 22:361–373
95. Long SP, Bernacchi CJ (2003) Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. J Exp Bot 54:2393–2401
96. Osmond CB, Badger MR, Maxwell K, Björkman O, Leegood RC (1997) Too many photons: photorespiration, photoinhibition and photooxidation. Trends Plant Sci 2:119–121
97. Brestic M, Cornic G, Fryer MJ, Baker NR (1995) Does photorespiration protect the photosynthetic apparatus in French bean leaves from photoinhibition during drought stress? Planta 196:450–457
98. Igamberdiev AU, Mikkelsen TN, Ambus P, Bauwe H, Lea PJ, Gardeström P (2004) Photorespiration contributes to stomatal regulation and carbon isotope fractionation: a study with barley, potato and Arabidopsis plants deficient in glycine decarboxylase. Photosynth Res 81:139–152
99. Boyer JS, Westgate ME (2004) Grain yields with limited water. J Exp Bot 55(407):2385–2394
100. Munns R, James RA, Sirault XR, Furbank RT, Jones HG (2010) New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. J Exp Bot 61:3499–3507
101. Saini HS, Lalonde S (1998) Injuries to reproductive development under water stress, and their consequences for crop productivity. J Crop Prod 1:223–248
102. Saini HS, Westgate ME (2000) Reproductive development in grain crops during drought. In: Spartes DL (ed) Advances in agronomy, vol 68. Academic, SanDiego, pp 59–96
103. Boyer JS, McLaughlin JE (2007) Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. J Exp Bot 58(2):267–277
104. García-Tejero I, Jiménez-Bocanegra JA, Martínez G, Romero R, Durán-Zuazo VH, Muriel-Fernández JL (2010) Positive impact of regulated deficit irrigation on yield and fruit quality in a commercial citrus orchard [*Citrus sinensis* (L.) Osbeck, cv. Salustiano]. Agr Water Manage 97(5):614–622
105. Winkel T, Renno JF, Payne WA (1997) Effect of the timing of water deficit on growth, phenology and yield of pearl millet (*Pennisetum glaucum* (L.) R. Br.) grown in Sahelian conditions. J Exp Bot 48:1001–1009
106. King RW, Evans LT (1977) Inhibition of flowering in *Lolium temulentum* L. by water stress: a role for abscisic acid. Aus J Plant Physiol 4:225–233
107. Saini HS, Aspinall D (1981) Effect of water deficit on sporogenesis in wheat (*Triticum aestivum* L.). Ann Bot 48:623–633
108. Rodrigo J, Hormaza IJ, Herrero M (2000) Ovary starch reserves and flower development in apricot (Prunus armeniaca). Physiol Plant 108(1):35–41
109. Engin H (2006) Scanning electron microscopy of floral initiation and developmental stages in 'Glohaven' peach under water deficit. Bangladesh J Bot 35(2):163–168
110. Alburquerque N, Burgos L, Egea J (2003) Apricot flower bud development and abscission related to chilling, irrigation and type of shoots. Sci Hortic 98(3):265–276
111. Southwick SM, Davenport TL (1986) Characterization of water stress and low temperature effects on floral induction in citrus. Plant Physiol 81:26–29
112. Jones HG (1987) Repeat flowering in apple caused by water stress or defoliation. Trees Struct Funct 1(3):135–138
113. Crisosto CH, Sugar D, Lombard P (1988) Effects of putrescine sprays at anthesis on 'Comice' pear yield component. Adv Hortic Sci 2:27–29
114. Koshita Y, Takahara T (2004) Effect of water stress on flower-bud formation and plant hormone content of satsuma mandarin (*Citrus unshiu* Marc.). Sci Hortic 99(3-4):301–307
115. Akhalalkatsi M, Losch R (2005) Water limitation effect on seed development and germination in *Trigonella coerulea* (Fabaceae). Flora 200:493–501
116. Mohan Ram HY, Rao IVR (1984) Physiology of flower bud growth and opening. Proc Indian Acad Sci Plant Sci 93: 253–274
117. Campbell DR (1996) Evolution of floral traits in a hermaphroditic plant: field measurements of heritabilities and genetic correlations. Evolution 50:1442–1453
118. Barnabas B, Jager K, Feher A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31:11–38
119. Fang X, Turner NC, Yan G, Li F, Siddique KHM (2010) Flower number, pod production, pollen viability, and pistil function are reduced and flower and pod abortion increased in chickpea (*Cicer arietinum* L.) under terminal drought. J Exp Bot 61(2):335–345
120. Porch TG, Jahn M (2001) Effect of high temperature stress on microsporogenesis in heat-sensitive and heat-tolerant genotypes of *Phaseolus vulgaris*. Plant Cell Environ 24:723–731
121. Blum A (1998) Improving wheat grain filling under stress by stem reserve mobilisation. Euphytica 100:77–83

122. Dorion S, Lalonde S, Saini HS (1996) Induction of male sterility in wheat by meiotic stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. Plant Physiol 111:137–145
123. Sheoran IS, Saini HS (1996) Drought-induced sterility in rice: changes in carbohydrate levels and enzyme activities associated with the inhibition of starch accumulation in pollen. Sex Plant Reprod 9:161–169
124. Koonjul PK, Minhas JS, Nunea C, Sheoran IS, Saini HS (2005) Selective transcriptional down-regulation of anther invertases precedes the failure of pollen development in water-stressed wheat. J Exp Bot 56:179–190
125. Xuemei JI, Shiran B, Wan J, Lewis DC, Jenkins CLD, Condon AG, Richards RA, Dolferus R (2010) Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. Plant Cell Environ 33:926–942
126. Liu JX, Bennett J (2010) Reversible and irreversible drought induced changes in the anther proteome of rice (*Oryza sativa* L.) genotypes IR64 and Moroberekan. Mol Plant. doi:10.1093/mp/ssq039
127. Saini HS, Sedgley M, Aspinall D (1984) Developmental anatomy in wheat of male sterility induced by heat stress, water deficit or abscisic acid. Aust J Plant Physiol 11:243–253
128. Turner LB (1993) The effect of water stress on florl characters, pollination and seed set in white clover (*Trifolium repens* L.). J Exp Bot 44:1155–1160
129. Nguyen GN, Hailstones DL, Wilkes M, Sutton BG (2009) Drought-induced oxidative conditions in rice anthers leading to a programmed cell death and pollen abortion. J Agron Crop Sci 195(3):157–164
130. Ruiz-Sanchez MC, Egea J, Galego R, Torrecillas A (1999) Floral biology of 'Bulida' apricot trees subjected to postharvest drought stress. Ann Appl Biol 135(2):523–528
131. Esparza G, DeJong TM, Weinbaum SA, Klein I (2001) Effects of irrigation deprivation during the harvest period on yield determinants in mature almond trees. Tree Physiol 21(14): 1073–1079
132. Matthews MA, Anderson MM (1989) Reproductive development in Vitis vinifera: responses to seasonal water deficits. Am J Enol Vitic 40:52–60
133. Johnson RS, Handley DF, DeJong TM (1992) Long-term response of early maturing peach trees to postharvest water deficits. J Am Soc Hortic Sci 117:881–886
134. Ussahatanonta S, Jackson DI, Rowe RN (1996) Effects of nutrient and water stress on vegetative and reproductive growth in *Vitis vinifera* L. Aust J Grape Wine Res 2:64–69
135. Torrecillas A, Domingo R, Galego R, Ruiz-Sánchez MC (2000) Apricot tree response to withholding irrigation at different phenological periods. Sci Hortic 85(3):201–215
136. Schussler JR, Westgate ME (1994) Increasing assimilate reserves does not prevent kernel abortion at low water potential in maize. Crop Sci 34:1569–1576
137. Boyer JS (2010) Drought decision-making. J Exp Bot 61(13):3493–3497
138. McLaughlin JE, Boyer JS (2004) Glucose localization in maize ovaries when kernel number decreases at low water potential and sucrose is fed to the stems. Ann Bot 94: 75–86
139. McLaughlin JE, Boyer JS (2004) Sugar-responsive gene expression, invertase activity, and senescence in aborting maize ovaries at low water potentials. Ann Bot 94:675–689
140. Hiyane R, Hiyane S, Ching Tang A, Boyer JS (2010) Sucrose feeding reverses shade-induced kernel losses in maize. Ann Bot 106(3):395–403
141. Yu L, Setter TL (2003) Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. Plant Physiol 131:568–582
142. Ji X, Shiran B, Wan J, Lewis DC, Jenkins CLD, Condon AG, Richards RA, Dolferus R (2010) Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. Plant Cell Environ 33:926–942
143. Kokubun M, Shimada S, Takahashi M (2001) Flower abortion caused by pre-anthesis water deficit is not attributed to impairment of pollen in soybean. Crop Sci 4:1517–1521
144. Lebon G, Wojnarowiez G, Holzapfel B, Fontaine F, Vaillant-Gaveau N, Clément C (2008) Sugars and flowering g in the grapevine (*Vitis vinifera* L.). J Exp Bot 59((10):2565–2578
145. Setter TL, Flannigan BA, Melkonian J (2001) Loss of kernel set due to water deficit and shade in maize: carbohydrate supplies, abscisic acid, and cytokinins. Crop Sci 41:1530–1540
146. Setter TL, Parra R (2010) Relationship of carbohydrate and abscisic acid levels to kernel set in maize under post-pollination water deficit. Crop Sci 50:980–988
147. Zinselmeier C, Westgate ME, Schussler JR, Jones RJ (1995) Low water potential disrupts carbohydrate metabolism in maize (*Zea mays* L.) ovaries. Plant Physiol 107:385–391
148. Young TE, Gallie DR (2000) Regulation of programmed cell death in maize endosperm by abscisic acid. Plant Mol Biol 42:397–414
149. Mäkela P, McLaughlin JE, Boyer JS (2005) Imaging and quantifying carbohydrate transport to the developing ovaries of maize. Ann Bot 96:939–949
150. Çakir R (2004) Effect of water stress at different developmental stages on vegetative and reproductive growth of corn. Field Crop Res 89:1–16
151. Brevedan RE, Egli DB (2003) Crop physiology and metabolism: short period of water stress during seed filling, leaf senescence, and yield of soybean. Crop Sci 43:2083–2088
152. Yang J, Zhang J, Wang Z, Zhu Q, Liu L (2003) Activities of enzymes involved in sucrose-to-starch metabolism in rice grains subjected to water stress during grain filling. Field Crop Res 81:69–81
153. Yang JC, Zhang JH, Wang ZQ, Xu GW, Zhu QS (2004) Activities of key enzymes in sucrose-to-starch conversion in wheat grains subjected to water deficit during grain filling. Plant Physiol 135:1621–1629
154. Samarah NH (2005) Effects of drought stress on growth and yield of barley. Agron Sustain Dev 25(1):145–149

155. Mi G, Chen F, Zhang F (2009) Grain filling rate is limited by insufficient sugar supply in the large-grain wheat cultivar. J Plant Breed Crop Sci 1(3):60–64
156. Yang J, Zhang J (2005) Grain filling of cereals under soil drying. New Phytol 169:223–236
157. Nicolas ME, Gleadow RM, Dalling MJ (1985) Effect of post-anthesis drought on cell-division and starch accumulation in developing wheat grains. Ann Bot 55:433–444
158. Gooding MJ, Ellis RH, Shewry PR, Schofield JD (2003) Effects of restricted water availability and increased temperature on the grain filling, drying and quality of winter wheat. J Cereal Sci 37(3):295–309
159. Younesi O, Moradi A (2009) The effect of water limitation in the field on sorghum seed germination and vigor. Aust J Basic Appl Sci 3(2):1156–1159
160. Rotundo JL, Westgate ME (2009) Meta-analysis of environment effects on soybean seed composition. Field Crops Res 110:147–156
161. Pinheiro C, Rodrigues AP, Saraiva de Carvalho I, Chaves MM, Ricardo CP (2005) Sugar metabolism in developing lupin seeds affected by a short-term water deficit. J Exp Bot 56:2705–2712
162. Saint Pierre C, Peterson CJ, Ross AS, Ohm JB, Verhoeven MC, Larson M, Hoefer B (2008) White wheat grain quality changes with genotype, nitrogen fertilization and water stress. Agron J 100(2):414–420
163. Kim KS, Park SH, Jenks MA (2007) Changes in leaf cuticular waxes of sesame (*Sesamum indicum* L.) plants exposed to water deficit. J Plant Physiol 164(9):134–1143
164. Larson KD, DeJong TM, Johnson RS (1988) Physiological and growth responses of mature peach trees to postharvest water stress. J Am Soc Hortic Sci 113:296–300
165. Girona J, Mata M, Arbones A, Alegre S, Rufat J, Marsal J (2003) Peach tree response to single and combined regulated deficit irrigation regimes under shallow soils. J Am Soc Hortic Sci 128:432–440
166. Naor A, Stern R, Peres M, Greenblat Y, Gal Y, Flaishman MA (2005) Timing and severity of postharvest water stress affect following-year productivity and fruit quality of field-grown 'Snow Queen' nectarine. J Am Soc Hortic Sci 130:806–812
167. Lopez G, Girona J, Marsal J (2007) Response of winter root starch concentration to severe water stress and fruit load and its subsequent effects on early peach fruit development. Tree Physiol 27(11):1619–1626
168. Goldhamer DA, Viveros M (2000) Effects of preharvest irrigation cutoff durations and postharvest water deprivation on almond tree performance. Irrigation Sci 19:125–131
169. Marsal J, Lopez G, Arbones A, Mata M, Vallverdu X, Girona J (2009) Influence of postharvest deficit irrigation and pre-harvest fruit thinning on sweet cherry (cv. New Star) fruit firmness and quality. J Hortic Sci Biotechnol 84:273–278
170. Fernandes-silva AA, Ferreira TC, Correia CM, Malheiro AC, Villalobos FJ (2010) Influence of different irrigation regimes on crop yield and water use efficiency of olive. Plant Soil 333:35–47
171. Moriana A, Orgaz F, Pastor M, Fereres E (2003) Yield responses of a mature olive orchard to water deficits. J Am Soc Hortic Sci 128:425–431
172. Greven M, Neal S, Green S, Dichio B, Clothier B (2009) The effects of drought on the water use, fruit development and oil yield from young olive trees. Agric Water Manage 96:1525–1531
173. Gucci R, Lodolini EM, Rapoport HF (2009) Water deficit-induced changes in mesocarp cellular processes and the relationship between mesocarp and endocarp during olive fruit development. Tree Physiol 29(12):1575–1585
174. Medrano H, Escalona JM, Cifre J, Bota J, Flexas J (2003) A ten-year study on the physiology of two Spanish grapevine cultivars under field conditions: effects of water availability from leaf photosynthesis to grape yield and quality. Funct Plant Biol 30:607–619
175. Savin R, Nicolas ME (1996) Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. Aust J Plant Physiol 23:201–21
176. Guttieri MJ, Bowen D, Gannon D, O'Brien K, Souza E (2001) Solvent retention capacities of irrigated soft white spring flours. Crop Sci 41:1054–1061
177. Tollenaar M, Lee ED (2002) Yield potential, yield stability and stress tolerance in maize. Field Crops Res 75:161–169
178. Gusta LV, Chen THH (1987) The physiology of water and temperature stress. In: Heyne EG (ed) Wheat and wheat improvement nº13, 2nd edn. ASA/CSSA/SSSA, Madison, pp 115–150
179. Coles GD, Jamieson PD, Haslemore RM (1991) Effects of moisture stress on malting quality in triumph barley. J Cereal Sci 14:161–177
180. Lozovaya VV, Lygin AV, Ulanov AV, Nelson RL, Daydé J, Widholm JM (2005) Effect of temperature and soil moisture status during seed development on soybean seed isoflavone concentration and composition. Crop Sci 45: 1934–1940
181. Ali Q, Ashraf M, Anwar F (2010) Seed composition and seed oil antioxidant activity of maize under water stress. J Am Oil Chem Soc 87(10):1179–1187
182. Lindeboom N, Chang PR, Tyler RT (2004) Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: a review. Starch Starke 56:89–99
183. Dai Z (2009) Starch granule size distribution in grains at different positions on the spike of wheat (*Triticum aestivum* L.). Starch Stärke 61(10):582–589
184. Zhao CX, He MR, Wang ZL, Wang YF, Lin Q (2009) Effects of different water availability at post-anthesis stage on grain nutrition and quality in strong-gluten winter wheat. CR Biol 332(8):759–764
185. Kumar V, Rani A, Solanki S, Hussain SM (2006) Influence of growing environment on the biochemical composition and physical characteristics of soybean seed. J Food Compos Anal 19:188–195

186. Carrera C, Martinez MJ, Dardanelli J, Balzarini M (2009) Water deficit effect on the relationship between temperature during the seed fill period and soybean seed oil and protein concentrations. Crop Sci 49:990–998
187. Carvalho IS, Ricardo CPP, Chaves MM (2005) Seeds chemical composition of two lupines (*Lupinus albus* and *Lupinus mutabilis*) influenced by water stress. J Agron Crop Sci 191:95–98
188. Nayyar H, Singh S, Kaur S, Kumar S, Upadhyaya HD (2006) Differential sensitivity of macrocarpa and microcarpa types of chickpea (*Cicer arietinum* L.) to water stress: association of contrasting stress response with oxidative injury. J Integr Plant Biol 48:1318–1329
189. Genard M, Souty M, Holmes S, Reich M, Breuils L (1994) Correlations among quality parameters of peach fruit. J Sci Food Agric 66:241–245
190. Mercier V, Bussi C, Lescourret F, Genard M (2009) Effects of different irrigation regimes applied during the final stage of rapid growth on an early maturing peach cultivar. Irrigation Sci 27(4):297–306
191. Gelly M, Recasens I, Mata M, Arbones A, Rufat J, Girona J, Marsal J (2003) Effects of water deficit during stage II of peach fruit development and postharvest on fruit quality and ethylene production. J Hortic Sci Biotechnol 78:324–330
192. Gelly M, Recasens I, Girona J, Mata M, Arbones A, Rufat J, Marsal J (2004) Effects of stage II and postharvest deficit irrigation on peach quality during maturation and after cold storage. J Sci Food Agric 84:561–568
193. Connor DJ (2005) Adaptation of olive (*Olea europaea* L.) to water environments. Aust J Agric Res 56:1181–1189
194. Proietti P, Famiani F, Tombesi A (1999) Gas exchange in olive fruit. Photosynthetica 36(3):423–432
195. Stefanoudaki E, Williams M, Chartzoulakis K, Harwood J (2009) Effect of irrigation on quality attributes of olive oil. J Agric Food Chem 57(15):7048–7055
196. Bravdo B, Hepner Y, Loinger C, Tabacman H (1985) Effect of irrigation and crop level on growth, yield and wine quality of Cabernet Sauvignon. Am J Enol Vitic 36:132–139
197. Kennedy JA, Matthews MA, Waterhouse AL (2002) Effect of maturity and vine water status on grape skin and wine flavonoids. Am J Enol Vitic 53:268–274
198. Deluc LG, Quilici DR, Decendit A, Grimplet J, Wheatley MD, Schlauch KA, Mérillon JM, Cushman JC, Cramer GR (2009) Water deficit alters differentially metabolic pathways affecting important flavour and quality traits in grape berries of Cabernet Sauvignon and Chardonnay. BMC Genomics 10:212
199. Coombe BG (1989) The grape berry as a sink. Acta Hortic 239:149–158
200. Gollop R, Farhi S, Perl A (2001) Regulation of the leucoanthocyanidin dioxygenase gene expression in Vitis vinifera. Plant Sci 161:579–588
201. Gollop R, Even S, Colova-Tsolova V, Perl A (2002) Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. J Exp Bot 53:1397–1409
202. Bindon KA, Dry PR, Loveys BR (2007) Influence of plant water status on the production of C13-norisoprenoid precursors in *Vitis vinifera* L. cv. Cabernet Sauvignon grape berries. J Agric Food Chem 55:4493–4500
203. Pérez-Pastor A, Ruiz-Sánchez MC, Martínez JA, Norte PA, Artés F, Domingo R (2007) Effect of deficit irrigation on apricot fruit quality at harvest and during storage. J Sci Food Agric 87(13):2409–2415
204. Gómez-Rico A, Salvador MD, Moriana A, Pérez D, Olmedilla N, Ribas F, Fregapane G (2007) Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. Food Chem 100(2):568–578
205. Cattivelli L, Rizza F, Badeck FW, Mazzucotelli E, Mastrangelo AM, Francia E, Mare C, Tondelli A, Stanca AM (2008) Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. Field Crops Res 105(1–2):1–14
206. Atlin G (2003) Improving drought tolerance by selecting for yield. In: Fischer KS, Lafitte R, Fukai S, Atlin Q, Hardy B (eds) Breeding rice for drought-prone environments. IRRI, Los Bańos, Philippines, pp 14–22
207. Jackson P, Robertson M, Cooper M, Hammer G (1996) The role of physiological understanding in plant breeding; from a breeding perspective. Field Crops Res 49:11–39
208. Evans LT (1993) Crop evolution, adaptation and yield. Cambridge University Press, Cambridge
209. Loss SP, Siddique KHM (1994) Morphological and physiological traits associated with wheat yield increases in Mediterranean environments. Adv Agron 52:229–276
210. Araus JL (1996) Integrative physiological criteria associated with yield potential. In: Reynolds MP, Rajaram S, McNab A (eds) Increasing yield potential in wheat: breaking the barriers. CIMMYT, Mexico DF, pp 150–167
211. Richards RA (1996) Defining selection criteria to improve yield under drought. Plant Growth Regul 20:157–166
212. Slafer GA, Araus JL (1998) Improving wheat responses to abiotic stresses. In: Slinkard AE (ed) Proceedings of the 9th international wheat genetics symposium vol 1. University of Saskatchewan Extension Press, Saskatoon, pp 201–213
213. Edmeades GO, Bolaños J, Chapman SC, Lafitte HR, Banziger M (1999) Selection improves drought tolerance in tropical maize populations: I. Gains in biomass, grain yield, and harvest index. Crop Sci 39:1306–1315
214. López-Castañeda CR, Richards RA (1994) Variation in temperate cereals in rainfed environments. I. Grain yield, biomass and agronomic characteristics. Field Crops Res 37:51–62
215. Richards RA, Rawson HM, Jonhson DA (1986) Glaucousness in wheat: its development and effect on water-use efficiency, gas exchange and photosynthetic tissues temperatures. Aust J Plant Physiol 13:465–473
216. Araus JL, Bort J, Steduto P, Villegas D, Royo C (2003) Breeding cereals for Mediterranean conditions: ecophysiological clues for biotechnology application. Ann Appl Biol 142:129–141
217. Slafer GA, Araus JL (2007) Physiological traits for improving wheat yield under a wide range of conditions. In: Spiertz JHJ, Struik PC, van Laar HH (eds) Scale and complexity in plant

systems research: gene-plant-crop relations. Springer, Dordrecht, pp 147–156
218. Webster T (2002) Dwarfing rootstocks: past, present and future. Compact Fruit Tree 35(3):67–72
219. Reighard GL, Loreti L (2009) Rootstock development. In: Layne DR, Bassi D (eds) The peach: botany production and uses. CABI, Cambridge, pp 193–220
220. Hammatt N (1992) Progress in the biotechnology of trees. World J Microbiol Biotechnol 8(4):369–377
221. Zarrouk O, Pinochet J, Gogorcena Y, Moreno MA (2006) Graft compatibility between peach cultivars and *Prunus* rootstocks. Hortic Sci 41:1389–1394
222. Zarrouk O, Testillano PS, Risueño MC, Moreno MA, Gogorcena Y (2010) Changes in cell/tissue organization and peroxidase activity as markers for early detection of graft incompatibility in peach/plum combinations. J Am Soc Hortic Sci 135:9–17
223. Steel KA, Virk DS, Kumar R, Prasad SC, Witcombe JR (2007) Field evaluation of upland rice lines selected for QTLs controlling root traits. Field Crops Res 101(2):180–186
224. Giuliani S, Sanguineti MC, Tuberosa R, Bellotti M, Salvi S, Landi P (2005) Root-ABA1, a major constitutive QTL, affects maize root architecture and leaf ABA concentration at different water regimes. J Exp Bot 56:3061–3070
225. Landi P, Sanguineti MC, Liu C, Li Y, Wang TY, Giuliani S, Belloti M, Salvi S, Tuberosa R (2007) Root-ABA1 QTL affects root lodging, grain yield, and other agronomic traits in maize grown under well-watered conditions. J Exp Bot 58:319–326
226. Harris K, Subudhi PK, Borrell A, Jordan D, Rosenow D, Nguyen H, Klein P, Klein R, Mullet J (2007) Sorghum stay-green QTL individually reduce post-flowering drought-induced leaf senescence. J Exp Bot 58:327–338
227. Sadok W, Naudin P, Boussuge B, Muller B, Welcker C, Tardieu F (2007) Leaf growth rate per unit thermal time follows QTL-dependent daily patterns in hundreds of maize lines under naturally fluctuating conditions. Plant Cell Environ 30(2):135–146
228. Welcker C, Boussuge B, Bencivenni C, Ribaut JM, Tardieu F (2007) Are source and sink strengths genetically linked in maize plants subjected to water deficit? A QTL study of the responses of leaf growth and of anthesis-silking interval to water deficit. J Exp Bot 58:339–349
229. Wang JW, Yang FP, Chen XQ, Liang RQ, Zhang LQ, Geng DM, Zhang XD, Song YZ, Zhang GS (2006) Induced expression of DREB transcriptional factor and study on its physiological effects of drought tolerance in transgenic wheat. Acta Genetica Sinica 33:468–476
230. Maccaferri M, Sanguineti MC, Corneti S, Araus JL, Ben Salem M, Bort J, DeAmbrogio E, Garcia del Moral L, Demontis A, El-Ahmed A, Elouafi I, Maalouf F, Machlab H, Martos V, Nachit MN, Nserallah N, Ouabbou H, Royo C, Slama A, Villegas D, Tuberosa R (2008) Quantitative trait loci for grain yield and adaptation of durum wheat (*Triticum durum* Desf.) across a wide range of water availability. Genetics 178: 489–511
231. Hall NM, Griffiths H, Corlett JA, Jones HG, Lynn J, King GJ (2005) Relationships between water-use traits and photosynthesis in Brassica oleracea resolved by quantitative genetic analysis. Plant Breed 124(6):557–564
232. Laza MR, Kondo M, Ideta O, Barlaan E, Imbe T (2006) Identification of quantitative trait loci for delta 13 C and productivity in irrigated lowland rice. Crop Sci 46:763–773
233. Spielmeyer W, Hyles J, Joaquim P, Azanza F, Bonnett D, Ellis ME, Moore C, Richards RA (2007) A QTL on chromosome 6A in bread wheat (*Triticum aestivum*) is associated with longer coleoptiles, greater seedling vigour and final plant height. Theor Appl Genet 115:59–66
234. Tuberosa R, Salvi S (2006) Genomics-based approaches to improve drought tolerance of crops. Trends Plant Sci 11:405–412
235. Vargas M, van Eeuwijk FA, Crossa J, Ribaut JM (2006) Mapping QTLs and QTL x environment interaction for CIMMYT maize drought stress program using factorial regression and partial least squares methods. Theor Appl Genet 112:1009–1023
236. Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? Plant Physiol 147:469–486
237. Bogeat-Triboulot MB, Brosché M, Renaut J, Jouve L, Le Thiec D, Fayyaz P, Vinocur B, Witters E, Laukens K, Teichmann T, Altman A, Hausman JF, Polle A, Kangasjärvi J, Dreyer E (2007) Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populus euphratica*, a poplar growing in arid regions. Plant Physiol 143:876–892
238. Hummel I, Pantin F, Sulpice R, Piques M, Rolland G, Dauzat M, Christophe A, Pervent M, Bouteillé M, Stitt M, Gibon Y, Muller B (2010) Arabidopsis plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. Plant Physiol 154:357–372
239. Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr Opin Plant Biol 9(2):189–195
240. Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG, Hinchey BS, Kumimoto RW, Maszle DR, Canales RD (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci 104: 16450–16455
241. Habash DZ, Kehel Z, Nachit M (2009) Genomic approaches for designing durum wheat ready for climate change with a focus on drought. J Exp Bot 60(10):2805–2815
242. Yang XH, Wen XG, Gong HM, Lu QT, Yang ZP, Tang YL, Liang Z, Lu CM (2007) Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. Planta 225:719–733
243. Zhu JM, Alvarez S, Marsh EL, LeNoble ME, Cho IJ, Sivaguru M, Chen SX, Nguyen HT, Wu YJ, Schachtman DP et al (2007) Cell wall

proteome in the maize primary root elongation zone. II. Region specific changes water soluble lightly ionically bound proteins water deficit. Plant Physiol 145:1533–1548
244. Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Natl Acad Sci 104:19631–19636
245. Vanderauwera S, De Block M, Van de Steene N, van de Cotte B, Metzlaff M, Van Breusegem F (2007) Silencing of poly(ADP-ribose) polymerase in plants alters abiotic stress signal transduction. Proc Natl Acad Sci 104:15150–15155
246. Tognetti VB, Palatnik JF, Fillat MF, Melzer M, Hajirezaei M-R, Valle EM, Carrillo N (2006) Functional replacement of ferredoxin by a cyanobacterial flavodoxin in tobacco confers broad-range stress tolerance. Plant Cell 18:2035–2050
247. Pasquali G, Biricolti S, Locatelli F, Baldoni E, Mattana M (2008) Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples. Plant Cell Rep 27: 1677–1686
248. Lopes MS, Reynolds MP (2010) Partitioning of assimilates to deeper roots is associated with cooler canopies and increased yield under drought in wheat. Funct Plant Biol 37:147–156
249. Saint Pierre C, Trethowan R, Reynolds M (2010) Stem solidness and its relationship to water-soluble carbohydrates: association with wheat yield under water deficit. Funct Plant Biol 37:166–174
250. Beis A, Patakas A (2010) Differences in stomatal responses and root: shoot signalling between two grapevine varieties subjected to drought. Funct Plant Biol 37:139–146
251. Collins MJ, Fuentes S, Barlow EWR (2010) Partial rootzone drying and deficit irrigation increase stomatal sensitivity to vapour pressure deficit in anisohydric grapevines. Funct Plant Biol 37:128–138
252. Kang S, Zhang J (2004) Controlled alternate partial root-zone irrigation: its physiological consequences and impact on water use efficiency. J Exp Bot 55:2437–2446
253. Dodd IC, Theobald JC, Bacon MA, Davies WJ (2006) Alternation of wet and dry sides during partial rootzone drying irrigation alters root-to-shoot signalling of abscisic acid. Funct Plant Biol 33:1081–1089
254. Kirda C, Topcu S, Cetin M, Dasgan HY, Kaman H, Topaloglu F, Derici MR, Ekici B (2007) Prospects of partial root zone irrigation for increasing irrigation water use efficiency of major crops in the Mediterranean region. Ann Appl Biol 150:281–291
255. Wang Y, Liu F, Andersen MN, Jensen CR (2010) Improved plant nitrogen nutrition contributes to higher water use efficiency in tomatoes under alternate partial root-zone irrigation. Funct Plant Biol 37:175–182
256. Davies WJ, Wilkinson S, Loveys B (2002) Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. New Phytol 153:449–460

Crop Responses to Nitrogen

Gilles Lemaire
INRA, Lusignan, France

Article Outline

Glossary

Critical plant N concentration Critical plant N concentration is defined as the minimum plant nitrogen concentration of a crop corresponding to its maximum crop mass.

Critical crop N uptake Critical crop N uptake is defined as the minimum crop nitrogen uptake for achieving maximum crop mass.

Harvest index (HI) Harvest index (HI) is the ratio between harvested biomass Y (grains, tubers) and aboveground crop mass W at crop maturity.

Intercepted photosynthetic active radiation (IPAR) Intercepted Photosynthetic Active Radiation (IPAR) is the proportion of the incident PAR which is intercepted by the crop at a given time. This proportion is related to the size of the canopy, the Leaf Area Index, and depends also on canopy structure: leaf angle and geometry.

Leaf area index (LAI) Leaf area index (LAI) is the total canopy leaf area of a crop per unit of soil area. LAI allows the estimation of the proportion of the incident light which is intercepted by the canopy, and then which can be used for photosynthesis of the whole crop.

Nitrogen absorption efficiency (NAE) Nitrogen absorption efficiency (NAE) is the increase in crop nitrogen uptake per unit of supplemental N supply rate.

Nitrogen conversion efficiency (NCE) Nitrogen conversion efficiency (NCE) is the increase in crop dry mass (dW) or in crop yield (dY) per unit of supplemental crop N uptake corresponding to an increase in nitrogen supply rate.

Nitrogen use efficiency (NUE) Nitrogen use efficiency (NUE) is the increase in crop dry mass (dW) or in crop yield (dY) per unit of supplemental N supply rate. So NUE = NAE × NCE.

N dilution N dilution is the process corresponding to more rapid accumulation of nitrogen-free compounds than nitrogen compounds within plant as plant grows, leading to decline in plant nitrogen concentration with plant mass accumulation.

Nitrogen nutrition index (NNI) Nitrogen nutrition index (NNI) is an index which allows the estimation of the crop nitrogen status. This index is calculated at any moment as the ratio between the actual plant nitrogen concentration of the crop and the critical plant N concentration (see this definition) corresponding to the actual crop mass.

Photosynthetic active radiation (PAR) Photosynthetic active radiation (PAR) is the part of solar radiation spectrum corresponding to wavelengths that are active for photosynthesis.

Radiation use efficiency (RUE) Radiation use efficiency (RUE) is the ratio between the quantity of biomass accumulated within a crop and the quantity of photosynthetic active radiation (PAR) intercepted by this crop during the same period of time.

RuBPc-o Ribulose bisphophate carboxylase/oxygenase, the enzyme located within chloroplasts which allows the carboxylation of CO_2.

Definition

A prerequisite for the analysis of crop responses to nitrogen (N) is the determination of the plant nitrogen content and repartition. How much N is incorporated within plants and crops? Within which plant tissue? For

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

which physiological function? Thus, according to the answers to these questions, it is possible to determine a *critical plant nitrogen status* as the minimum plant N concentration that allows the maximum plant (or crop) growth rate. It has been demonstrated that this critical plant N concentration decreases as plant grows as the result of an ontogenetic plant architecture development leading to a *dilution of N* compounds within increasing proportion of free-N compounds as plant gets bigger. This N dilution process can be formulated through a negative power relationship between plant N concentration and crop mass. This critical N dilution curve allows the discrimination of situations of N deficiency (below the curve) and situations of N luxury consumption (above the curve). So a Nitrogen Nutrition Index (NNI) can be calculated for quantifying the intensity of N deficiency or N luxury of any crop at any stage of its life cycle. This possibility for determination of crop N status and for quantification of N deficiency either in terms of intensity or timing allows the complete inversion of the approach: instead of response of crop to N supply, the problem is to study the response of crop to N deficiency. By this way, the check treatment is the non-limiting N conditions where crop growth potential is limited only by genetics and climate. Then the effect of intensity and timing of N deficiency period on the different plant growth processes and yield component elaboration can be studied with a more generic approach. This new method for analysis of the effect of plant N nutrition on crop yield allows the identification of physiological, agronomical, and genetical ways for improving nitrogen use efficiency of crops.

Introduction

Nitrogen is considered to be the most important limiting factor, after water deficit, for crop production worldwide. Over the last 50 years, the worldwide use of mineral fertilizers is one of the key elements for producing sufficient food to meet the demand of increasing human population [1, 2]. During this period, the use of mineral N fertilizers in agriculture systems increased sevenfold in parallel with the doubling of agricultural food production. This huge amount of mineral N is provided by industrial processes of chemical reduction of atmospheric nitrogen. So even if this resource can be considered as quantitatively non-limited, the energy cost of the Haber–Bosch process necessary to obtain mineral N fertilizers and the large greenhouse gas emission associated with oblige to reconsider the sustainability of the use of mineral N in agriculture. Moreover, the use of large amounts of N in intensive agriculture production systems has led to important environmental problems such as the eutrophication of freshwater [3] and marine ecosystems [4], pollution of ground water, and gaseous emissions of N oxides and ammonia in the atmosphere [5, 6]. In consequence, problems associated with sustainable development, global changes, environment protection, and global food security are now questioning the efficiency of use of N fertilizers in agricultural systems [7].

During the last decades, the relatively high products/fertilizer price ratio, incited farmers to adopt an insurance strategy in fertilizer management: applying excess of N to avoid any restriction in crop N supply and then any penalty in crop yield. These practices, when they were generalized on large agricultural areas led to a progressive increase in soil N surplus accumulation and an elevated risk of N leaching with dramatic consequences on ground water quality [8]. Adoption of a more restricted strategy for supplying and timing of N fertilizers is now a prerequisite for a more sustainable agriculture development. But such a strategy is difficult to be adopted in practice because of the non-predictable variations in weather which determines both soil N mineralization and crop growth potential. In consequence, a reduction in N application rates to avoid surplus of N in soils would increase the probability of temporary crop N nutrition deficiency, and then, as a consequence, an increased risk for a lower crop production and then a deterioration of the economic outputs the farmers are expecting. Therefore, optimizing crop production with the goal of reducing environment hazards requires an improvement in the understanding of the regulatory mechanisms by which crops absorb nitrogen from the soil and use it efficiently for yield component and quality elaboration. But it requires also a better understanding of soil microbial processes and the interaction between C and N cycles in order to optimize crop N residue cycling, soil organic matter dynamics, and to improve the capacity to predict soil N supply.

Fertilization management has to be conceived as a mean to match as closely as possible crop N supply with crop N demand in order to limit accumulation of mineral N within soils, and then emissions to hydrosphere and atmosphere. By this way, matching timing of N supply with timing of crop N demand appears very necessary. It is fundamental to underline here the necessity for a high precision for adjustment of N supply to N demand: for an intensive wheat crop producing about 8 t ha^{-1} of grain, the total N demand is about 240 kgN ha^{-1} and the loss of only 20 kgN ha^{-1} through leaching (i.e., less than 10% of the total N demand) can lead to pass over the admissible limit of nitrate concentration (50 mg l^{-1}) in drainage water! Moreover, the loss of only 2–3 kgN ha^{-1} through emission of N_2O to atmosphere (that corresponds to 1% of the total N demand) is considered as highly detrimental for greenhouse gas effect. So the problem to face now is not to understand how the yield of the different crop species can be improved by addition of N fertilizers, but (i) to determine the dynamics of crop N demands all along the crop development cycle; (ii) to determine the timing of the soil N supply according to soil characteristics, climate, and soil agronomic management; (iii) to determine the crop responses to different intensity and timing of N nutrition deficiency; and then (iv) to manage timing of crop N fertilization using diagnostic and decision tools for optimizing trade-off between minimizing crop yield reduction and minimizing environmental impacts.

N in Plants

For most of the crop species, plant life cycle can be roughly divided into two main phases: (i) the vegetative growth phase, when young developing roots and leaves behave as sink organs that absorb and assimilate inorganic nitrogen for amino acid and protein synthesis, and (ii) the remobilization phase when senescing tissues start to behave as source organs translocating organic molecules to ensure formation of new developing and/or storage organs [9]. The first phase is dominated by the dynamic of leaf area expansion as a mean for light capture, and then the role of nitrogen in both leaf growth and leaf photosynthesis is capital. The second phase is dominated by the development of reproductive organs such as seeds, fruits, tubers, and bulbs, or by storage organs allowing survival for perennial species such as trunks for trees or roots and stubbles for herbaceous species like grasses. As a consequence, the same N absorbed by the plant can be used successively for different functions and then analysis of crop response to N nutrition cannot be simply reduced to an addition of different elementary functions, but as a complex and integrated adaptive system with strong interactions among different processes.

In plants, N is required primarily for the synthesis of proteins, both structural and enzymatic, as the more important components of cells. There is large variation in the composition of the different cell types within a plant according to the different types of tissues. Cells which store carbohydrates or are part of support and transport tissues have relatively lower protein and nucleic acid concentration than metabolically active cells within meristems and photosynthetic parenchyma [10]. The relative numbers of each type of cells determine the organ composition. So organs that have relatively slow metabolism but are specialized in support and transport, such as roots and stems, contain little protein and thus have small N concentration in dry matter. Organs which store starch or other carbohydrate, such as grains, fruits, and tubers, have also low N concentration. On the contrary, leaves having a high metabolic activity through photosynthesis have high N concentrations. So, plants are very heterogeneous systems in terms of N concentration at every level of organization: cells, tissues, and organs. Therefore plant N demand, i.e., the quantity of N required for the plant to achieve its potential growth and development, depends on the morphologic and histologic plant composition.

Leaves are essential organs by which plants capture light and assimilate carbon. Light capture, photosynthesis, and the associated respiratory processes require a large number of different enzymes, pigments, and proteins [11]. About 50% of the soluble protein of the leaf is in RuBPc-o enzyme alone, another 25% is in the light harvesting and electron transport components [11]. Thus the chloroplast, as the location of RuBPc-o and other photosynthetic enzymes, and according to its high protein membrane component, contains the major part of the total leaf N. Composition of leaves and their N concentration varies with age, development

stage, and environment. Leaves of cereals have the greatest concentration of N just after their full expansion that corresponds to their maximum in photosynthetic activity. After a period the N concentration in the leaves decreases in parallel with metabolic activity as senescence progresses [12]. Remobilization of N-components occurs during the senescence process, and then amino acids from proteolysis are transported to younger developing leaves, or reproductive organs. A consequence of this ageing and senescing processes is that older leaves situated in the lower canopy where light intensity is limiting for photosynthesis, provide N for new leaf production at the top of the canopy. This recycling process thus tends to optimize photosynthesis in relation to N supply and light at the level of the canopy [13].

To achieve large rates of photosynthesis in well-illuminated conditions, leaves require a large concentration of RuBPc-o and other N-components and, hence, large N concentration [14]. As N supply rises, the amount of N per unit area in leaves increases, enhancing the rate of CO_2 assimilation [12, 15]. Thus, there is a good correlation between CO_2 assimilation rate and leaf N content. However, with very large N supply, leaf N content may increase without any increase of photosynthetic rate, unless larger CO_2 concentrations are used [16]. Plants with C_3 photosynthetic pathway contain more N per unit leaf area than those with C_4 metabolism. So C_4 crops use N more efficiently than C_3 ones [17]. Ample N increases the number of chloroplast compared with deficient N. Also the density of protein in the stroma is increased by large N supply. All these processes contribute to accumulate N within leaf tissues as plant N supply increases.

Although the primary role of RuBPc-o in plants is to assimilate CO_2, a storage function for N has been mooted for this enzyme [18, 19]. Thus, N of RuBPc-o have two successive functions within plants: CO_2 assimilation and source of N supply for reproductive and storage organs. Storage of N within the plants for further reuse can be considered as an adaptive mechanism for buffering plant N nutrition in highly variable soil N supply conditions. In this way, plants can store N reserves during periods of vegetative growth and large soil N availability, and then to reuse these N compounds at the end of their life cycle when mineral soil N is exhausted. Accumulation of RuBPc-o in excess within leaf tissues is not the only way for plant to store N. Accumulation of vegetative storage proteins (VSP) within different plant tissues or organs is a complementary way for storing N reserves, as demonstrated in alfalfa [20] and in perennial grasses [21]. For perennial plants, the storage of N reserves within perennial organs such as roots, rhizomes, tubers, or trunks is a prerequisite for regrowth after defoliation or after winter.

Due to the importance of light capture and photosynthesis for plant growth, much attention has been paid to allocation of N to leaves within canopies. However, allocation of N to other vegetative tissues is also quantitatively important. Green leaf N content represents only 53% of total shoot N in a lucerne crop [22], and only 30% of shoot N in a wheat crop at the beginning of the grain filling period [23]. Lemaire et al. [24] showed that for a large number of crop species there exists trade-off between accumulation of N in leaves for optimizing crop photosynthesis and accumulation of N within stem tissues for optimizing the plant architecture development and the elaboration of reproductive organs.

N compounds as amino acid and proteins are used within meristematic tissues for initiation and expansion of new organs: leaves, stem internodes, tillers, and branches and roots, and also inflorescences, fruits, and seeds. The use of N for leaf area expansion during vegetative growth period is of first importance because it determines the dynamics of LAI expansion of the crop and then the capacity of the crop to intercept light and to accumulate biomass. The rate at which N is supplied to meristems greatly determines cell production rate while the final cell size is only little affected [25–27]. As cells enlarge in size, their N concentration decreases through a dilution by accumulation of greater quantities of N-free compounds until final size is reached. So any limitation of N supply to meristem tissues leads to a reduction in the cell production rate and in the size of the final organ produced. For reproductive meristems, shortage of N reduces more the number of grains than the grain size [28–30].

When analyzed at the level of a plant population, it has been clearly demonstrated that N is allocated to individual plants according to their own contribution to interception of light [31]. N resources are allocated preferentially to dominant plants and then stressed

plants cannot respond to N supply because of the lack of light. In consequence, competition for light among individual plants within a dense canopy determines greatly competition for N resources.

In conclusion, plants and canopies are very heterogeneous systems in terms of N content, concentration, and repartition. This heterogeneity can be analyzed at different levels of organization: organite, cell, tissue, organ, whole plant, and plant population. N compounds, mainly as proteins are involved in all metabolic processes and then they are used successively for different functions such as cell division, organ growth and development, light capture, photosynthesis and respiration, reserve formation and recycling. Once absorbed by the plant, N is used several times through internal recycling processes and reallocation to the different plant parts. So analysis of plant N nutrition is very complex because it involves several interdependent metabolic functions and due to constant feedback mechanisms, it can be studied only through a dynamic approach.

Crop N Demand

Crop N demand (N expressed in kg ha^{-1}) at any time of the crop cycle can be defined as the result of critical crop mass (Wc) that is the maximum crop mass attainable in a given environment without any limitation of nutrients and its critical plant N concentration %Nc [32]:

$$N = \%Nc\, Wc \quad (1)$$

The critical plant N concentration (%Nc) has been defined as the minimum plant N concentration corresponding to maximum crop mass [33]. This concept can be applied in dynamic terms, such that the daily crop N demand (or critical N uptake rate expressed in kg ha^{-1} day^{-1}) is the quantity of N the crop has to absorb each day to maintain its potential growth rate over a given period of time.

Many studies conducted on a large range of crop species (see [34] for a review) have shown that the critical plant N concentration (%Nc) is regularly decreasing as crop mass accumulates during the crop growth period. Moreover, it has been shown that this dynamic of decline in %Nc could be represented by a unique and constant relationship whatever the conditions and the genotypes for a same species [35]:

$$\%Nc = a(Wc)^{-b} \quad (2)$$

Coefficient a represents the critical plant N concentration for Wc = 1t ha^{-1}. Coefficient b is dimensionless, and it represents the dynamic of the dilution of N within the dry matter.

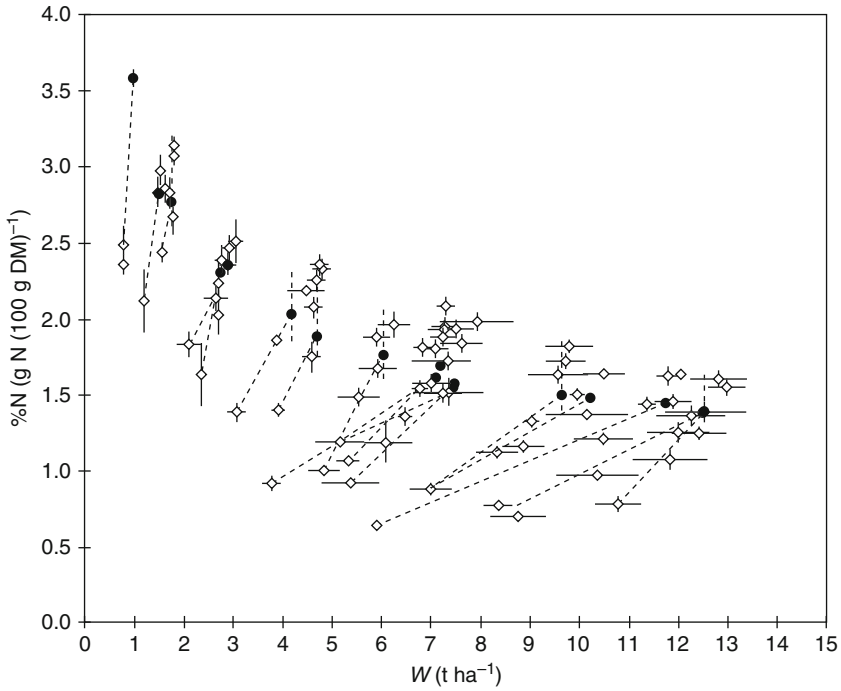
Mixing Eqs. 1 and 2 allows the expression of the dynamics of the critical crop N uptake, i.e., the crop N demand, in relation with the dynamics of potential crop biomass accumulation:

$$Nc = a'(Wc)^{1-b} \quad (3)$$

where coefficient a' is the crop N demand (or the critical N uptake) for a potential crop mass accumulation of 1 t ha^{-1}. Value of a' is equal to $10a$, when a is expressed in % and a' is expressed in kg ha^{-1}.

Values of a (and a') and b have been established for the main cultivated species according to the method developed on wheat by Justes et al. [36]. This method as illustrated on Fig. 1 requires a family of response curves of plant N concentration (%N) vs crop mass (W) across increasing N supply rates. For each response curve, the critical plant N concentration (%Nc) is estimated as the intersection point between the oblique line representing the response of both %N and W to increased rate of N supply, and the vertical line representing the increase in %N with further N supply rates once the maximum crop mass (Wc) is reached that correspond to luxury N accumulation. As the determination of %Nc is done at different stages of growth for a large range of value of W, then it is possible to fit the series of %Nc–Wc data points for getting the critical N dilution curve and to calculate the value of coefficients a and b.

These values are presented in Table 1. For a given species, coefficients a (and a') and b remained constant whatever the climatic conditions (years and locations). Moreover, Lemaire et al. [37] showed that for wheat, maize, canola, sorghum, and sunflower the same value of coefficients holds either in temperate or in subtropical conditions. Comparison among species allows a clear distinction between C_3 and C_4 groups for the value of coefficient a (and a') while coefficient b is unaffected by the type of the metabolic pathway. Within each of the metabolic groups, it is difficult to



Crop Responses to Nitrogen. Figure 1
Determination of the critical plant nitrogen (N) concentration of maize crops as the intersection point between the *oblique line* corresponding to the response of both plant nitrogen concentration (%N) and crop mass (*W*) to increasing nitrogen supply rates, and the *vertical line* corresponding to the increases in plant N concentration without corresponding increase in crop mass that corresponds to luxury nitrogen accumulation [46]

establish clear differences between species because of the high correlation between the coefficient a (or a') and b. Then the fitted curves corresponding to Eq. 2 for the different crop species are relatively close to each other [38].

The derivative of Eq. 3 allows the expression of the crop N demand in dynamic terms:

$$\frac{dN}{dt} = \frac{dN}{dW}\frac{dW}{dt} = a_c(1-b)W^{-b}\left(\frac{dW}{dt}\right) \qquad (4)$$

Equation 4 shows that the daily crop N demand follows the daily crop growth rate, but for a similar daily crop growth rate, the daily crop N demand declines as the crop mass increases.

A theory has been developed for explaining the close relationship between crop N demand and crop biomass accumulation rate [33, 35]. The hypothesis is that plant mass W is composed of two compartments: Wm, the metabolic tissues involved directly in

Crop Responses to Nitrogen. Table 1 Values of coefficient a' and b of Eq. 3, $N = a'(Wc)^{1-b}$, for different crop species

Crop species	a' (kgN ha^{-1})	b (dimensionless)	References
Temperate grasses (C_3)	48	0.32	[39, 40]
Alfalfa (C_3)	48	0.33	[41]
Pea (C_3)	51	0.32	[42]
Wheat (C_3)	53	0.44	[36]
Canola (C_3)	45	0.25	[43]
Rice (C_3)	52	0.52	[44]
Tomato (C_3)	45	0.33	[45]
Maize (C_4)	34	0.37	[46]
Sorghum (C_4)	39	0.39	[47]
Tropical grasses (C_4)	36	0.34	[48]

plant growth processes (photosynthesis and meristem activity) with a high N concentration %Nm; and Ws, the structural tissues involved in plant architecture and transport with a low N concentration %Ns. Then:

$$W = \mathrm{Wm} + \mathrm{Ws} \tag{5}$$

and:

$$\%\mathrm{N} = \frac{1}{\mathrm{W}}(\%\mathrm{NmWm} + \%\mathrm{NsWs}) \tag{6}$$

Supposing that Wm increases allometrically with *W*, then:

$$\mathrm{Wm} = kW^{\alpha} \tag{7}$$

and then:

$$\%\mathrm{N} = k(\%\mathrm{Nm} - \%\mathrm{Ns})\,W^{\alpha-1} + \%\mathrm{Ns} \tag{8}$$

This equation is very close to the empirical Eq. 2. The main difference is the asymptotic value of %Nc which is equal to %Ns and not to zero. But the difference is very little for the range of *W* from 1 to 15 t ha^{-1}[35].

Following Hardwick [49] assumptions, it can be postulated that because Wm is associated mainly with photosynthesis it scales for plant area, and then to crop Leaf Area Index (LAI):

$$\mathrm{Wm} = p(\mathrm{LAI}) \tag{9}$$

and then:

$$\mathrm{LAI} = \frac{k}{p}W^{\alpha} \tag{10}$$

So Eqs 3 and 10 indicate that both N uptake and LAI are allometrically related to crop mass. For a large range of crop species, cultivated either under temperate or sub-tropical climate, the hypothesis for a common value of coefficients *b* and α cannot be rejected, but then a direct relation of proportionality could be established between crop N uptake and crop LAI [37]:

$$\mathrm{N} = \frac{a'p}{k}\mathrm{LAI} \tag{11}$$

So, at crop level, the dynamic of expansion of LAI is driving the crop N demand.

Lemaire et al. [37] showed that the two coefficients *b* and α evolves in parallel during the time course of the crop growth. Just after seedling, and until a crop mass of approximately 1.5 t ha^{-1} is reached the value of *b* and α is high, close to 0.90–0.95 while it decreases rapidly to a value of 0.6–0.7 after this stage when competition for light between individual plants within the canopy is established.

Lemaire et al. [37] tried to determine among the two parameters, crop mass or LAI, who is the fundamental driver of crop N demand. They concluded that Eq. 3 was the more robust across genotypes and environments, but in fact the two Eqs. 3 and 11 each represent an incomplete expression of the feedback regulation by shoot growth of N absorption capacity of the roots and of N partitioning within the canopy as described within the above section. A more complex analysis of N allocation between leaf and stem and of remobilization of N from old leaves would be necessary to better capture genotypic differences in N uptake capacities. Nevertheless, the robustness of the relationship between critical crop N uptake (Nc) or critical crop N concentration (%Nc) with crop mass accumulation (W) across environments and genotypes allows the use of these empirical relationships as a base for the determination of crop N demand and for diagnosis of crop N status.

Regulation of Crop N Uptake

The theory developed above indicates that plant N uptake seems to be regulated by plant growth itself. In steady state N supply conditions, plant N uptake is feedback regulated by shoot signals, with a positive signal from photosynthetic C supply and a negative one from reduced N recirculation to roots [50–52]. So the relationship between N uptake and LAI as attested by Eq. 11 can be explained by the fact that LAI expansion allowing increased light interception provides larger C supply to roots, and also increases N storage capacities within expanding leaves as RuBPc-o that avoids the depletion of N uptake by recirculating reduced N compounds in the phloem. This leads to the proportionality between crop N uptake and LAI for most of the species, but the slope of the relationships, i.e., the N uptake per unit LAI, is variable across species according to their morphology and, more particularly, their leaf/stem ratio. So leaf area expansion is not the only way for plant to store

reduced N. Stem growth and leaf thickness increase are also a possibility for sequestrating N to avoid the repression of N uptake capacity of roots. In consequence, a more general and stable relationship between N uptake and crop mass can be obtained as attested by Eq. 3. Nevertheless, this relationship is not linear because N uptake per unit of crop mass decreases as the LAI per unit of crop mass, i.e., the leaf area ratio (LAR) of the crop, decreases, that determines the N dilution effect.

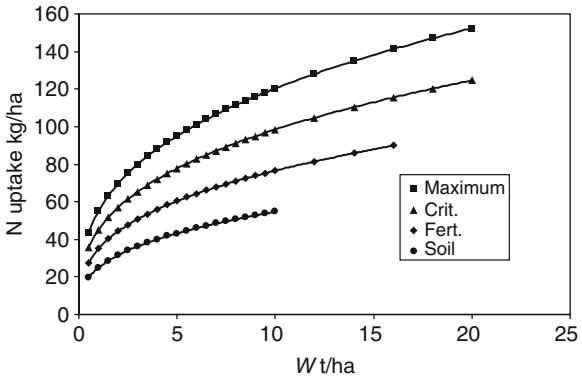
In the field, in a variable N supply condition, plant N uptake is co-regulated by both crop growth rate potential and the soil N availability. Two groups of transport systems, with low and high affinity for nitrate, operate in plants [53]. Devienne-Barret et al. [54] have proposed a model accounting for this co-regulation of plant N uptake by soil nitrate concentration and plant growth:

$$\frac{dN}{dt} = a_c(1-b)\,W^{-b}\left(\frac{dW}{dt}\right)_{max}\left[V_H\frac{C}{K_H+C}+V_L\frac{C}{K_L+C}\right] \quad (12)$$

where V and K are coefficients of the Michaelis–Menten formula and subscripts describe the high (H) and the low (L) affinity transport systems for nitrate; C is the actual concentration of nitrate in soil solution, W is the crop mass in t ha^{-1} and b is the allometric coefficient of Eq. 3.

Figure 2 represents the crop N uptake vs crop mass trajectories for different steady-state N supply conditions, i.e., C = constant. Among these curves, it is then possible to identify the critical N uptake curve as defined above. It is also possible to imagine that the maximum N uptake curve corresponds to the higher quantity of N a crop is able to accumulate at a given crop mass. The difference between this curve and the critical curve correspond to the quantity of luxury N the crop is able to store.

So whatever the cause of the variation in crop mass provided that N supply remains at steady state, any increase in crop mass (ΔW) is accompanied by a corresponding increase in crop N uptake (ΔN). As soil N supply increases, plant N uptake increases as a consequence of both (i) the increase in soil N concentration (C) and (ii) the increase of plant growth rate itself. So, at any moment, crop growth rate is the consequence of crop N uptake and vice versa.

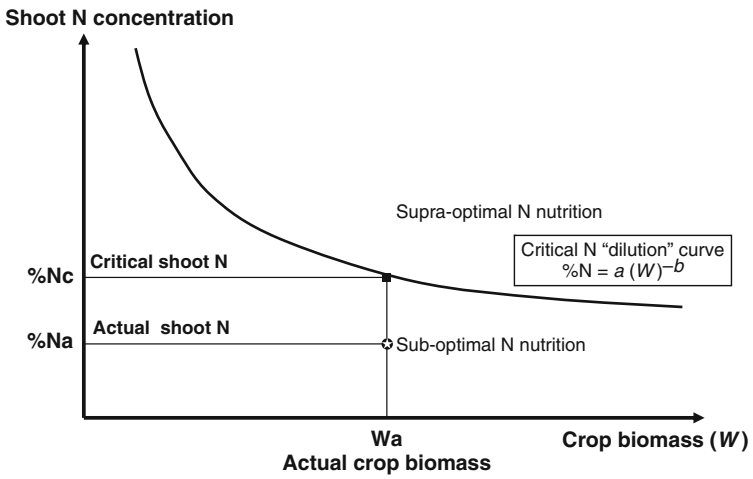


Crop Responses to Nitrogen. Figure 2
Nitrogen (N) Uptake – Crop Mass (W) trajectories for different steady states N supply: critical N uptake (Crit.), maximum N uptake (Maximum), non-fertilized (Soil), and suboptimal N application (Fert.). The two lines represent either (i) two growth stages of the same crop, (ii) two crops having different growth rate, or (iii) an environmental effect. The lines represent the response curves to increased N supply

Diagnosis of Crop N Status: Nitrogen Nutrition Index

The main consequence of the theory developed above is that neither the plant N concentration nor the crop N uptake per se can indicate unequivocally the crop N nutrition status. Eqs. 2 and 3 indicate that these two variables have to be interpreted in relation with crop mass.

As shown in Fig. 2, the critical N uptake curve separate situations where N supply is limiting crop mass accumulation for situations where N is accumulated in excess without any supplemental increase in crop mass. For a given situation and at any time course of the growth period of the crop characterized by an actual crop N uptake (Na) corresponding to an actual crop mass Wa, it is possible to determine a Nitrogen Nutrition Index (NNI) as the ratio between Na and the critical N uptake, Nc, corresponding to the same crop mass provided the critical N uptake of the crop species has been determined. NNI can also be



Crop Responses to Nitrogen. Figure 3
The use of the critical N dilution curve for the determination of the nitrogen nutrition index

determined directly from actual plant N concentration and dilution curves as illustrated in Fig. 3:

$$\mathrm{NNI} = \frac{\%\mathrm{Na}}{\%\mathrm{Nc}} \tag{13}$$

Values of NNI close to 1 indicate that at the date of the determination of Na or %Na the crop were in situation of non-limiting N supply. Values more than 1 indicate a luxury consumption of N. Values less than 1 indicate an N deficiency, the intensity of which can be estimated by the value of the NNI: a value of 0.6 indicating that crop N availability was only 60% of the critical level. Such an index of crop N status has been used as a diagnostic tool for analyzing a posteriori agronomical data from field experiments or farm observations in order to explain variations in yield by differences in crop N status [55].

Nevertheless, this approach does not take into account that the minimum plant N concentration is not 0 but is equal to %Ns (Eq. 8), which is the minimum plant N concentration for the plant to stay alive as postulated by Angus and Moncur [56]. So a more complex nitrogen nutrition index can be calculated:

$$\mathrm{NNI}' = \frac{\%\mathrm{Na} - \%\mathrm{Ns}}{\%\mathrm{Nc} - \%\mathrm{Ns}} \tag{14}$$

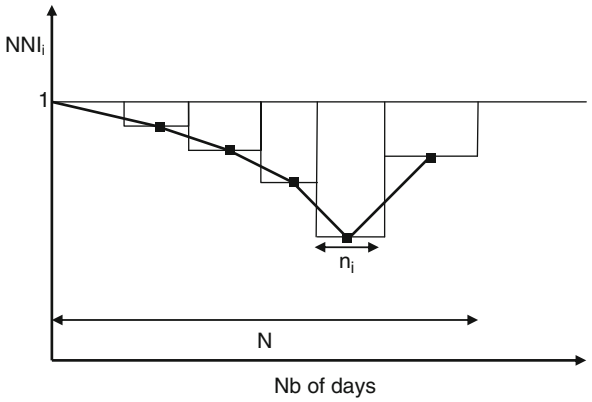
This new index, NNI′, is therefore physiologically more relevant than NNI, but it involves a greater degree of uncertainty related to the value of %Ns which has not been documented for many crop species. Lemaire and Gastal, [35] by using Eq. 8 derived a value of 0.77% and 0.82% for %Ns for wheat and maize, respectively. So variation in %Ns across crop species could be not very important. Therefore, the use of Eq. 2 for determining crop N status can be recommended owing to its simplicity.

NNI estimates the instantaneous crop N status at the period of time when actual plant N concentration %Na and actual crop mass Wa are estimated. But under changing N supply in the field, it is necessary to determine NNI several times during the growth cycle. An integrated value of NNI can be obtained by the weighted mean of NNI during the different growth periods, each time interval representing the duration in days or degree-days. Lemaire and Gastal [35] showed that it was possible to establish a linear relationship between the $\mathrm{NNI}_{\mathrm{int}}$ and the relative biomass accumulation as expressed as the ratio between actual (Wa) and potential (Wm) biomass:

$$\mathrm{Wa}/\mathrm{Wm} = K(\mathrm{NNI}_{\mathrm{int}} - \mathrm{NNI}_0) \tag{15}$$

where K is the response of the crop to increment in its average N nutrition status and NNI_0 is the minimum crop N status to allow plant growth. This minimum theoretically corresponds to %Ns.

Jeuffroy and Bouchard [57] characterizes the N deficiency period of a wheat crop by both its length



Crop Responses to Nitrogen. Figure 4
Estimation of NNIint from a sequential determination of NNI_i : $NNN_{int} = 1/N\ \Sigma NNI_i n_i$ The intensity of N deficiency (ID) is estimated by the lowest value of INN_i, and the duration of the deficiency (DD) is equal to the number of days with $NNI_i < 1$

(deficit N duration, (DD) and its intensity (ID) by means of the 1-NNI minimum observed value, and they calculated an integrated index of crop N status by the mean of the product ID × DD = IDD that represents about twice the area between the curve of NNIi dynamic and the horizontal line NNIi = 1 (Fig. 4). They showed that IDD explained 96% of the variation in grain number of wheat within a large experimental sites x years data set.

So it is clear that NNI is a good basic tool for analyzing actual plant status in crops, and then to interpret agronomical data in field conditions in order to detect if or not plant N deficiency periods occurred, with which intensity and timing and to analyze the consequences on crop growth and the crop yield components. But despite its high informative value as diagnostic tool for crop N status, NNI is difficult to use practically in field conditions and it remains more a research than an agronomical management tool. NNI determination is time consuming because of the necessity to determine crop mass. Then it is necessary to use noninvasive and cost-effective methods for a rapid and operational determination of plant N status, and then to use NNI as a reference for calibration of these indirect methods.

The theory developed above showed that the N dilution effect and then the dependency of plant N concentration from the crop mass is the result of two processes: (i) the decline in plant leaf area ratio (LAR) as crop mass increases, and (ii) the preferential allocation of N to the well-illuminated upper layer of leaves as canopy develops. Therefore, Lemaire et al. [58] suggested that while plant N concentration declines with crop mass accumulation, the N content per unit of leaf area within the upper layer of leaves was more stable and would correlate well with the crop NNI. Then, it would be possible to use the N concentration of the well-illuminated leaf layer as an indicator of crop N status independently of the crop mass. Farrugia et al. (2004), using this correlation, were able to develop a method of diagnostics of grassland N status [59] and of maize crop N status [60]. The leaf N concentration can be measured directly from leaf samplings, but it can also be estimated indirectly by noninvasive methods. The leaf color chart (LCC) is an easy-to-use and inexpensive diagnostic tool for monitoring the relative greenness of a rice leaf as an indicator of the leaf and then of the plant N status [61]. The estimation of chlorophyll content of leaves by portable systems based on leaf transmittance or leaf reflectance in specific wave bands is also well correlated with leaf N content, and can be a method for crop N status diagnosis [62, 63]. But these predictions are in general dependant on cultivars and years [64]. Houlès et al. [65] demonstrated that through remote sensing it was possible to estimate both crop LAI and the quantity of chlorophyll per unit of soil area, and then the quantity of N within the canopy per unit of soil area. Therefore it would be possible to recalculate the NNI of the crop. Such an estimate can be intensively repeated in space and time that allows very precise information on the spatiotemporal dynamics of crop N status that is very useful for precision agriculture.

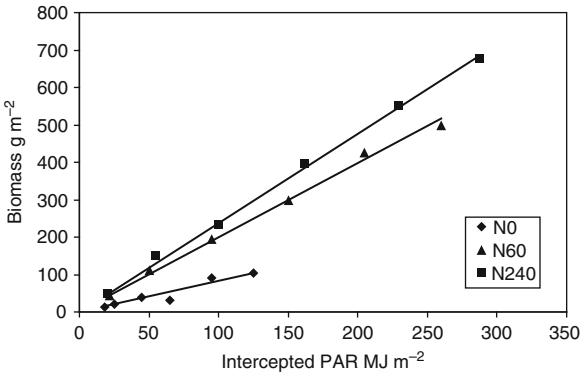
Crop Responses to N Deficiency

The use of the concept of critical crop N concentration and the possibility for diagnosis of the actual crop N status totally reversed the approach of the response of crop to N fertilizers. The response of crop to N was studied by analyzing the response curve of yield to increasing fertilizer rates.

The difficulty for such an approach is that (i) the actual N supply for the crop is not known, because the

quantity of N provided directly by the soil, and then the yield in absence of any fertilization is very variable, and (ii) the potential yield, i.e., the yield achievable in a given climatic condition when N supply can be considered as non-limiting is unknown. So, the crop N demand and the total crop N supply are unknown variables and it is possible to get only the regression between increment in yield and increments in N fertilization rates. This approach led to provide huge families of crop response curves to N fertilizers very variable to each other according to soils, climates, years, species, and cultivars, and the only way was to analyze this variability with statistic approaches with any possibility to identify and to quantify processes in order to develop prediction models.

The possibility for diagnosis crop N status allowed the identification of situations where crop growth and development were not limited by N deficiency, and then to develop crop potential growth models as resulting from climate conditions. Then using the critical N uptake concept, it was possible to derive from these models the dynamics of crop N demand all along the crop cycle. So instead of having the check treatment with no N fertilizer and to analyze response of crop to increments in N supply, the check treatment is now the crop with critical N status, and then the response of the crop to the occurrence of periods of N nutrition deficiency of different timing and intensity all along the crop growth cycle and the consequences on yield components and quality of the yield can be analyzed.



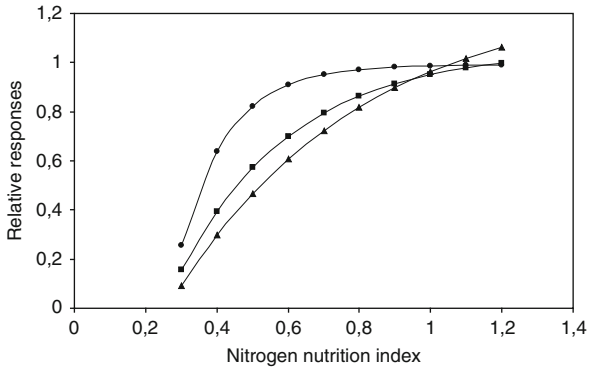
Crop Responses to Nitrogen. Figure 5
Relationships between the quantity of photosynthetic active radiation (*PAR*) intercepted by a tall fescue sward and the accumulation of aboveground biomass for three contrasted N supply rate: N0: no N application; N60: 60 kgN ha^{-1}; N240: 240 kgN ha^{-1}. The slope of the regression represents the radiation use efficiency [67]

N Deficiency Effects on Crop Mass Accumulation

According to Monteith [66], crop mass accumulation is linearly related to the quantity of photosynthetic radiation (PAR) intercepted by a crop during its life cycle. The slope of this regression can be interpreted as the radiation use efficiency (RUE).

As illustrated in Fig. 5, and as shown by Bélanger et al. [67], N deficiency affects both the quantity of PAR intercepted by the crop and the efficiency with which the intercepted radiation is used for biomass elaboration (RUE). These authors, using NNI, have shown that in relative terms, RUE was more affected by moderate N deficiency (NNI = 0.6–0.8) than the quantity of PAR intercepted while for more severe deficiency, the response of the two variables converged. This type of response has been confirmed on sunflower [68] and on sorghum and maize [69]. LAI expansion seems a little bit more responsive to N deficiency than RUE: for an NNI of 0.6, RUE was reduced by 30% while LAI was reduced by 40% (Fig. 6).

RUE is an integrated variable accounting for photosynthesis and respiration, but also the allocation of assimilates to root [70]. These authors showed that, in fact, canopy gross photosynthesis was less affected by N deficiency than the accumulation of total biomass reflecting higher respiration losses in N-deficient crops, and the accumulation of shoot biomass was more affected than the accumulation of total biomass reflecting then an important increase of allocation of biomass to roots in N deficient situations. The lower shoot/root ratio in N deficient crops is widely documented [71, 72]. In fact this increased allocation of assimilate to roots is the consequence of the lower activity of shoot meristems (leaf and stem extension) that allows a greater quantity of carbon to be used for root growth. So the more sensible plant growth process to N deficiency appears to be the leaf area expansion rate, with two major consequences: (i) a reduction



Crop Responses to Nitrogen. Figure 6
Effects of crop N status determined by the nitrogen nutrition index (*NNI*) of tall fescue swards receiving different N application rates and (i) the relative quantity of intercepted PAR, PAR_{act}/PAR_{max} (●), (ii) the relative Radiation Use Efficiency, RUE_{act}/RUE_{max} (■), and the relative LAI, LAI_{act}/LAI_{max} (▲). (Redrawn from [67])

in the dynamic of light interception and then in the canopy photosynthesis and crop C supply and (ii) a preferential allocation of C for root growth that contributes to increase root foraging activity for a further increase in N uptake capacity.

The response of leaf area of plants and canopies to N deficiency is brought about by a decline in the expansion rate and size of individual leaves combined with reduction of branching or tillering. The accumulation of nonstructural carbohydrates in N-deficient leaves indicates that C supply is not the cause of the leaf area expansion under N deficiency [38].

N deficiency decrease the rates of cell division and cell expansion with little effect on final cell length [26], so the reduction of leaf size in N-deficient plants is mainly due to a reduction in cell number. Gastal et al. [73] proposed a quantitative relationship between the leaf elongation rate (LER) of tall fescue and the NNI of the sward:

$$\frac{LER_{actual}}{LER_{critical}} = 1.39 - 1.9e^{-1.49NNI} \qquad (16)$$

Subscript "actual" corresponds to any suboptimal N condition, and subscript "critical" refers to a non-limiting N nutrition. For a severe limitation in N nutrition, NNI = 0.4, LER is then reduced to about 30% of its maximum value in non-limiting conditions. That demonstrates the high responsiveness of leaf expansion to N deficiency.

Lemaire et al. [24] used the relationship between LAI and crop mass (W) of Eq. 10 for studying the different types of response of crop species to N deficiency. This approach allows the separation of the reduction of LAI being directly associated with the reduction in crop mass (i.e., simply a crop size effect) from any specific reduction of LAI at similar crop mass (i.e., a modification of plant architecture through a reduction of leaf area ratio. They demonstrated that under a similar intensity of N deficiency as estimated through NNI, the reduction of LAI of maize is totally allometrically related to its reduction in crop mass (crop size effect), while for wheat N deficiency provokes in supplement a reduction of LAI at same crop mass, i.e., a reduction in LAR. These two types of responses represented two extremes and tall fescue behaves like maize while canola behaves like wheat; sorghum and sunflower having intermediate responses [24]. Hence classification of crops in either metabolic (C_3 vs C_4) or botanical (monocots vs dicots) groups does not correspond to any particular response type. In response to N deficiency, some species such as maize or tall fescue tend to maximize light interception by minimizing the reduction in LAI, at the expanse of N concentration per unit leaf area, and then leaf photosynthesis, while other species such as wheat and canola tend to develop an inverse response. Do these two opposite strategies mean that there exists a trade-off between photosynthesis per unit of LAI and leaf area expansion as already proposed by Sinclair and Horie [74]?

N Deficiency Effects on Leaf and Canopy Photosynthesis

The response of leaf photosynthesis to irradiance largely depends on the leaf N content. Leaf photosynthesis at saturating light intensity (A_{max}) increases asymptotically with leaf N content [75]. This relationship shows a positive intercept on the leaf N content axis, indicating that when leaf photosynthesis rate becomes zero, leaves would still contain significant amount of N, corresponding the structural leaf

N (N_s) of Eq. 8. The variation in the A_{max}/SLN (leaf N content per unit leaf area) relationship seems relatively limited among cultivated species of the same metabolic group [10, 76], despite Sinclair and Horie [74] showed a lower A_{max} at similar SLN for soybean. This variation among species could be due to (i) differences in nitrogen costs of PEP-carboxylase and RuBPc-o and the relative amounts of these two enzymes in leaves, and/or (ii) the possible accumulation of vegetative storage proteins within leaves of legume species such as soybean.

There is a large discussion whether A_{max} has to be related to either leaf N content per unit mass or per unit leaf area [75]. Lemaire and Gastal [32] indicated that none of these two relationships are completely right. The two components of photosynthesis, light harvesting by chlorophyll and CO_2 reduction by RuBPc-o, are affected by leaf N status, and these two processes have to be expressed on a leaf tissue volume basis, and not only on an area basis. Then leaf thickness is an important parameter to take into account. Specific leaf area (SLA) is the parameter allowing correspondence between leaf N content per unit area and per unit dry matter basis. But relationship between leaf thickness and SLA is weak because of the variations of nonstructural carbohydrate content within leaves.

The quantum efficiency that is the response of leaf photosynthesis to light at low irradiance is only little affected by N deficiency [77, 78]. Moreover, the dark respiration of leaves seems to increase with increasing leaf N [78]. Hence, as the leaves are progressively shaded within the canopy the effect of N deficiency on leaf net photosynthesis becomes lower and lower and then negligible.

Gastal and Bélanger [77] showed that canopy gross photosynthesis of a tall fescue sward at high irradiance (CGP_{max}) only responds smoothly to N deficiency: a reduction in NNI from 1 to 0.4 reduced the relative CGP_{max} from 1 to 0.6 only. This low responsiveness of canopy photosynthesis to N deficiency is due to the fact that as canopy develops, a greater number of leaves are shaded and then their photosynthesis does not respond to N shortage. When irradiance becomes more limited, the responsiveness of canopy photosynthesis to N deficiency becomes more limited. So when canopy photosynthesis is integrated over day and for a long period where crop LAI is high, the response of canopy photosynthesis to N deficiency appears relatively limited, which explains the limited influence of N deficiency on RUE.

N deficiency Effects on Harvest Index and Components of Grain Yield

For grain crops, yield is closely related to grain number per unit area of soil. The elaboration of grains depends on flows of C and N compounds to reproductive meristems during a narrow window period around flowering, anthesis, and very initial grain development, and also on environmental conditions (temperature, radiation, and water stress) during this period. This critical period coincides with the maximum rate of crop N uptake [79]. So any limitation of crop growth rate at this period by N deficiency decreases grain number and then grain yield on cereals such as maize [28], wheat [29, 80]. Jeuffroy and Bouchard [57] established for wheat a relationship between grain number and the severity and duration of the N deficiency before anthesis as calculated by NNI method. The effect of N deficiency on grain number is the consequence of two simultaneous effects: (i) a lower crop growth rate at anthesis restricting C supply to spikelets and then a spikelet abortion [81]; and (ii) a decrease in the N content in the spike stems [29] corresponding to a direct effect of N deficiency on floret fertility.

The other grain yield component, grain weight, is generally less affected by N deficiency at anthesis than the grain number [47]. However, it is necessary to take into account the negative correlation between grain weight and grain number: a reduced grain number resulting from pre-anthesis N deficiency can lead to a more favorable source:sink ration during grain filling period and then to an unaffected grain weight. Grain filling in both carbohydrates and proteins depends on (i) the recycling of C and N compounds previously accumulated within vegetative organs during pre-anthesis growth period, and (ii) post-anthesis photosynthesis and N absorption. The relative importance of these two components depends on plant species and their capacities to store C and N compounds in their vegetative organs. So some species such as wheat or rice are able to develop large LAI [7–9] and then to store large quantities of N, then they are able to feed 80–90%

of the N demand for grain development by recycling N stored within vegetative biomass, while other species such as maize, because they develop less LAI [4, 5] with lower N content because they have C_4 metabolism, are obliged to feed 40–60% of their grain N demand through post-anthesis N absorption. As a consequence, crop species like maize are more susceptible to terminal soil N shortage than crop species like wheat or rice.

Delaying leaf senescence for species like maize or sorghum should allow these species to continue to maintain high N absorption rate after anthesis. The supplement of carbohydrates allocated to roots permitted by this delayed leaf senescence allows the maintenance of root absorption capacity [82]. So using stay-green genotypes in low N supply conditions seems to be beneficial for both sorghum [83] and maize [84].

Nitrogen Use Efficiency in Crops

From an agronomic point of view, nitrogen use efficiency (NUE) of a crop represents its capacity to produce a supplement of yield (dY) for each added unit of N fertilizer (dNf) that corresponds to the derivative of the crop yield response curve to the rate of N supply: $Y = f(Nf)$. As this response curve is asymptotic, NUE generally declines with the higher rate of N supply, indicating that the first N unit applied is more efficiently converted into yield than later applications. According to the crop species types, Y can represent either the aboveground biomass, as for forage crops, or the grain part as for cereals, grain legumes, or oil seed crops.

Such a global approach does not allow a clear mechanistic analysis of the physiological traits controlling NUE between different species and cultivars because crops respond to total N supply (Nt) that include fertilizer supply (Nf) and soil N mineralization (Ns). So according to variations in Ns due both to soil and climatic conditions and to previous crop management, different value of Nt can be obtained with the same Nf, leading to large differences in NUE. For this reason, Moll et al. [85] propose to define NUE as the yield produced per unit of N available in the soil, considering two components:

1. The Nitrogen Absorption Efficiency (NAE) which measures the ability of a crop to uptake N from soil
2. The nitrogen conversion efficiency (NCE) which measures the ability of a crop to use absorbed N for dry matter and grain production

$$\mathrm{NUE} = (\mathrm{NAE})(\mathrm{NCE}) \tag{17}$$

Nitrogen Absorption Efficiency

Genotypic differences in N uptake at different levels of N supply have been shown in rice [86], wheat [87], and maize [88]. Modern cultivars have a higher N uptake capacity because they have a higher biomass production. This effect is accounted by Eq. (x) as it shows that the crop N uptake rate increases with crop growth rate. So any factor enhancing the potential crop growth rate, genotype or environment, increase de facto the N uptake capacity of the crop. More interesting from a plant breeding point of view would be to increase the N uptake capacity of crop at similar crop biomass production. Lemaire and Gastal [32] reported data comparing N uptake of tall fescue and cocksfoot swards at similar biomass. These data show that cocksfoot had a higher N uptake capacity than tall fescue under a sub-limiting N supply condition, while the two species had similar N uptake capacity under non-limiting N supply. Similarly, Lemaire et al. [89] showed that grain sorghum had a higher N uptake capacity than maize under limiting N supply, while the two species had similar N uptake under non-limiting N supply.

The fact that N uptake capacity and then nitrogen absorption efficiency (NAE) does not vary too much among crop species is due to the fact that the critical N uptake curve, as described by Eq. 2 is not very different among species of the same metabolic group (C_3 vs C_4). Then differences between crop species in NAE under non-limiting N supply conditions would reflect their differences in their potential biomass accumulation according to their respective metabolic group. Under limiting N supply conditions, clear differences emerge among species. As for cocksfoot vs tall fescue, sorghum appears to have a greater root length density than maize. This would confer these species a better capacity of interception of soil mineral N. Soil N recovery that determines NAE is the result of the nitrogen balance between crop N uptake rate, immobilization by soil microbial communities and losses through leaching, denitrification, and

volatilization [90]. Root architecture and biochemical composition, and perhaps root exudates could play an important role in this balance, and then on NAE. So because of a permanent turnover of N in soil, plants having a dense root system are able to compete more efficiently against microbes for capturing mineral N when the principal source of N is the mineralization of organic matter, i.e., in limiting N supply conditions. In non-limiting N supply conditions, there is ample mineral N in soil, the immobilization capacity of microbial community is saturated and then the differences among species tend to disappear. Only the difference in N uptake capacity linked to difference in crop growth capacity can then be observed.

Some coincidences between quantitative trait loci (QTL) for root architecture and NAE have been detected on wheat [91]. A large genotypic variability has been identified across maize lines for the density and length of lateral roots [92], suggesting that it would be possible to breed this species for improving its NAE under low N supply conditions. Similarly, genetic studies showed that NAE was the most important component of the Nitrogen use Efficiency in rice [93] and wheat [87].

Nitrogen Conversion Efficiency

The efficiency of conversion of absorbed N by crop into yield has to be analyzed at two levels:

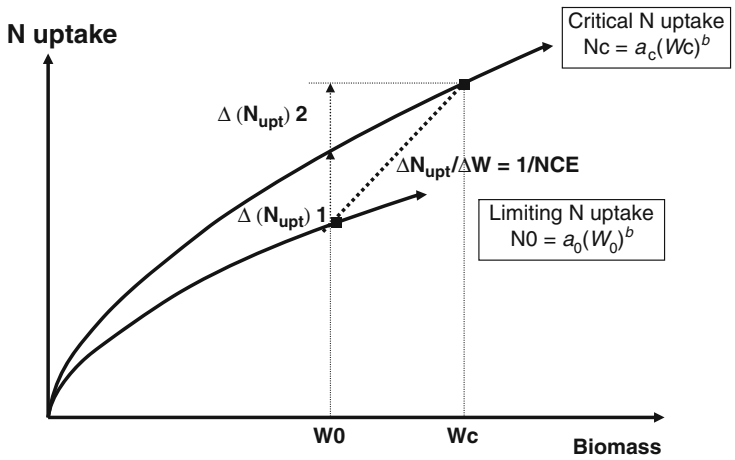
- The efficiency for crop biomass production (W), i.e., the supplement of biomass (dW) associated to the supplement of N uptake (dN) when N supply increases
- The harvest index HI, i.e., the proportion of crop mass allocated to grain

Figure 7 allows a more detailed analysis of the NCE for crop mass production. This figure represents two N uptake vs crop mass (W) trajectories for crops at two level of N supply: a limiting N supply rate and a non-limiting one corresponding to critical N status. At each date the slope of the line joining data points corresponding to the two N supply rates is equal to $\Delta N_{upt}/\Delta W$ which is the reciprocal of NCE. The supplement of nitrogen taken up by the crop, ΔN_{upt}, due to the increase in N supply rate, can be defined as the sum of two components (i) $(\Delta N_{upt})_1$, the quantity of N the limiting N supply treatment should have been absorb in supplement for reaching the corresponding critical level, and (ii) $(\Delta N_{upt})_2$, the supplemental increase in N uptake associated to the accelerated growth rate resulting to the higher N supply level. The NCE of $(\Delta N_{upt})_1$ is 0 as the increment in N uptake is made at a constant crop mass. The NCE of $(\Delta N_{upt})_2$ is not constant and can be approached only for a given crop mass by the derivative of Eq. 3:

$$\frac{dN_{upt}}{dW} = ab(W)^{b-1} \tag{18}$$

This equation indicates that the efficiency of conversion of N absorbed into crop mass dW/dN_{upt} increases as crop mass increases. Such an analysis allows the identification of two different sources of variation for NCE: (i) when W is increased only by other factors than N supply, climate and/or genotype, then NCE increases with crop mass, and (ii) when W is increased by N supply, then NCE is lowered by the cost in N uptake for restoring the plant N status. This approach allows the study of NCE as a dynamic process where the time has to be explicitly taken into account through crop growth rate: the higher crop growth rate, the higher NCE of the crop. Then it allows the identification of the trivial effect of plant mass per se: a small crop, either because genetically small or because of unfavorable environmental conditions will have a lower NCE as compared to a bigger crop. This reason explains why a good correlation is observed between genotypes growing either in high or low N supply conditions [88]. In consequence, breeding for a high NCE must lead to the selection of genotypes to higher growth potential. There is no clear indication until now if NCE variation among genotypes would persist when comparisons are made at same crop mass. Since coefficients a and b of Eq. 2 differ little or not at all between species within C_3 or C_4 groups, the chances to find intraspecific differences in NCE when comparing plants having a similar growth potential remains very limited.

When considering yield (Y) and not only crop biomass (W), NCE can be highly variable among crop species as resulting to large genotypic variations in



Crop Responses to Nitrogen. Figure 7

Schematic representation of the components of the nitrogen conversion efficiency. The first component $\Delta(N_{upt})1$ corresponds to the quantity of N necessary for crop to reach its critical N status, and its NCE is 0. The second component $\Delta(N_{upt})2$ corresponds to the quantity of N necessary for the synthesis the supplement of biomass ΔW and whose the NCE is $dW/dN = 1/ab(W)^{1-b}$ that increases with crop mass

harvest index (HI). For grain crops as cereals, grain legumes, and oil seed species, HI is an important source of variation in grain yield per unit of N uptake. The repartition of N between the harvested and non-harvested plant parts that is the nitrogen harvested index (NHI) is then an important aspect of the grain nutritional quality [95]. Grain produced per unit of N uptake and grain N concentration are in general inversely related [96], and a variable proportion of the variation in yield per unit N uptake is accounted for by grain protein concentration or by NHI, according to crop type.

Grain protein N concentration is the result of two concomitant processes: (i) the rate of accumulation of proteins during grain filling and (ii) the rate of accumulation of free-N compounds (carbohydrates) within grains that lead to a dilution of N as grains develop. So a low grain N concentration can result in both a N deficiency during grain filling period and a large accumulation rate of starch. Thus, late application of N fertilizer to avoid any N shortage during grain filling can lead to an increase of the grain protein concentration. But as the capacity of roots for N absorption at this stage of the crop is largely impaired by the beginning of leaf senescence and the shortage of C allocated to roots, the recovery of this N application is low, and then, large mineral N residues in soil increase the risk for subsequent N leaching.

Conclusion

Since soil N availability, N uptake and distribution within plant and crop and finally crop growth are permanently interrelated during crop development and growth, the traditional view where crop N uptake was totally regulated by soil N supply must be reconsidered and replaced by a more dynamic approach where plant N uptake rate at any moment is co-regulated by both soil N supply and plant growth capacity itself. This co-regulation lead to the concept of critical plant N concentration and N dilution that link plant and crop N status to plant and crop mass. Such an approach allows the determination of the dynamics of plant N demand and plant N status all along their life-cycle. Critical N-curves are now available for most of the crop species growing either in temperate or tropical conditions. This allows the determination of the nitrogen nutrition index of any crop in any condition for diagnosis of its actual N status and estimating the necessity for applying N fertilizers.By this way, it would be possible

now to adjust more precisely the quantity and the timing of N fertilizer supply for matching the crop N demand according to target yields. As a consequence, a reduction of the risk for N losses to atmosphere and hydrosphere should be obtained while the crop productivity would be maintained.

One other perspective is to improve the efficiency of use of N within agro-systems. It appears that improving the ability of crops to absorb and accumulate N efficiently from soil (nitrogen absorption efficiency) is the first objective for high nitrogen use efficiency in cropping systems. This ability of plants and crops to take up mineral N from soil has to be investigated both at high or low N supply conditions. As demonstrated above, in both conditions, N uptake capacity of a crop is directly dependent upon its growth capacity as determined by (i) its own genetic potential, (ii) environmental conditions such as soil and climate, (iii) cropping management techniques, and (iv) interactions between these variables. So any improvement in crop growth capacity by both breeding cultivars and crop management (irrigation, P, K, S fertilization, planting density) will increase NAE of the crop and then will reduce the risk of accumulation of soil N mineral surplus with environmental hazards. But more important would be to improve NAE of crop species at similar crop growth potential. This would allow achieving a given target yield with less N fertilizer. Such an objective requires a more efficient root system to increase the competitive ability of the plant for using soil mineral N against microbial communities. This objective could be reached through a combination of plant breeding strategy and crop management techniques such as soil tillage and soil structure conservation.

Future Directions

The improvement of the ability of plants and crops to absorb and accumulate N efficiently from soil, that is measured by NAE, appears to be the first objective for a plant breeding strategy. As demonstrated above, the N uptake capacity of a crop primarily depends on its growth potential under given climatic conditions. So adaptation of genotypes for fast growth potential is the first way for improving its N uptake capacity. However, it should be also possible to increase the plant N uptake capacity at similar growth potential. By this way, it could be possible to increase the quantity of N a crop is able to uptake from soil in low N supply conditions and then to reduce the quantity of N fertilizers necessary to obtain a given target yield. Under low N supply conditions, root development and architecture as well as the interactions with rhizosphere through root exudates may be of major importance for the determination of the N uptake capacity of crops. Under high N supply conditions, it is the down regulation of root absorption capacities by shoot signals which determine the crop N uptake capacity. So for breeding genotypes with higher N uptake capacities both in low and in high N supply conditions, it is necessary to analyze more deeply all the regulation processes of N absorption at physiological and molecular levels. This kind of approach should be one of the major tasks in the next decade. The development of large-scale genomic, proteomic, and metabolomic studies is necessary for such an objective. However, the difficulty will be to integrate the huge amount of data generated by these studies. All elementary processes studied at cellular or molecular level have to be scaled up to the level of whole plant and crop where they are agronomically relevant. The nitrogen nutrition of crops and its efficiency for yield production is controlled by elementary physiological processes such as nitrate or ammonium transport through cell membranes in roots, nitrate reduction, ammonium assimilation, and protein synthesis. Each of these metabolic processes is regulated at molecular level, and then is susceptible to have more or less genetic variability. But when all processes are scaled up to whole plant or crop level, a large part of this elementary variability is buffered because of existing trade-off among elementary processes and feedback mechanisms. Quantitative genetics using both mutants and genetic engineering appears to be one of the most promising tools to allow the identification of key regulatory genes that are likely to control a variety of physiological and developmental processes involved in the determination of crop nitrogen absorption efficiency. But this approach must be always accompanied by a modeling approach in order to analyze all the feedback and trade-off at whole plant and crop level.

Breeding plants to increase nitrogen conversion efficiency for aboveground dry matter production appears to be more difficult because, apart from the difference between C_4 and C_3 groups, no clear

interspecific differences are observed among cultivated crops. So it seems that intraspecific variability should be very low. However, if the N efficiency for grain yield is considered, it should be possible to detect inter- and intraspecific differences. Detailed analysis of grain development and grain-filling processes should provide information about the genetic variability. The development of models describing both N partitioning and translocation within plant, and dynamics of grain development, taking into account the coupling between N and C fluxes between plant organs are necessary for detecting the key regulation processes for optimizing grain yield and quality. This approach will require a cooperative and integrated effort between plant molecular physiologists, geneticists, crop physiologists, and agronomists and the intensive use of bioinformatics.

Bibliography

Primary Literature

1. Angus JF (2001) Nitrogen demand and supply in Australian agriculture. Aust J Exp Agr 41:277–288
2. Eikhout B, Bouwman AF, Zeijts VH (2006) The role of nitrogen in world food production and food sustainability. Agric Ecosyst Environ 116:4–14
3. London JG (2005) Nitrogen study fertilizes fears of pollution. Nature 433:791
4. Beman JM, Arrigo K, Matson PM (2005) Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. Nature 434:211–214
5. Ramos C (1996) Effect of agricultural practices on the nitrogen losses in environment. In: Rodriguez-Barrueco C (ed) Fertilizer and environment. Kluwer, Dordrecht, pp 335–361
6. Stulen I, Perez-Soba M, De kok LJ, Van Der Eerden (1998) Impact of gaseous nitrogen deposition on plant functioning. New Phytol 139:61–70
7. Cassman KG (2007) Climate change, biofuels, and global food security. Environ Res Lett 2:11–12
8. Addiscott TM, Withmore AP, Powlson DS (1991) Farming, fertilizers and nitrate problem. CAB International, Wallingford, p 170
9. Hirel B, Lemaire G (2005) From agronomy and ecophysiology to molecular genetics for improving nitrogen use efficiency in crops. J Crop Imp 15:213–257
10. Evans JR (1983) Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum* L.). Plant Physiol 72:297–302
11. Lawlor DW (1995) Photosynthesis, productivity and environment. J Exp Bot 46:1449–1461
12. Lawlor DW, Konturri M, Young AT (1989) Photosynthesis by flag leaves of wheat in relation to protein, ribulose biphosphate carboxylase activity and nitrogen supply. J Exp Bot 40:43–52
13. Werger MJA (1991) Leaf nitrogen distribution and whole canopy photosynthetic carbon gain in herbaceous stands. Vegetatio 97:11–20
14. Nakano HM, Mae T (1997) The effects of elevated partial pressure of CO2 on the relationship between photosynthetic capacity and N content in rice leaves. Plant Physiol 115: 191–198
15. Makino A, Mae T, Ohira K (1988) Differences between wheat and rice in the enzymic properties of ribulose-1, 5-biphosphate carboxylase/oxygenase and the relationship to photosynthetic gas exchange. Planta 174:30–38
16. Theobald JC, Mitchell RAC, Parry MAJ, Lawlor DW (1998) Estimating the excess investment in ribulose-1, 5-bisphophate carboxylase/oxygenase in leaves of spring wheat grown under elevated CO_2. Plant Physiol 118:945–955
17. Sage RF (1987) The nitrogen use efficiency of C3 and C4 plants. I- Leaf nitrogen, growth, and biomass partitioning in *Chenopodium album* (L.) and *Amaranthus retroflexus* (L.). Plant Physiol 84:954–958
18. Lawlor DW, Boyle FA, Keys AJ, Kendall AC, Young AT (1988) Nitrate nutrition and temperature effects on wheat: a synthesis of plant growth and nitrogen uptake in relation to metabolic and physiological processes. J Exp Bot 39:329–343
19. Millard P (1988) The accumulation and storage of nitrogen by herbaceous plants. Plant Cell Environ 11:1–8
20. Avice JC, Ourry A, Lemaire G, Volenec JJ, Boucaud J (1997) Root protein and vegetative storage protein are key organic nutrients for alfalfa shoot regrowth. Crop Sci 37:1187–1193
21. Ourry A, McDuff JH, Ougham H (1996) The relationship between mobilisation of N reserves and changes in translatable messages following defoliation in *Lolium temulentum* L. and *Lolium perenne* L. J Exp Bot 47:739–747
22. Lemaire G, Khaity M, Onillon B, Allirand JM, Chartier M, Gosse G (1992) Dynamics of accumulation and partitioning of N in leaves, stems and roots of lucerne in dense canopy. Ann Bot 70:429–435
23. Grindlay DJC, Sylvester-Bradley R, Scott RK (1995) The relationship between canopy green area and nitrogen in the shoot. In: Lemaire G, Burns IG (eds) Diagnostic procedures for crop N management. INRA Publication, Paris, pp 53–60, INRA-Editions, Collection "Les Colloques"
24. Lemaire G, van Oosterom E, Jeuffroz MH, Gastal F, Massignan A (2008) Crop species present different qualitative types of response to N deficiency during their vegetative growth. Field Crop Res 105:253–265
25. McAdam JW, Volenec JJ, Nelson CJ (1989) Effects of nitrogen on mesophyll cell division and epidermal cell elongation in tall fescue leaf blades. Plant Physiol 89:549–556
26. Gastal F, Nelson CJ (1994) Nitrogen use within growing leaf blade of tall fescue. Plant Physiol 105:191–197
27. Fricke W, McDonald AJS, Matson-Djos L (1997) Why do leaves and leaf cells of N-limited barley elongate at reduced rates? Planta 202:522–530

28. Uhart SA, Andrade FH (1995) Nitrogen deficiency on maize. I- Effects on crop growth, development, dry matter partitioning and kernel set. Crop Sci 35:1376–1383
29. Desmotes-Mainard S, Jeuffroy MH, Robin S (1999) Spike dry matter and nitrogen accumulation before anthesis in wheat as affected by nitrogen fertilizer: relationship to kernel per spike. Field Crop Res 64:249–259
30. Mae T (1997) Physiological nitrogen efficiency in rice: nitrogen utilization, photosynthesis, and yield potential. In: Ando T (ed) Plant nutrition for sustainable food production and environment. Kluwer, Dordrecht, pp 51–60
31. Lemaire G, Avice JC, Kim TH, Ourry A (2005) Development changes in shoot N dynamics of lucerne in relation to leaf growth dynamics as a function of plant density and hierarchical position within the canopy. J Exp Bot 56:935–943
32. Lemaire G, Gastal F (2009) Quantifying crop responses to nitrogen deficiency and avenues to improve nitrogen use efficiency. In: Sadras VO, Calderini DF (eds) Crop physiology: applications for genetic improvement and agronomy. Academic/Elsevier, Amsterdam, pp 171–199
33. Greenwood DJ, Lemaire G, Gosse G, Cruz P, Draycott A, Neeteson JJ (1990) Decline in percentage N of C3 and C4 crops with increasing plant mass. Ann Bot 66:425–436
34. Lemaire G, Jeuffroy MH, Gastal F (2008) Diagnostis tool for plant and crop N status in vegetative stage. Theory and practices for crop N management. Eur J Agron 28:614–624
35. Lemaire G, Gastal F (1997) N uptake and distribution in plant canopies. In: Lemaire G (ed) Diagnosis on the nitrogen status in crops. Springer, Heidelberg, pp 3–43
36. Justes E, Mary B, Meynard JM, Machet JM, Thellier-Huché L (1994) Determination of a critical nitrogen dilution curve for winter wheat crops. Ann Bot 74:397–407
37. Lemaire G, van Oosterom E, Sheehy J, Jeuffroy MH, Massignan A, Rossato L (2007) Is crop demand closely related to dry matter accumulation of leaf area expansion during vegetative growth? Field Crop Res 100:91–106
38. Gastal F, Lemaire G (2002) N uptake and distribution in crops: an agronomical and ecophysiological perspective. J Exp Bot 53:789–799
39. Lemaire G, Salette J (1984) Relation entre dynamique de croissance et dynamique de prélèvement d'azote par un peuplement de graminées fourragères. 1- Etude de l'effet du milieu. Agronomie 4:423–430
40. Lemaire G, Salette J (1984) Relation entre dynamique de croissance et dynamique de prélèvement d'azote par un peuplement de graminées fourragères. 2- Etude de la variabilité entre génotypes. Agronomie 4:431–436
41. Lemaire G, Cruz P, Gosse G, Chartier M (1985) Etude des relations entre la dynamique de prélèvement d'azote et la dynamique de croissance en matière sèche d'un peuplement de luzerne. Agronomie 5:685–692
42. Ney B, Doré T, Sagan M (1997) The nitrogen requirement of major agricultural crops: grain legumes. In: Lemaire G (ed) Diagnosis of the nitrogen status in crops. Springer, Heidelberg, pp 107–117
43. Colnenne C, Meynard JM, Reau R, Justes E, Merrien A (1998) Determination of a critical nitrogen dilution curve for winter oilseed rape. Ann Bot 81:311–317
44. Sheehy JE, Dionara MJA, Mitchell PL, Peng S, Cassman KG, Lemaire G, Williams RL (1998) Critical concentrations: implications for high-yielding rice (*Oryza sativa*, L.) cultivars in tropics. Field Crop Res 59:31–41
45. Tei F, Benincasa P, Guidici M (2002) Critical nitrogen concentration in processing tomato. Eur J Agron 18:45–56
46. Plénet D, Lemaire G (1999) Relationships between dynamics of nitrogen uptake and dry matter accumulation in maize crops. Determination of critical N concentration. Plant Soil 216:65–82
47. Plénet D, Cruz P (1997) The nitrogen requirement for major agricultural crops: maize and sorghum. In: Lemaire G (ed) Diagnosis of the nitrogen status in crops. Springer, Heidelberg, pp 93–106
48. Duru M, Lemaire G, Cruz P (1997) The nitrogen requirement for major agricultural crops: grasslands. In: Lemaire G (ed) Diagnosis of nitrogen status in crops. Springer, Heidelberg, pp 56–72
49. Hardwick RC (1987) The nitrogen content of plants and the self thinning rule in plant ecology: a test of the core-skin hypothesis. Ann Bot 60:439–446
50. Ismande J, Touraine B (1994) N demand and regulation of nitrate uptake. Plant Physiol 105:3–7
51. Lejay L, Tillard P, Petit M, Olive FD, Filleur S, Daniel-Vedele F, Gojon A (1999) Molecular and functional regulation of two NO3- uptake systems by N and C status of *Arabidopsis* plants. Plant J 18:509–519
52. Forde BG (2002) The role of long-distance signaling in plant response to nitrate and other nutrients. J Exp Bot 53:39–43
53. Glass ADM, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, Okamoto M, Rwat S, Siddiqi MY, Unkless SE, Vidmar J (2002) The regulation of nitrate and ammonium transport systems in plants. J Exp Bot 53:855–864
54. Devienne-Barret F, Justes E, Machet JM, mary B (2000) Integrated control of nitrate uptake by crop growth rate and soil nitrate availability under field conditions. Ann Bot 86:995–1005
55. Lemaire G, Meynard JM (1997) Use of the nitrogen nutrition index for the analysis of agronomical data. In: Lemaire G (ed) Diagnosis of the nitrogen status in crops. Springer, Heidelberg, pp 45–56
56. Angus JF, Moncur MW (1985) Models of growth and development of wheat in relation to plant nitrogen. Aust J Agr Res 36:537–544
57. Jeuffroy MH, Bouchard C (1999) Intensity and duration of nitrogen deficiency on wheat grain number. Crop Sci 39:1385–1393
58. Lemaire G, Plénet D, Grindlay D (1997) Leaf N content as an indicator of crop N nutrition. In: Lemaire G (ed) Diagnosis on the nitrogen status in crops. Springer, Heidelberg, pp 189–199
59. Farrugia A, Gastal F, Scholefield D (2004) Assessement of nitrogen status of grasslands. Grass Forage Sci 59:113–120

60. Ziadi N, Bélanger G, Gastal F, Claesses A, lemaire G, Tremblay N (2009) Leaf nitrogen concentration as an indicator of corn nitrogen status. Agron J 101:947–957
61. Alam MM, Ladha JK, Rahman Khan S, Harun-Ur-Rashid Khan AH, Buresh RJ (2005) Leaf color chart for managing nitrogen fertilizer in lowland rice in Bangladesh. Agron J 97:949–959
62. Peng S, Garcia FV, Laza RC, Sanico AL, Visperas RM, Cassman KG (1996) Increased N-use efficiency using a chlorophyll meter on high-yielding irrigated rice. Field Crops Res 47:243–252
63. Feibo W, Lianghuan W, Fuha X (1998) Chlorophyll meter to predict nitrogen side dress requirements for short-season cotton. Field Crop Res 56:309–314
64. Le Bail M, Jeuffroy MH, Bouchard C, Barbotin A (2005) Is it possible to forecast the grain quality and yield of different varieties of winter wheat from minolta SPAD meter measurements? Eur J Agron 23:379–391
65. Houlès V, Guérif M, Mary B (2007) Elaboration of a nutrition indicator for winter wheat blades on leaf area index and chlorophyll meter content for making nitrogen recommendations. Eur J Agron 27:1–11
66. Monteith JL (1972) Solar radiation and productivity in tropical ecosystems. J Appl Ecol 9:747–766
67. Bélanger G, Gastal F, Lemaire G (1992) Growth analysis of a tall fescue sward fertilized with different rates of nitrogen. Crop Sci 6:1371–1376
68. Trapani N, Hall AJ (1996) Effects of leaf position and nitrogen supply on the expansion of leaves of field-grown sunflower. Plant Soil 184:331–340
69. Muchow RC, Davis R (1998) Effect of nitrogen supply on the comparative productivity of maize and sorghum in a semi-arid tropical environment. II- radiation interception and biomass accumulation. Field Crop Res 18:17–30
70. Bélanger G, Gastal F, Warembourg F (1994) Carbon balance on tall fescue: effects of nitrogen and growing season. Ann Bot 74:653–659
71. Robson MJ, Parsons AJ (1978) Nitrogen deficiency in small closed communities of S24 ryegrass. I-Photosynthesis, respiration, dry matter production and partition. Ann Bot 42:1185–1197
72. Jarvis SC, McDuff JH (1989) Nitrate nutrition of grasses from steady state supplies in flowing solution culture following nitrate deprivation and/or defoliation. J Exp Bot 40:695–975
73. Gastal F, Bélanger G, Lemaire G (1992) A model of the leaf extension rate of tall fescue in response to nitrogen and temperature. Ann Bot 70:437–442
74. Sinclair TR, Horie T (1989) Leaf nitrogen, photosynthesis, and crop radiation use efficiency: a review. Crop Sci 29:90–98
75. Grindlay DJC (1997) Towards an explanation of crop nitrogen demand based on leaf nitrogen per unit leaf area. J Sci Food Agric 63:116–123
76. Van Keulen H, Goudrian J, Seligman NG (1989) Modelling the effects of nitrogen on canopy development and crop growth. In: Rusell G, Marshall LB, Jarvis PG (eds) Plant canopies: their growth, form and function. Cambridge University Press, Cambridge, pp 83–104
77. Gastal F, Bélanger G (1993) The effects of nitrogen fertilization and the growing season on photosynthesis of fied-grown tall fescue canopies. Ann Bot 72:401–408
78. Connor DJ, Hall AJ, Sadras VO (1993) Effects of nitrogen content on the photosynthetic characteristics of sunflower crops during grain filling. Aust J Plant Physiol 20:251–263
79. Fischer RA (1993) Irrigated spring wheat and timing and amount of nitrogen fertilizer. II-Physiology of grain yield response. Field Crop Res 33:57–80
80. Martre P, Porter JR, Jamieson PD, Triboï E (2003) Modelling grain nitrogen accumulation and protein composition to understand the sink/source regulations of nitrogen utilization in wheat. Plant Physiol 133:1959–1967
81. Desmotes-Mainard S, Jeuffroy MH (2004) Effects of nitrogen and radiation on dry matter and nitrogen accumulation in spike of winter wheat. Field Crop Res 87:221–233
82. Dreccer MF (2005) Nitrogen use at the leaf and canopy level: a framework to improve N use efficiency. J Crop Imp 15: 97–125
83. Borell A, Hammer GL (2000) Nitrogen dynamics and the physiological basis for stay-green in sorghum. Crop Sci 40: 1295–1307
84. Mi C, Liu J, Chen F, Zhang F, Cui Z, Liu X (2003) Nitrogen uptake and remobilization in maize hybrids differing in leaf senescence. J Plant Nutr 26:447–459
85. Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron J 74:562–564
86. Borell A, Garside AL, Fukaï S, Reid DJ (1998) Season nitrogen rate and plant type affect nitrogen uptake and nitrogen use efficiency in rice. Aust J Agr Res 49:829–843
87. Le Gouis J, Béghin D, Heumez E, Pluchard P (2000) Genetic differences for nitrogen uptake and nitrogen utilization efficiencies in winter wheat. Eur J Agron 12:163–173
88. Bertin P, Gallais A (2001) Physiological and genetic basis of nitrogen use efficiency on maize. Maydica 46:53–68
89. Lemaire G, Charrier X, Hébert Y (1996) Nitrogen uptake capacities of maize and sorghum crops in different nitrogen and water supply conditions. Agronomie 16: 231–246
90. Lemaire G, Recous S, Mary B (2004) Managing residues and nitrogen in intensive cropping systems. New understandings for efficient recovery by crops. In: Proceedings of the 4th international crop science congress, Brisbane, Australia, 2004
91. Laperche A, Devienne-Barret F, Maury O, Le Gouis J, Ney B (2007) A simplified conceptual model of carbon and nitrogen functioning for QTL analysis of winter wheat adaptation to nitrogen deficiency. Theor Appl Genet 113: 1131–1146
92. Camus-Kulandaivelu L, Veyreiras JB, Madur D, Combes V, Fourman M, Barraud S, Dubreuil P, Gouesnard B,

Manicacci D, Charcosset A (2006) Maize adaptation to temperate climate: relationship between population structure and polymorphism in the Dwarf 8 gene. Genetics 172: 2449–2463

93. Singh U, Ladha JK, Castillo EG, Punzalan G, Tirol-Padre A, Duqueza M (1998) Genotypic variation in nitrogen use efficiency. I-medium and long-duration rice. Field Crop Res 58: 35–53
94. Sadras VO (2006) The N:P stoichiometry of cereal, grain legume and oilseed crops. Field Crop Res 95:13–29

Books and Reviews

Gastal F, Lemaire G (2002) N uptake and distribution in crops: an agronomical and ecophysiological perspective. J Exp Bot 53:789–799

Grindlay DJC (1997) Towards an explanation of crop nitrogen demand based on leaf nitrogen per unit leaf area. J Sci Food Agric 63:116–123

Hirel B, Lemaire G (2005) From agronomy and ecophysiology to molecular genetics for improving nitrogen use efficiency in crops. J Crop Imp 15:213–257

Justes E, Mary B, Meynard JM, Machet JM, Thellier-Huché L (1994) Determination of a critical nitrogen dilution curve for winter wheat crops. Ann Bot 74:397–407

Lemaire G, Gastal F (1997) N uptake and distribution in plant canopies. In: Lemaire G (ed) Diagnosis on the nitrogen status in crops. Springer, Heidelberg, pp 3–43

Lemaire G, Gastal F (2009) Quantifying crop responses to nitrogen deficiency and avenues to improve nitrogen use efficiency

Lemaire G, Jeuffroy MH, Gastal F (2008) Diagnostis tool for plant and crop N status in vegetative stage. Theory and practices for crop N management. Eur J Agron 28:614–624

Lemaire G, Recous S, Mary B (2004) Managing residues and nitrogen in intensive cropping systems. New understandings for efficient recovery by crops. In: Proceedings of the 4th international crop science congress, Brisbane, Australia, 2004

Lemaire G, van Oosterom E, Jeuffroz MH, Gastal F, Massignan A (2008) Crop species present different qualitative types of response to N deficiency during their vegetative growth. Field Crop Res 105:253–265

Lemaire G, van Oosterom E, Sheehy J, Jeuffroy MH, Massignan A, Rossato L (2007) Is crop demand closely related to dry matter accumulation of leaf area expansion during vegetative growth? Field Crop Res 100:91–106

Sinclair TR, Horie T (1989) Leaf nitrogen, photosynthesis, and crop radiation use efficiency: a review. Crop Sci 29:90–98

Sinclair TR (1998) Historical changes in harvest index crop N accumulation. Crop Sci 38:638–643

Van Keulen H, Goudrian J, Seligman NG (1989) Modelling the effects of nitrogen on canopy development and crop growth. In: Rusell G, Marshall LB, Jarvis PG (eds) Plant canopies: their growth, form and function. Cambridge University Press, Cambridge, pp 83–104

Crop Science and Technology, Introduction

Paul Christou[1,2], Roxana Savin[3]
[1]Department de Produccio Vegetal i Ciencia Forestal, Universitat de Lleida/ICREA, Lleida, Spain
[2]Institució Catalana de Recerca i Estudis Avançats, (ICREA), Barcelona, Spain
[3]Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain

Article Outline

Population growth in the coming decades will put severe pressure on human food, animal feed, and fiber production. Bioenergy applications are already exerting increasing pressure on agricultural commodities and land use with severe economic consequences, particularly in the developing world. Any crop productivity increases must come necessarily from enhancing crop performance as further land expansion for agriculture is unlikely to take place. Environmental sustainability and social justice issues are becoming increasingly key elements in debates on how to assure adequate food for the ever-increasing global population. It is likely that the efficiency of increasing productivity would benefit by complementing the conventional empirical approaches with opportunities presented by the development of new knowledge and technologies in the field of Crop Science. Therefore, in this section a number of seminal articles by world experts in the field are featured. Through this collection of articles key advances in the field are highlighted and the reader is pointed to future directions in terms of opportunities and constraints for a more productive and sustainable agriculture. This is not meant to be an exhaustive list of topics; rather the aim is to highlight particular technologies and topics that have a real potential to make a substantial contribution to a more productive and environmentally friendlier agriculture in the short to medium term. This section comprises 45 articles that can be divided into two parts: (a) molecular approaches and (b) crop physiology and agronomy.

Transgenic Crops and Molecular Breeding

World food and feed security are increasingly dependent on continuous crop improvement and in particular the development of crops with increased resistance to abiotic stresses (► Abiotic Stress Tolerant Crops: Genes, Pathways and Bottlenecks). Plants unlike animals are not mobile, consequently they need to develop strategies to combat natural enemies such as herbivores and environmental stresses. Drought, salinity, submergence, and temperature stresses amongst others are all important abiotic constraints which limit crop yields. Significant advances in our understanding of molecular mechanisms underpinning a plant's ability to combat abiotic stresses has resulted in the creation of transgenic plants with improved resistance to such stresses.

Sustainable, renewable resources are those derived from biological sources, primarily plant biomass which can be regenerated with minimal inputs using energy from the sun (► Biomass Crops for Biofuels and Bio-based Products). Biomass for biofuels includes many sources of material derived from agricultural harvests including grains, agricultural residues such as stalks and leaves, perennial crops such as hay and trees, animal manures, building waste wood, municipal solid waste such as paper, and various food industry wastes. Humans currently consume at least 25% more raw materials every year than are replaced through biological growth. In order to sustain quality of life and maintain adequate environmental resources, those resources must be balanced and renewable. Pressure on those resources has never been greater with the world population currently at seven billion people, and estimated to plateau at 10.5 billion by 2050.

Once a gene is introduced into a plant its expression may be controlled by a number of different factors (► Transgene Expression in Plants, Control of). Among these, promoters are most important as they regulate expression temporally and spatially. Transcriptional fusions of genes, with the Cauliflower Mosaic Virus 35S promoter when integrated into the plant genome (mostly dicotyledonous plants), result in

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

transgenic plants with high and constitutive expression of the transgene. However, 35S-driven transgene expression is very variable with ca: 20% of the transformants exhibiting high expression levels, while the majority (ca: 80%) display an intermediate or low and unstable transgene expression. The possible causes of variation in transgene expression in a population of transgenic plants need to be identified and addressed in order to generate useful plants with stable and predictable levels of expression of introduced transgenes.

Plant transformation is a fundamental component of both basic and applied plant biology (► Crop Plants Transformation Methods). For basic research, transformation allows scientists to study how genes function and allows the study of both endogenous genes and transgenes. This has increased our understanding of how plants grow, develop, and defend themselves against pests, diseases, and harsh environments; how photosynthesis is controlled; and the basis of primary and secondary metabolism. For applied research, transformation can be used to improve the agronomic performance of crops, making them hardier, more nutritious, more productive, or converting them from conventional crops into green factories producing chemical precursors, novel oils, industrial enzymes, and pharmaceuticals. Plants have been cultivated for food and animal feed, fibers and structural materials, and small molecules that can be used as dyes, scents, and medicines since the dawn of history, and for the same length of time people have sought to improve plants by breeding them and selecting the better-performing and most useful varieties. The limitations of this approach, that is, the fact that breeders are restricted to the existing gene pool in each group of sexually compatible species, and that breeding takes a long time to achieve its goals, can be overcome by plant transformation, thus accelerating the development of plants with novel, beneficial traits. Plant transformation includes both the uptake of naked DNA (direct DNA transfer) and the transfer of DNA by the conjugation-like method adopted by *Agrobacterium tumefaciens* and *A. rhizogenes* (*Agrobacterium*-mediated transformation).

Genetics, molecular biology, genomics, and other disciplines have now provided us with an insight to the genes that encode important crop traits on which humans now depend. Knowledge of the molecular genetic basis of valuable crop traits will help provide solutions to our requirements for sustainable existence, faced with a growing global population and diminishing natural resources (► Crop Traits: Gene Isolation). Isolated genes encoding useful traits and their use in plant transformation experiments allow the development of an in-depth understanding of the mechanisms that control such important crop traits. Thus, through such investigations that utilize isolated genes, one is able to understand and harness traits involved in plant domestication such as plant architecture, to those offering solutions for high crop production purposes such as growth under unfavorable environments or disease outbreaks. Cloning or identification of the genes involved in crop traits provide an insight into factors which determine gene–trait relationships. Understanding the molecular basis of crop traits provides a route to their controlled modulation ultimately leading to the development and implementation of novel genetic engineering solutions to create plants with superior and sustainable characteristics.

Conventional plant breeding practices alone will not be able to achieve sustainability in today's agriculture. Advances in plant genomics research are opening up a new era in plant breeding where the linkage of genes to specific traits will lead to more efficient and predictable breeding programs (► Crop Breeding for Sustainable Agriculture, Genomics Interventions in). Plant genomics is a rapidly developing field, which is radically improving our understanding of plant biology by making available novel tools for the improvement of plant properties relevant to sustainable agricultural production. Recent advances in high throughput genomics technologies including next generation sequencing and high-throughput genotyping have helped immensely in understanding the functions and regulation of genes in crop plants. The ever-increasing availability of genome sequences in crop plants has facilitated greatly the development of genomic resources that will allow us to address biological functions and a number of basic processes relevant to crop production leading to sustainable agriculture. It is therefore expected that genomics will be an integral part of the agricultural/plant breeding practices of the future for improving crop productivity to achieve food security and sustainable production.

Plants have been used as sources of small molecular weight compounds (secondary metabolites) with applications as medicines, flavors, and fragrances for millennia. However, many plants containing such high-value secondary metabolites are difficult to cultivate or are becoming endangered because of overharvesting. In addition, the chemical synthesis of plant-derived compounds is often uneconomical due to their highly complex structures. The biotechnological production of valuable secondary metabolites is an attractive alternative to the extraction of whole plant material (► Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures). Functional genomics may open entirely new avenues to screen unexplored medicinal plant species for their pharmacological value.

Molecular breeding (MB) allows the stacking of favorable alleles, or genomic regions, for target traits in a desired genetic background, thanks to the use of polymorphic molecular markers (MM) that monitor differences in genomic composition among cultivars, or genotypes, at specific genomic regions, or genes, involved in the expression of those target traits (► Molecular Breeding Platforms in World Agriculture). The use of MM generally increases the genetic gain per crop cycle compared to selection based on plant phenotyping only, and therefore reduces the number of needed selection cycles, hastening the delivery of improved crop varieties to the farmer. In contrast to the private sector, MB adoption is still limited in the public sector, and is hardly used at all in developing countries. The situation is critical in developing countries due to resource-limited breeding programs. As a result, the developing world has yet to benefit from the MB revolution, and most countries indeed lack the fundamental prerequisites for a move to informatics powered breeding. A sustainable web-based Molecular Breeding Platform (MBP) as a one-stop-shop for information, analytical tools and related services to help design and conduct marker-assisted breeding experiments in the most efficient way will alleviate many of these bottlenecks in the developing world. Such a platform will enable breeding programs in the public and private sectors in developing countries to accelerate variety development using marker technologies for different breeding objectives.

With the advent of molecular techniques, plants have the potential to serve as production vehicles for natural or engineered products that were previously limited to other hosts. Plant molecular pharming of industrial proteins refers to recombinant proteins used in industrial processes and produced in plants (► Plant Molecular Pharming, Industrial Enzymes). Enormous quantities of a variety of enzymes go into making products such as paper, leather, detergents, pharmaceuticals, food, beverages, chemicals, and fabric, to name a few, and economical production of these industrially important enzymes is crucial to commerce. This production must be balanced with the need for sustainability and environmental stewardship. Sustainable production of industrial enzymes requires that resources are not overexploited and wastes are not polluting. The use of plants as "green" factories can meet both criteria. Combined with modern farming and containment methods, transgenic plants have the potential to produce large quantities of target material safely and sustainably.

The demand for recombinant medical proteins has increased in recent years and modern biotechnological methods have, until recently, ensured the production of safe and effective biopharmaceuticals to meet this demand. Various production platforms are currently used in the pharmaceutical industry, most based on the fermentation of engineered pro- and eukaryotic microorganisms, insect cells, or mammalian cells (► Plant Molecular Pharming, Pharmaceuticals for Human Health). The growth of the market for biopharmaceuticals is predicted to outpace production capacity using these platforms in the next decade, so alternatives are necessary. The production of pharmaceutical proteins in plants began with a monoclonal antibody expressed in transgenic tobacco plants more than 20 years ago. Since then many different plant species have been genetically engineered to produce valuable pharmaceutical proteins. Major progress has been achieved in transformation and expression technology, downstream processing of transgenic plant material, and the adaptation of regulatory procedures to encompass the new production platforms, allowing the first plant-made pharmaceuticals to begin clinical trials.

Since the successful expression of complete antibodies in transgenic plants and the first report of plant-based vaccine production in 1992 a large number of different vaccines, antibodies, as well as antibody fragments have been produced in plants for medical

or veterinary purposes (► Plant Molecular Pharming, Veterinary Applications). Novel processing methods have been developed over the past several years to facilitate the development of recombinant proteins for veterinary applications.

Humankind has had an ever-increasing impact on the environment. With the increasing intensification of agriculture, particularly during the twentieth century, this impact has become even more pronounced, often with undesirable or unacceptable consequences, including water pollution, soil erosion, and loss of habitat, often accompanied by a loss of biodiversity (► Transgenic Crops, Environmental Impact). Pests, particularly weedy plants, demonstrate a remarkable ability to adapt to agricultural production systems. The practice of growing monocultures, typically used in intensive agriculture, increases the number of pests; these are currently predominantly controlled through use of pesticides. However, with increasing exposure to pesticides many pest populations are evolving resistance to these compounds. An additional problem encountered with many pesticides, and particularly insecticides, are their nontarget effects on beneficial insects. Transgenic crops expressing genes conferring resistance to insect pests and/or herbicides are becoming increasingly more widely grown and have the potential to eliminate many of the problems in conventional agriculture thus reducing agriculture's negative impact on the environment.

Annual losses worldwide due to plant diseases are estimated to be ~14% of total losses and about $220 billion. In addition, the need for measures to control diseases limits the acreage of land available for cultivation; restricts the crops that can be grown in fields already contaminated with certain pathogens; and necessitates the use of agrochemicals for treating seeds, fumigating soils, spraying plants, and applying postharvest treatments. Such control measures add to the cost of food production and toxic chemicals can be harmful to human health and the environment (► Transgenic Crops Resistant to Fungal, Bacterial and Viral Pathogens). Resistance to pathogens can be achieved by application of disease-suppressing cultural practices, use of plant defense-promoting substances, deployment of biological agents antagonistic to the pathogens that cause disease, agrochemicals, conventional breeding strategies, and genetic engineering. The need for controlling plant diseases effectively is not only a major challenge, but also a necessity to reduce food losses while improving food quality and safeguarding the environment.

Genetic Engineering can increase the nutritional quality of crops by increasing the availability of essential nutrients, which are often limited in human diets and lead to specific deficiency diseases (► Biotechnology and Nutritional Improvement of Crops). Food insecurity is one of the most important social issues faced today, with nearly one billion people enduring chronic hunger and an additional two billion people suffering from nutrient deficiencies, most in the developing world. Strategies to address food insecurity must ultimately address underlying problems such as poverty and poor governance/infrastructure, but the improvement of agricultural productivity in the developing world is an important goal, and biotechnology is one of a raft of measures being considered to achieve it.

The economic impact of transgenic crops has been immense (► Global Economic Impact of Transgenic/Biotech Crops (1996–2008)). This has been a major driver in their rates of adoption amongst farmers in the USA and other industrialized countries but perhaps more importantly by small holders in the developing world. Analysis on farm income effects through extensive analysis of existing farm level impact data for biotech crops confirms this to be a major reason for their broad adoption worldwide.

Biotechnology offers efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. The first generation of commercialized biotechnology products were crops focusing largely on input agronomic traits whose value was often opaque to consumers. The coming generations of crop plants can be grouped into broad areas each presenting what, on the surface, may appear as unique challenges and opportunities (► Transgenic Crops, Next Generation). The present and future focus is on continuing improvement of agronomic traits such as yield and abiotic stress resistance in addition to the biotic stress tolerance of the present generation, crop plants as biomass feedstocks for biofuels and "bio-synthetics," value-added output traits such as improved nutrition and food functionality, and plants as production factories for therapeutics and industrial products. From a consumer perspective,

the focus on value added traits, especially improved nutrition, is undoubtedly one of the areas of greatest interest.

The rapid development and deployment of modern biotechnology in the last decades has made biosafety a major issue. Although modern biotechnology can benefit agricultural productivity in developing countries transgenic crops remain an issue with regard to the conservation and sustainable use of biodiversity, as well as to human health. The perceived risks, which relate to the release of transgenic crops into the environment as well as the placement of genetically engineered crops onto the market, have as much to do with social values as scientific concerns. Regulatory frameworks for transgenic crops and the underpinning legislative frameworks have been or are being developed worldwide (► Commercialisation of GM Crops: Comparison of Regulatory Frameworks). These are viewed as essential components for the prudent deployment of transgenic crops worldwide.

The safety measures associated with transgenic crop deployment are embedded in process- or product-based regulatory approaches. The EU regulatory approach is process-based, precautionary, and includes mandatory labeling and traceability requirements for transgenic crops and their derived food and feed products (► Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union). During its development, the EU regulatory system has become increasingly more stringent and unduly onerous. In the EU, the risk analysis consists of three components: risk assessment, risk management, and risk communication. When analyzing potential risks, it is important to bear in mind that the real choice is not between transgenic crops that are inherently risky and traditionally bred ones that are completely safe. The cultivation of existing crops and those with novel traits (including transgenic crops) will have both positive and negative consequences. To fully acknowledge the overall outcome of adopting specific crops, and to assess and manage more effectively the environmental footprint of agriculture as a whole, broader and more balanced legislative oversight is needed in the EU.

A framework for a better communication about science and regulation and production of GM crops is described in ► GM Crop Risk Debate, Science and Socioeconomics.

While transgenic herbicide resistant crops have been a boon to agriculture, reducing both production costs and ecological impacts of farming, weeds have rapidly evolved resistance to the major herbicide used in transgenic crops (glyphosate), rapidly rendering the technology less sustainable than had been thought (► Sustainable Herbicide-Resistant Crops). While no practice in agriculture has been sustainable forever, the period of sustainability can be extended. Methods to extend both the usefulness to crops where needed as well as the sustainability of transgenic herbicide technologies such as rotations of crops and herbicides, increasing the targets of herbicide action, suppressing herbicide targets in rotation, are needed.

Crop Physiology and Agronomy

Seed dormancy is a means of restricting germination to the season when environmental conditions are suitable for plant establishment. From an agricultural perspective, dormancy is a problem (► Seed Dormancy and Agriculture, Physiology). Many important challenges face agriculture in relation to dormancy and these apply to cultivated crops as well as noxious weeds. The physiological mechanisms responsible for the expression of the character are now better understood and molecular information underpinning the process is gradually being generated and incorporated into strategies to solve dormancy-related problems.

One of the first decisions a farmer needs to make is to choose the particular genotype to be grown in the fields based on anticipated or projected economic returns. This is a critical choice that determines the sustainability of the agricultural system (► Genotype by Environment Interaction and Adaptation). Identifying breeding implications on specific adaptive traits and the different statistical approaches for genotype by environment interaction characterization is important.

Attaining global food security by means of increased crop productivity will require an increase in gains from selection achieved through conventional breeding. The identification of molecular markers associated with loci controlling traits of agronomic interest coupled with the exploitation of marker-assisted breeding (MAB) approaches provides the opportunity to accelerate gain from selection (► Marker-Assisted Breeding in Crops). Genomic selection is already having a positive

impact on the improvement of crop yield, mainly in the private sector where high-throughput infrastructures allow breeders to handle the large number of molecular data points that are needed for deploying genomic selection effectively. Ultimately, an effective exploitation of MAB to enhance crop performance will rely on a closer integration between molecular approaches and conventional breeding.

The next generation of highly productive crops in an increasingly variable and changing climate, will rely on genetic interventions based on process understanding, selection of target traits in managed environments, and high-throughput phenotyping and genotyping (► Plant Breeding Under a Changing Climate). Therefore, it is crucial to understand the recent advances in plant breeding for high yield potential environments and also those where abiotic stress is a major limitation to productivity.

Agronomic systems are defined as site-specific management of soils and crops on the basis of eco-regional and physiographic characteristics, and in the context of socioeconomic and policy environments. These systems are strong determinants of agricultural production, sustainable use of resources, and their environmental impact. Agricultural soils and ecosystems can also be used for sequestration of atmospheric CO_2 by enhancing photosynthesis, increasing net primary productivity (► Agronomic Interactions with CO_2 Sequestration).

Crop management comprises a set of agronomic practices such as tillage systems, methods of fertilization, and crop rotations (► Cropping Systems: Shaping Nature). Cropping system may vary among farms depending on availability of resources and particular constraints. The different cropping systems may determine water and nutrient availabilities, carbon cycle, erosion, and the pathogen inoculum in the soil.

Understanding plant development or the progression of plants through their life cycle is important because of the need to know and predict when harvestable products are at their optimum (► Crop Development Related to Temperature and Photoperiod). Current knowledge on how temperature (including vernalization) and photoperiod regulate crop development is of major interest and determines how crops adapt in a wide range of environments. Thus, it is critical to understand the physiology and genetic basis of crop development, and to predict as accurately as possible the timing of key developmental events.

Reduced crop productivity from the theoretical potential maximum commonly occurs because of high temperatures. In addition, air temperatures are predicted to increase during the twenty-first century. Crop physiological and developmental processes are sensitive to temperature so that high temperatures do frequently affect negatively crop productivity (► Sustainable Productivity, Heat Tolerance for). The effects of high temperature, elevated CO_2 and their interaction on crops, are therefore important to understand and subsequently to mitigate potential negative effects of genes that confer heat tolerance.

Since plants are immobile, their distribution greatly influences the ability of a crop to capture and use environmental resources (radiation, water, and nutrients), which are necessary for growth and yield. The spatial arrangement of plants and the temporal development of their structures (mainly leaves and roots) define the ► Spatial Crop Structure in agricultural systems. Density and spatial arrangement of crops may affect intraspecific competition and resource use efficiency, allowing full or partial use of available resources.

The rate of accumulation of dry plant matter is entirely dependent on the interception of energy from the sun in the wavelength range 400–700 nm. This energy is utilized by photosynthesis to synthesize carbohydrates and other biological molecules needed for essential plant processes (► Crop Radiation Capture and Use Efficiency). Given the current emphasis on global food security, there is currently much interest in raising the radiation use efficiency of key crops in important agro-ecosystems.

Scarcity of water resources is an increasingly important issue since it will dictate global production of food and feed for the next generations (► Crop Responses to Available Soil Water). Key factors responsible for sustained plant growth and production under water scarcity, for annual as well as perennial (fruit) crops are of paramount importance.

Irrigated agriculture is currently responsible for over 40% of total production on 17% of all cultivated land area. It is therefore imperative that irrigated agriculture not only sustains its current rates of productivity but also increases (► Irrigation Management for

Efficient Crop Production). Irrigation expansion is currently under pressure from other sectors to reduce its share of the fresh water resources. Efficient crop production under irrigation in the future would be essential to produce more food with less water. This is an immense challenge, not easy to achieve without novel and innovative approaches in irrigation management and crop productivity.

Sustainability of fertilizer use is very important (► Fertilizer Science and Technology), as fertilizers are indispensable because nutrient supplies from the soil are normally inadequate for high-yielding crops and compensate for nutrient removals by previously cultivated crops. In addition, fertilizer may also improve the quality of human food and animal feed.

Nitrogen is the most important limiting factor, after water deficit, for crop production worldwide (► Crop Responses to Nitrogen). Therefore, understanding how the yield of different crops can be improved by addition of nitrogen (N) fertilizers is critical. There are several important issues regarding the dynamics of crop N demands during the crop development cycle, the timing of the soil N supply according to soil characteristics, climate and soil agronomic management, the crop responses to different intensity and timing of N nutrition deficiency, and the time management of crop N fertilization using diagnostic and decision-making tools to optimize trade-offs between minimizing crop yield reduction and minimizing environmental impacts.

A substantial increase in the effectiveness with which available water and nutrients are used is required to ensure food security and environmental protection. An essential component of crop improvement is breeding for deeper or denser root systems. These characteristics promote soil moisture and nutrient capture and high dry matter production in cultivars subjected to water and/or nutrient stresses (► Roots and Uptake of Water and Nutrients). The current understanding of the structure and functions of crop root systems and the avenues for the optimization of root anatomy and morphology traits that could be applied to the genetic and agronomic improvement of crop root systems for more effective below-ground resource capture are thus very important.

Lodging is the process by which the shoots of small grained cereals are permanently displaced from their vertical stance (► Lodging Resistance in Cereals). The reduced lodging risk of shorter varieties enabled them to respond to greater amounts of fertilizers and this was a significant reason for the steady improvement in global cereal grain yields starting in the late 1960s. However, lodging is still a major problem in many countries and there is an urgent need to improve lodging resistance to further increase the yield of cereal species.

Plant growth and yield are severely affected by saline soils. High concentrations of salt in the soil make it difficult for plants to take up water, whilst the accumulated salts in cells, particularly sodium and chloride ions, are toxic to plant metabolism (► Increasing Salinity Tolerance of Crops). These two factors result in a reduction in plant growth, an increase in the rate of leaf senescence, and a loss in crop yield. Crop salinity tolerance can be improved, but a more in-depth understanding of osmotic and ionic stresses is needed.

Agroecology provides the basic ecological principles needed for studying, designing, and managing agroecosystems that are productive, sustainable, and economically viable (► Agroecological Basis for Managing Biotic Constraints). Rather than focusing on one particular component of the agroecosystem, agroecology emphasizes the interrelatedness of all of its components and the complex dynamics of ecological processes including all environmental and human elements. From a management perspective, the agroecological objective is to provide balanced environments, sustained yields, biologically mediated soil fertility, and natural pest regulation through the design of diversified agroecosystems and the use of low-input technologies.

Plant diseases cause substantial crop losses every year (► Crop Diseases, Management and Control of). Controlling plant diseases is therefore vital to maintaining crop productivity. Crop diseases can be controlled using a variety of methods; however, plant pathogens are genetically adaptable and can overcome plant resistance, and the toxic effects of pesticides. Ensuring that crops are adequately protected from diseases depends therefore on being a step ahead of the pathogens by improving existing control measures and developing new approaches.

Increase of crop yields may be achieved by maximizing the proportion of sunlight energy that is fixed

by the crop or by reducing the amount of energy that is lost by insect pests, diseases, and weeds. More than 50% of the potential yield of agricultural crops is lost by the three constraints. ►Integrated Pest Management (IPM) aims to diminish losses caused by insect pests in agriculture, in an economically, ecologically, and sociologically acceptable manner. A major challenge for ecology is the development of a scientific approach to better understand processes in agroecosystems in order to implement more rapidly sustainable IPM systems.

Yield increases will continue to play a dominant role in world food security (►Crop Yields Around the World: Closing the Gap and Raising the Potential). Understanding the gaps between potential and actual yield is of paramount importance in order to increase actual yields. These include several aspects with respect to the natural resource base of the plot (climate, soil type, topography) and long-term management investments.

Grain quality of field crops is related to seed structure and composition. Grain composition is the major reason why only a limited number of plant species are used for food and fiber (►Grain Quality in Oil and Cereal Crops). It is impossible to put forward a unique grain quality definition for any species because this depends on the specific product end use. Therefore, understanding the physiological bases of seed composition and structure is essential to produce grains with a particular quality specification.

Agricultural production takes place under erratic and unpredictable conditions, particularly the availability and timing of radiation and rainfall patterns which are extremely difficult to predict. Their effects are compensated to some extend by the qualities of the land and the interventions of the farmer. Any methodology that would improve the predictability of the availability of resources and their impact on the performance of the production system could in principle improve performance and reduce the level of uncertainty (►Simulation Models as Tools for Crop Management). Crop growth simulation models are viewed as excellent tools for the reduction of this uncertainty. Advantages and limitations of such models need to be understood in the context of past experiences and the current state of the art in order to ascertain their best possible uses.

Crop Traits: Gene Isolation

Kurniawan Rudi Trijatmiko[1], Andy Pereira[2]
[1]International Rice Research Institute, Manila, Philippines
[2]University of Arkansas, Fayetteville, AR, USA

Article Outline

Glossary

Backcross To cross the progeny of a hybrid to one of its parents, which when repeated for many generations would yield advanced backcross progeny.

cDNA Complementary DNA (cDNA) refers to the DNA copy of RNA transcripts, which can be cloned into vectors to generate a library (collection) of different cDNAs.

Chromosomal recombination Breakage and rejoining of parental homologous chromosomes during meiosis, resulting in the exchange of chromosomal segments.

Cloning vector DNA molecule into which another DNA fragment can be integrated and replicated to produce large quantities of the cloned DNA. Examples are plasmids, lambda phage, cosmids, yeast artificial chromosomes (YACs), and bacterial artificial chromosomes (BACs).

Complementation A method of validating a gene cloning by using wild-type allele to rescue the function of mutant allele through genetic transformation.

Crop trait Any morphological, physiological, or other biological feature measurable at the plant level, present in different forms in different individuals that enable genetic analysis.

Domestication An artificial selection process conducted by humans to produce plants that have more desirable traits than wild plants.

EST Expressed sequence tag is a short subsequence from a transcribed cDNA sequence.

Forward genetics A strategy to identify or clone genes that are responsible for a phenotype of interest.

Gene A gene is an ordered sequence of nucleotides that encodes a specific functional product (i.e., a protein or RNA molecule).

Marker Molecular or genetic marker is a DNA sequence at a known location on a chromosome.

Mutation Changes in a genomic sequence, occurring naturally or artificially, that can either have no effect, alter the product of a gene, or prevent the gene from functioning properly or completely.

NIL Near-isogenic lines (NILs) refers to genotypes or lines that are genetically almost identical except for a small chromosome fragment or DNA sequence by which they differ.

ORF Open reading frame (ORF) refers to a DNA sequence that does not encode a stopcodon and predicted to encode a protein.

PCR Polymerase chain reaction, a method in which DNA is amplified or increased in number of copies, using oligonucleotide primers flanking the DNA sequence and an enzyme that carries out the reaction.

Polyploid Organism that has more than two paired sets of chromosomes that is present in a diploid.

Positional cloning A method of cloning genes based on their position in the genome, using molecular markers in a genetic map located close to the gene and then identifying cloned DNA fragments containing the gene of interest.

Quantitative trait locus A region on the genome associated with a particular phenotype showing continuous and measurable variation such as height or weight.

Reverse genetics A strategy to identify the function of genes revealed by DNA sequencing, by analyzing the phenotypic effects of the gene sequences by mutations or changes in expression.

RFLP Restriction fragment length polymorphism (RFLP) is a molecular marker revealed by differences in restriction fragments between homologous (similar) fragments of DNA.

STS marker A sequence-tagged site (STS) marker is a short DNA sequence at known location in the genome, with nucleotide differences/polymorphisms that make it a useful marker for mapping.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Transposon A piece of DNA that can move within the genome of an organism, and when inserted in genes can cause a mutation.

Definition of the Subject and Its Importance

Crop plants have been selected from their wild ancestors by humans for food, fiber, health, recreation, industry, and other special purposes. Crop breeding has then added on desired traits for convenience in crop production. Genetics, molecular biology, genomics, and other disciplines have now provided us an insight to the genes that encode these very important crop traits on which the human race now depends. Knowledge of the molecular genetic basis of valuable crop traits will help provide solutions to our requirements for sustainable existence, faced with the threats of growing population needs, climate change, and dwindling natural resources. This entry describes the historical and conceptual discoveries underlying the basis of important crop traits, leading from plant domestication such as in plant architecture to those offering solutions for high crop production purposes such as growth under unfavorable environments or disease outbreaks. Detailed descriptions are provided on the cloning or identification of the plant genes involved in crop traits that give an insight on the evidence required for establishing the relationship. In addition, the molecular basis of the crop trait provides an understanding of the biological processes involved, the interaction to other traits, and a framework that can be built on to engineer novel solutions to the future needs from crop plants.

Introduction

The domestication of the major crop plants from around 10,000 to 4,000 years ago resulted in the selection of crops on which humans are now dependent [1]. The crop traits involved in domestication, transformed the wild species to a crop species catered to the needs of this paradigm shift of human civilization to an agricultural lifestyle. These early crop traits that made agriculture possible were selections from wild species that allowed humans to collect grain from the sown crop involving non-shattering, larger grain and fruit, determinate growth with more synchronous harvest, easier accessibility to the grain from protective hard outer coat, and an overall increase in edibility along with a plethora of many specialized traits. The genetic analysis and molecular isolation of key domestication traits is now uncovering the gene, the regulatory processes, and the selective sweeps from the nearest wild ancestors that accompanied the domestication process [2].

Gene cloning and DNA sequencing from the simple bacterial organisms to that of the complex polyploids such as wheat, has enabled researchers to examine gene sequences one at a time and make conclusions on similarity between close and distantly related species, propose functions based on molecular and biological properties. In fact the generation of gene sequences of a multitude of organisms is the fastest growing dataset, which promises to reveal the identity and function of all living forms. Genome sequencing started with model and standard genomes. The model plant *Arabidopsis thaliana*, a common weed, was selected for genome sequencing and molecular genetics analysis [3], with the DNA sequence of a specific ecotype Columbia [4]. Now, the 1001 genomes project intends to finish off high-quality resequencing of Arabidopsis [5] that will provide sequences of genes from the ecotypes adapted to diverse environments, which might reveal the effect of natural selection and adaptation of this very effective weed species. This also opens up a new era of going beyond a "reference" genome sequence of an organism toward sequence-based maps of closely related genotypes, which can provide a more accurate landscape of differences between genes and genomes and the relationships to expressed traits. The power of this technology was demonstrated by revealing the low level of mutations that occur between plants following a few generations of self-fertilization [6].

Gene isolation and cloning in higher plants has now come a long way since the early days of struggling with genome size and complexity. Isolation of mRNA and characterization of translated proteins was one of the primary methods to demonstrate the function of specific nucleic acids as shown for soybean leghemoglobin mRNA [7], which was then proven to be transcribed from the soybean genome [8]. One of the first approaches in plants to characterize the different components of a plant cell in terms of the complexity of nuclear and polysomal RNA, was using tobacco an

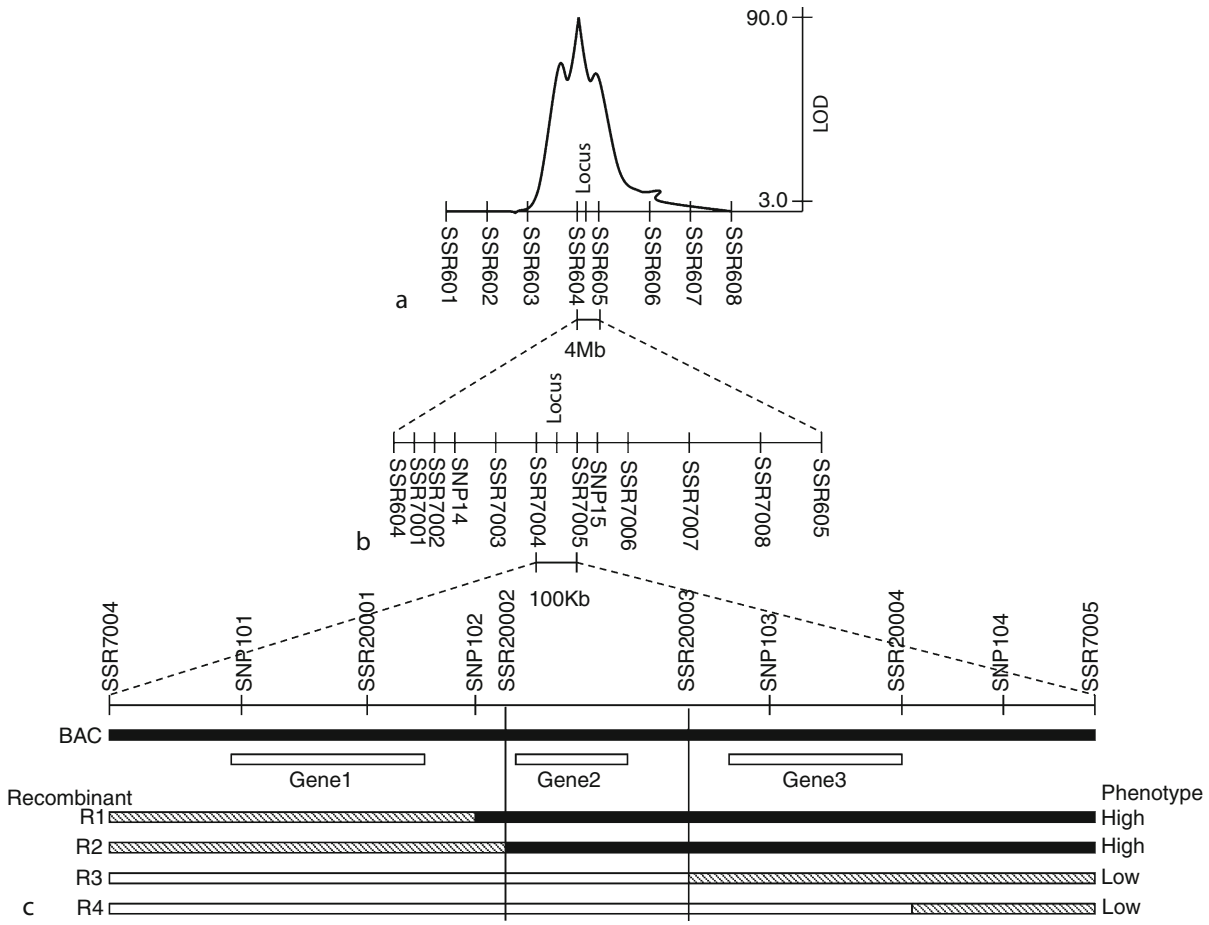
early amenable model for biological studies [9], and then analysis of the proportion of the genome and the genes that were transcribed [10]. One of the first plant genes cloned from genomic DNA was a soybean ribosomal DNA (rDNA) gene from a lambda genomic DNA library [11]. Soybean continued to be a species of interest, resulting in the generation of a complementary DNA (cDNA) library for the analysis of auxin response [12]. In maize, differential expression of the gene for the chloroplast encoded large subunit of ribulose bisphosphate carboxylase was shown in bundle sheath cells and absent in mesophyll cells [13], describing the regulation of enzymes distinguishing C4 plant cell types. These early studies showed the potential to address different plant traits by the analysis of cloned or isolated characterized genes, and the novel insights they provided.

In the course of improvement in techniques for the isolation of plant genes, methods were developed to make a correlation or causative association between specific functions and gene sequences. These functions could be defined on the basis of specific proteins with a biological function or genes determining a phenotype as revealed by isolated mutants or naturally occurring variants.

One of the important technologies that helped bridge the gap between genes and functions was the development of transformation technologies that allowed the expression of isolated genes in plants and the monitoring of the resultant phenotype. Following early demonstrations of the transfer and expression of the *Agrobacterium tumefaciens* Ti-plasmid into plant cells [14], these Ti-plasmids were engineered to transfer DNA (T-DNA) and express genes in transformed plant cells, demonstrated by a number of research groups around the same time in 1983 [15–19]. In addition to *Agrobacterium*-mediated transformation systems, other methods were developed such as direct DNA transfer to plant protoplasts [20], electroporation [21], and particle bombardment [22].

Cloning of plant genes can presently be accomplished by a variety of high-throughput ways. The cloning of expressed genes in the form of cDNA libraries has been done in plasmids, lambda vectors, and other expression systems that allow the selection of specific genes based on hybridization, DNA sequencing, or functional expression assays. The plant genome can be fractionated into representative fragments and cloned in a variety of sizes in appropriate cloning vectors. Genome fragments, in progressively increasing sizes from a few kilobases (Kb) to megabases (Mb), can be cloned in vectors such as plasmids, lambda phage, cosmids, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and modifications to these. While the smaller clones are useful for characterizing single genes or fragments thereof, the larger clones are used in positional cloning methods to identify genes using molecular markers located close to the gene of interest.

To enable the isolation of plant genes that are associated with plant biological functions/processes or crop traits, a causal relationship has to be made between DNA sequence and observed phenotype. Genetic analysis of mutants or other genetic variants that are localized on the chromosome can be used to clone the corresponding genes by methods such as map-based cloning (Fig. 1) and transposon tagging (Fig. 2), which are the two most commonly used methods for cloning genes by a forward genetics strategy centered around the phenotype of interest. In positional or map-based cloning, with a general strategy described in Fig. 1, the mapped position of a gene on the chromosome/genome, which specifies a specific trait or phenotype is the starting point to identify molecular/genetic markers close to the gene and then using integrated physical and genetic maps "walk" or "land" closer to the gene. The proof of isolating the gene is by recombination analysis, complementation by transformation, analysis of genetic variants by sequencing, or mutational analysis of the candidate genes. In transposon tagging, described in a scheme in Fig. 2, an insertion sequence from a native or heterologous transposon is used to generate mutants of a specific phenotype or genetic locus, and analysis of the transposon/insertion-tagged mutant by co-segregation analysis to a transposon probe, transposon revertant analysis or complementation of the mutant by transformation, and among the many ways evidence can be obtained to prove the identity of the tagged gene. With the availability of genome sequences of many plant species, these quite anonymous sequences need to be assigned a function, generally through a reverse genetics strategy. In reverse genetics strategies many tools are available, in most models and important crops, to



Crop Traits: Gene Isolation. Figure 1

Example of map-based cloning of a gene. (**a**) Locus mapped to a chromosome region between markers SSR604 and SSR605 via rough-scale mapping, with LOD (log of odds) score indicating linkage. (**b**) The gene further delimited to a 100-kb genomic region between markers SSR7004 and SSR7005 via fine mapping and physical mapping. (**c**) Precision mapping identified break points flanking a region containing a single gene. *Black bars* represent DNA from parent 1 (phenotype: high), *white bars* represent DNA from parent 2 (phenotype: low), and *patterned bars* represent DNA of heterozygote. The *vertical lines* show where recombination break points are positioned along the chromosome

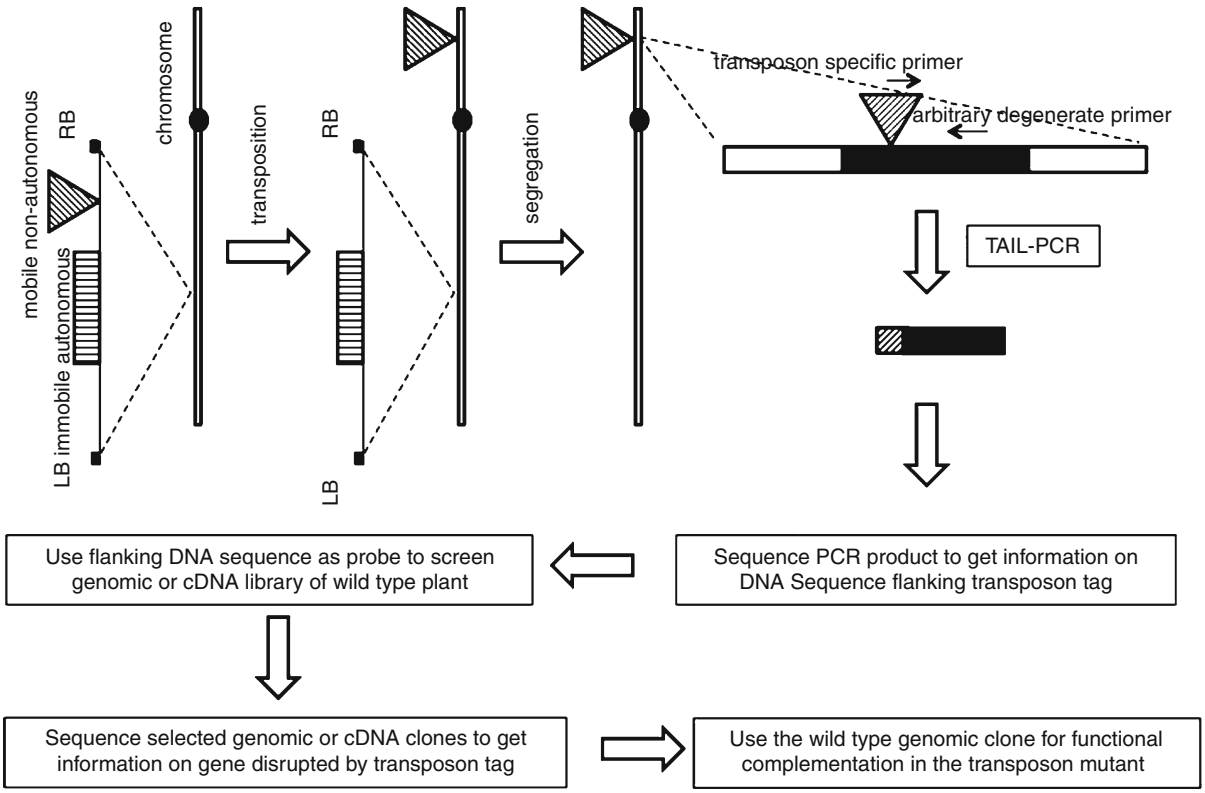
generate or select mutations in specific genes and thus attribute functions by the analysis of the mutant phenotypes.

This entry describes the specific methods that have been used in the isolation and characterization of genes that determine important crop traits that agriculture is based on. The crop traits identified in many plants have homologues in the model plant Arabidopsis that can provide clues to the function. The examples described are described in some detail and others summarized in a table (Table 1) for the reader to refer to.

Crop Traits: Gene Isolation

Dwarfing Genes in Cereals

The biggest increase in agricultural production in the modern era, around the middle of the last century, is attributed to the "Green Revolution" where grain yields of the major world cereals, wheat and rice were improved along with complementary crop production practices [23, 24]. The spectacular increases in wheat and rice yields during the Green Revolution were enabled by the introduction of dwarfing traits



Crop Traits: Gene Isolation. Figure 2

Example of gene tagging using heterologous transposons. An "in cis two-component transposon construct" integrated into plant chromosome via *Agrobacterium*-mediated transformation. The construct contains a mobile nonautonomous transposon and an immobile transposase source. The transposase mediates transposition of the mobile nonautonomous transposon into new chromosomal positions. Segregation in the next generation creates stable mutants containing the transposon inserted in a gene with no transposase source. These stable mutants can be used in forward or reverse genetics. Identification of integration position is typically done by sequencing of DNA flanking the transposon isolated using techniques, for example, TAIL-PCR, Inverse PCR, or plasmid rescue. The flanking sequence is used as a probe to screen genomic and cDNA clones. Functional complementation is done by transforming wild-type genomic clone containing the tagged gene into the insertional mutant line

into the crop varieties. The founder of this Green Revolution, Norman Borlaug received the Nobel Peace prize in 1970 in recognition for his efforts in bringing food security to major parts of the developing world [25]. Identification of the genes responsible for these traits showed that they interfere with the action or production of the gibberellin (GA) plant hormones [26].

The dwarfing gene of wheat, *Reduced Height-1* (*Rht-1*), was isolated based on sequence similarity to the previously isolated Arabidopsis *GIBBERELLIN INSENSITIVE* (*GAI*) gene [27]. GAI is a member of the DELLA proteins and a repressor of GA responses [28]. The wheat *Rht-B1b* and *Rht-D1b* dwarfing mutants had similar characteristics to the Arabidopsis *gai* mutant, including reduced plant height, reduced responses to gibberellin, and increased *in planta* gibberellin levels. Compared to the wild-type *Rht-1* allele, the *Rht-B1b* and *Rht-D1b* dwarfing alleles each contained a nucleotide substitution that created a stop codon near the N-terminus of the protein [27]. It was proposed that translational reinitiation after these introduced stop codons might result in an N-terminally truncated product that confers the mutant phenotype.

Crop Traits: Gene Isolation. Table 1 Some isolated genes in crops

Gene (s)	Crop	Molecular and phenotypic function	Cloning method[a]	Validation method[b]	Causative change[c]	Reference (s)
1. Dwarfing gene						
Rht-1	Wheat	Transcriptional regulator (SH2); plant height	SS	Mut, HE	EStp	[27]
sd1	Rice	GA20 oxidase; plant height	SS, MBC	Var	Coding	[30–32]
d	Tomato	Cytochrome P450 enzyme; plant height	TT	FC		[141]
2. Flowering/heading date						
Hd6	Rice	Protein kinase; flowering time	MBC	FC	EStp	[33]
Hd1	Rice	Transcriptional regulator (zinc finger); flowering time	MBC	FC	Coding	[34]
Hd3a	Rice	Phosphatidylethanolamine-binding protein; flowering time	MBC	OE, Sil	-	[37]
Ehd1	Rice	B-type response regulator; flowering time	MBC	FC	AC	[142]
Vgt1	Maize	Transcriptional regulator (AP2); flowering time	MBC, AM	OE, Sil	Reg	[143]
E3	Soybean	Phytochrome A; flowering time	MBC	Mut	AC?	[144]
Vrn1	Wheat	Transcriptional regulator (MADS); vernalization	MBC	Var	Reg	[145]
Vrn2	Wheat	Transcriptional regulator (ZCCT); vernalization	MBC	Sil	AC	[146]
3. Fruit ripening, shape, and weight						
Rin	Tomato	Transcriptional regulator (MADS-box); fruit ripening	MBC	FC, Sil	Coding	[147]
Ovate	Tomato	Regulatory protein (OVATE); fruit shape	MBC	FC	EStp	[148]
fw2.2	Tomato	Cell signaling; fruit weight	MBC	FC	Reg	[40]
4. Grain yield						
Gn1	Rice	Cytokinin oxidase/dehydrogenase; grain number	MBC	Sil	Reg/EStp	[42]
GS3	Rice	VWFC module-containing protein; grain weight	MBC	Var	EStp	[44]
GW2	Rice	RING-type protein with E3 ubiquitin ligase activity; grain width	MBC	OE, Sil	EStp	[47]
5. Disease resistance						
Hm1	Maize	HC toxin reductase; resistance to the fungus *Cochliobolus carbonum*	TT	Co	Reg	[49]
Cf-9	Tomato	Leucine-rich repeat family of proteins; resistance to leaf mold fungus *Cladosporium fulvum*	TT	Co	Wild	[54]
RB/Rpi-blb1	Potato	CC–NBS–LRR-class R-gene analog; resistance to oomycete pathogen *Phytophthora infestans*	MBC	FC	Wild	[58, 59]
Rpi-blb2	Potato	NBS–LRR protein; resistance to oomycete pathogen *Phytophthora infestans*	MBC	FC	Wild	[60]

Crop Traits: Gene Isolation. Table 1 (Continued)

Gene (s)	Crop	Molecular and phenotypic function	Cloning method[a]	Validation method[b]	Causative change[c]	Reference (s)
mlo	Barley	Membrane-anchored protein; resistance against the fungal pathogen *Blumeria graminis* f. sp. *hordei* (Bgh)	MBC	Mut, RA	AC/EStp	[62]
Mi	Tomato	NBS–LRR protein; resistance to root-knot nematodes (*Meloidogyne* spp.)	MBC	FC	Wild	[72]
Tm-2^2	Tomato	CC–NBS–LRR class of R proteins; resistance to tomato mosaic virus (ToMV)	TT	FC	Wild	[76]
Pto	Tomato	Protein kinase; resistance to *Pseudomonas syringae* pv. *tomato*	MBC	FC	Wild	[149]
Xa21	Rice	Protein kinase; resistance to *Xanthomonas oryzae* pv. *oryzae*	MBC	FC	Wild	[150]
Cf-2	Tomato	LRR protein; resistance to leaf mold fungus *Cladosporium fulvum*	MBC	FC	Wild	[151]
N	Tobacco	LRR protein; resistance to tobacco mosaic virus (TMV)	TT	FC	Wild	[152]
Hs1^{pro-1}	Sugar beet	LRR protein; resistance to beet cyst nematode	MBC	FC	Wild	[153]
I2C	Tomato	NBS–LRR protein; resistance to *Fusarium oxysporum* f sp *1ycopersici*	MBC	Sil, OE	Wild	[154]
Ve	Tomato	Cell surface-like receptors; resistance to *Verticillium dahliae*	MBC	FC	-	[155]
R1	Potato	Leucine-zipper/NBS/LRR protein; resistance to *Phytophthora infestans*	MBC	FC	Wild	[156]
Rpg1	Barley	Receptor kinase; resistance to *Puccinia graminis* f. sp. *tritici*	MBC	Var	EStp, AC, FS	[157]
Hero	Tomato	NBS–LRR protein; resistance to potato cyst nematodes *Globodera rostochiensis*	MBC	FC	Wild	[158]
Lr10	Wheat	CC–NBS–LRR protein; resistance to *Puccinia triticina*	MBC, HS	OE	CD	[159]
Lr21	Wheat	NBS–LRR protein; resistance to *Puccinia triticina*	MBC	FC	Wild	[160]
Pm3b	Wheat	CC–NBS–LRR protein; resistance to *Blumeria graminis* f. sp. *tritici*	MBC	STA	-	[161]
Pi9	Rice	NBS–LRR protein; resistance to *Magnaporthe grisea*	MBC	FC	Wild	[162]
Rpg5	Barley	Protein kinase; resistance to *Puccinia graminis* f. sp. *secalis*	MBC	Sil	FS	[163]
Yr36 (WKS1)	Wheat	Kinase-START protein; resistance to *Puccinia striiformis* f. sp. *tritici*	MBC	FC	Wild	[164]
Rdg2a	Barley	CC–NB–LRR protein; resistance to *Pyrenophora graminea*	MBC	FC	Reg	[165]

Crop Traits: Gene Isolation. Table 1 (Continued)

Gene (s)	Crop	Molecular and phenotypic function	Cloning method[a]	Validation method[b]	Causative change[c]	Reference (s)
6. Plant and inflorescence architecture						
tb1	Maize	Transcriptional regulator (TCP); plant and inflorescence structure	TT	Co	Reg	[80]
tga1	Maize	Transcriptional regulator (SBP); seed casing	MBC	Mut	AC	[85]
Q	Wheat	Transcriptional regulator (AP2); inflorescence structure	MBC	Mut	Reg/AC	[92]
vrs1	Barley	Transcriptional regulator (HD-ZIP); spikelet morphology	MBC	Mut	AC/FS	[98]
nud	Barley	Transcriptional regulator (ERF); seed casing	MBC	Var, Mut	CD	[103]
MOC1	Rice	Transcriptional regulator (GRAS); tillering	MBC	FC	TE	[107]
PROG1	Rice	Transcriptional regulator (ZF); growth habit	MBC	FC	Reg/AC	[109]
7. Seed quality and color						
opaque-2	Maize	Transcriptional regulator (bZIP); endosperm characteristic	TT	Co	TE	[111]
Wx	Maize	Starch synthase; sticky grains	TT	Rev	TE	[113]
Wx	Rice	Starch synthase; sticky grains	SS	Var	Splice	[115, 116]
Sh2	Maize	Pyrophosphorylase; supersweet sweet corn	TT	Rev	TE	[118]
su1	Maize	Isoamylase; sweet corn gene	TT	Co	AC	[119]
Rc	Rice	Transcriptional regulator (bHLH); seed color	MBC	Mut	Coding	[121]
c1	Maize	Transcriptional regulator (MYB); kernel color	TT	Co	Reg	[166]
y1	Maize	Phytoene synthase; carotenoid content	TT	Rev	Reg	[167]
R	Pea	Starch branching enzyme; seed sugar content	IS	Co	TE	[168]
Brix9-2-5	Tomato	Invertase; fruit-soluble solid content	MBC	IR	Reg	[169]
8. Seed shattering						
sh4	Rice	Transcriptional regulator (Myb3); abscission layer formation, shattering	MBC	FC	Reg/AC	[128]
qSH1	Rice	Transcriptional regulator (homeodomain); abscission layer formation, shattering	MBC	FC	Reg	[129]
sh-h	Rice	CTD phosphatase; abscission layer differentiation, shattering	MBC	Mut, Sil	Splice	[131)
Jointless	Tomato	Transcriptional regulator (MADS); abscission zone development, shedding	MBC	FC, Sil	Coding	[170]
9. Tolerance to abiotic stresses						
SKC1	Rice	HKT-type transporters; salt tolerance	MBC	FC	AC	[134]
Sub1	Rice	Transcriptional regulator (ERF); submergence tolerance	MBC	FC	CD	[137]

Crop Traits: Gene Isolation. Table 1 (Continued)

Gene (s)	Crop	Molecular and phenotypic function	Cloning method[a]	Validation method[b]	Causative change[c]	Reference (s)
Alt1	Wheat	Aluminum-activated malate transporter; tolerance to aluminum toxicity	SH	Co, Var, HE	Reg/AC	[140]
Snorkel1, Snorkel2	Rice	Transcriptional regulator (ERF); deepwater tolerance	MBC	Var, OE	CD	[171]

[a]*MBC* map-based cloning, *TT* transposon tagging, *AM* association mapping, *HS* haplotype study, *SS* sequence similarity, *SH* subtractive hybridization, *IS* immunological screening

[b]*FC* functional complementation, *Rev* transposon revertant analysis, *Co* co-segregation analysis, *RA* recombination analysis, *Var* analysis of genetic variants by sequencing, *Mut* mutational analysis of the candidate genes, *OE* overexpression, *Sil* silencing, *IR* intragenic recombination, *HE* heterologous expression, *STA* single-cell transient assay

[c]*AC* amino acid change, *Coding* disrupted coding sequence, *FS* frame shift, *EStp* early stop codon, *Reg* regulatory change, *Splice* intron splicing defect, *CD* complete deletion, *Wild* introgression from wild relative,*TE* transposon insertion (Following Doebley et al. [1] with modification)

The rice *sd-1* dwarfing allele, with origin from cultivar Dee-Geo-Woo-Gen and used in the Green Revolution dwarf variety IR8, was mapped to the long arm of chromosome 1 [29]. Characterization and isolation of this gene was reported in 2002 by three different groups [30–32]. Biochemical analysis of the *sd-1* mutant showed that the activity of GA20 oxidase (GA20ox), a key enzyme in the biosynthesis of gibberellin, did not function effectively in the mutant. A wild-type *GA20ox* gene (*GA20ox-2*) was amplified by PCR using primers based on the conserved domain of *GA20ox* genes. Linkage mapping showed that this gene mapped to the long arm of chromosome 1, tightly linked to the *sd1* locus. Compared to the wild-type *Sd-1* allele, the *sd-1* dwarfing allele (in Dee-Geo-Woo-Gen and IR8) contained a 383-base-pair deletion which produces a frame shift that creates a stop codon [30]. Another study showed RFLP markers flanking the *sd-1* locus were positioned on a physical segment of chromosome 1 covered by contiguous BAC clones by using the physical map of the reference genome available in the database. A candidate gene search identified the *GA20ox-2* gene in this region. This gene was amplified in the wild type and mutant, and sequence comparison revealed a 280-bp deletion in the coding region of this gene in the mutant allele [31]. A third independent analysis of 3,477 segregants using several PCR-based markers localized the *sd-1* locus in a 6-kb candidate interval on chromosome 1, containing only one predictable ORF, that of *GA20ox-2*. The 3,477 segregants were derived from selfing of a backcross inbred line (BIL) having close chromosomal similarity to Sasanishiki (normal-type parent) over the whole genome length with the only heterozygous sequences located on *sd-1* locus. Sequence comparison showed that Habataki (semidwarf parent) has a 383-bp deletion from the middle of exon 1 to upstream of exon 2, including a 105-bp intron, resulting in a frame shift that produces a termination codon in exon 3 [32]. These evidence clearly defined the identity of the *Sd-1* dwarfing gene and the mutant *sd-1* alleles.

Flowering or Heading Date

Heading date or flowering time is an important trait for the adaptation of crops to different cultivation areas. In rice, heading date is determined mainly by two factors: duration of the basic vegetative growth and photoperiod sensitivity (PS) [33]. Genetic analysis of heading date in rice cultivars Kasalath and Nipponbare showed that a number of quantitative trait loci, termed Hd (heading date) determine the genetic variation for flowering time [34]. Day-length treatment sensitivity tests with an NIL of *Hd6* [NIL(*Hd6*)] revealed the *Hd6* PS phenotype, with the Kasalath allele increasing days-to-heading under natural and long-day conditions but not under short-day conditions. Linkage analysis of an advanced backcross progeny mapped *Hd6* on the long

arm of chromosome 3 as a single Mendelian factor [35]. High-resolution mapping using 2,807 segregating plants derived from an advanced backcross progeny, in which the region around *Hd6* was heterozygous and almost all other regions were homozygous for Nipponbare, delimited *Hd6* to a 26.4-kb genomic region. The sequence analysis of this region identified one gene with deduced amino acid sequence having high homology (>90%) to the α subunit of protein kinase CK2 (CK2α) in maize and Arabidopsis. Sequence comparison showed a single-nucleotide substitution within the coding region, which changed the lysine codon (AAG) in Kasalath to a premature stop codon (TAG) in Nipponbare. Functional complementation was performed by introducing the Kasalath genomic fragment carrying the CK2α gene into Nipponbare and the transgenic plants scored under natural day-length condition showed late heading, indicating that the Kasalath allele of CK2α increases days-to-heading [33].

Another heading date locus *Hd1* was characterized by high-resolution mapping using 1,505 early heading BC3F3 segregants derived from a cross between Nipponbare and Kasalath, which resolved the locus to a genomic region of 12 kb. Sequence analysis of the region identified one putative gene with considerable similarity to the *CONSTANS* (*CO*) gene, known to be involved in photoperiod response in Arabidopsis. Sequence comparison showed a 2-bp deletion in the second putative exon of the Kasalath allele resulting in a premature stop codon and a predicted shorter protein than the wild-type Nipponbare protein. Functional complementation was performed by transferring the candidate *Hd1* region from Nipponbare into a NIL of Nipponbare carrying an introgression of Kasalath nonfunctional *Hd1* allele. Transgenic plants showed earlier heading under short-day conditions than the NIL control and null-segregants. These results provide clear evidence that the *Hd1* sequence in the candidate genomic region retains the function of photoperiod response [34].

The *Hd3a* locus was roughly mapped on chromosome 6. NIL(*Hd3a*), an NIL homozygous for the Kasalath allele at the *Hd3a* locus in the genetic background of Nipponbare, headed earlier than Nipponbare under SD conditions and headed at almost the same date as Nipponbare under LD and natural field conditions [36]. High-resolution mapping using 2,207 segregants delimited *Hd3a* to a 20-kb genomic region, whose sequence revealed one putative gene with high similarity to the *FLOWERING LOCUS T* (*FT*) gene, which promotes flowering in Arabidopsis. In this study, the nucleotide polymorphism that caused the allelic difference between Kasalath and Nipponbare *Hd3a* could not be clarified [37].

Fruit Weight and Grain Yield

The domestication and improvement of crops has been accompanied by increase in size and yield of harvestable products. Cultivated tomatoes (*Solanum lycopersicum*) have hence undergone more than a 100-fold increase in mass over their wild relatives. A major quantitative trail locus (QTL) for fruit weight, *fw2.2*, was mapped to the same position on chromosome 2 in an introgression line F2 derived from *S. lycopersicum* x *Lycopersicon pennellii* and a backcross 1 (BC1) population derived from *S. lycopersicum* x. *Lycopersicon pimpinellifolium*. The *fw2.2* locus accounts for 30% and 47% of the total phenotypic variance in the *L. pimpinellifolium* and *L. pennellii* populations, respectively, indicating that this is a major QTL controlling fruit weight in both species. The small-fruit *L. pennellii* allele for *fw2.2* is semidominant to the large-fruit *S. lycopersicum* allele [38]. High-resolution mapping using 3,472 F2 plants derived from a cross between *S. lycopersicum* and a NIL containing a small introgression from *L. pennellii* narrowed down *fw2.2* to a less than 150-kb region [39]. A yeast artificial chromosome (YAC) containing *fw2.2* was used to screen a cDNA library constructed from the small-fruit *L. pennellii*. Four unique transcripts were identified and used to screen a *L. pennellii* cosmid library, identifying four positive, nonoverlapping cosmids (cos50, cos62, cos69, and cos84), one corresponding to each unique transcript. These four cosmid clones were transformed into two tomato cultivars carrying the partially recessive large-fruit allele of *fw2.2*. R1 progeny of primary transformants carrying cos50, but not other cosmids, showed a statistically significant reduction in fruit weight compared to null-segregants, indicated that *fw2.2* is contained within cos50. Sequence analysis of cos50 revealed two open reading frames (ORFs): one

corresponding to cDNA44, which was used to isolate cos50, and another 663-nucleotide (nt) gene, *ORFX*, for which no corresponding transcript was detected in the initial cDNA library screen. Analysis on a single recombination event used in the previous mapping showed that *ORFX* is the likely cause of the *fw2.2* QTL phenotype. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that the relative level of the *ORFX* transcript in the carpels of the small-fruited NIL was significantly higher than in the large-fruited NIL. Analysis of the predicted amino acid sequence of *ORFX* indicated that it is a soluble protein with alpha/beta-type secondary structure, and has a structural similarity to the human oncogene c-H-ras p21. Sequence comparison of *L. pennellii* and *S. lycopersicum ORFX* regions indicated that the *fw2.2* phenotype is probably not caused by differences within the coding region of *ORFX*, but by one or more changes upstream in the promoter region of *ORFX* [40].

Grain yield is a complex crop trait determined mainly by the three component traits, number of panicles, number of grains per panicle, and grain weight; all of which are typical quantitative traits. In rice, the developments in genome mapping, sequencing, and functional genomic research have provided the necessary tools for dissecting the genetic and molecular bases of these quantitative traits [41].

The rice grain number gene *Gn1* was first roughly mapped on the short arm of chromosome 1 using 96 F2 individuals derived from heterozygote (*Gn1*/*gn1*) plants of NIL-*Gn1* carrying the *Gn1* region from cultivar Habataki in the Koshihikari background. Habataki plants have been known to produce more grains in their main panicle than Koshihikari plants. It was found that *Gn1* consisted of two loci, *QTL-Gn1a* and *QTL-Gn1b*. High-resolution mapping using 13,000 F2 plants derived from heterozygotes (*Gn1a*/*gn1a*) of NIL-*Gn1a* narrowed down the *Gn1a* region into a 6.3-kb interval containing one reading frame with high similarity to cytokinin oxidase/dehydrogenase (CKX), named *OsCKX2*. Sequence comparison between Habataki and Koshihikari revealed several nucleotide changes, including a 16-bp deletion in the 5′-untranslated region, a 6-bp deletion in the first exon, and three nucleotide changes resulting in amino acid variation in the first and fourth exons of the Habataki allele. However, protein expression study in *Saccharomyces cerevisiae* showed both Habataki and Koshihikari alleles of *OsCKX2* encode functional enzymes. Sequence analysis of 5150, a rice variety from China producing higher grain number, detected 11-bp deletion in the coding region of this gene creating a premature stop codon. The coincidence of the *OsCKX2* null allele and a higher grain number suggested that a reduction or loss of function of *OsCKX2* enhanced grain production. Confirmation was done by introducing antisense strands of *OsCKX2* into an easily transformable cultivar Taichung 65 (TC65), which possesses the Koshihikari allele of *OsCKX2*. Transgenic plants with reduced levels of expression developed higher grain numbers. Reverse Transcription (RT)-Southern blotting showed that the highest levels of *OsCKX2* expression in inflorescence meristems were found in Koshihikari, but were less abundant in Habataki and NIL-*Gn1a* and extremely low in 5150. These results suggested that the phenotypic differences observed might have been caused by differential transcription of *OsCKX2* [42].

The rice *GS3* grain weight gene was mapped to the pericentromeric region of chromosome 3 [43]. Near-isogenic lines (NILs) of *GS3* were developed by successive crossing and backcrossing Minghui 63 (large grain) with Chuan 7 (small grain), using Minghui 63 as the recurrent parent. High-resolution mapping using 1,384 BC3F2 individuals with the recessive phenotype (large grain) derived from one BC3F1 plant heterozygous for the *GS3* region and containing the least genetic background from Chuan 7 delimited the *GS3* region to 7.9 kb. A full-length cDNA was identified that matched well with the region, and sequence analysis of the predicted GS3 protein revealed the presence of a combination of multiple domains. Comparative sequencing analysis using six cultivars including three with long grains and three with short to medium grains indicated that all the large-grain varieties tested share the same nonsense mutation in the second exon of the *GS3* gene that causes a 178-aa truncation in the C-terminus of the predicted protein. These findings suggest that *GS3* may function as a negative regulator to prevent the growth and size of the grain [44].

Two QTLs for grain width were mapped to the short arm of chromosome 2 [45, 46). Subsequently, a major QTL for grain width GW2, was mapped to the same

region using an F2 population derived from a cross between WY3 (1,000-grain weight, 41.9 ± 1.3 g) and Fengaizhan-1 (FAZ1) (1,000-grain weight, 17.9 ± 0.7 g). High-resolution mapping using 6,013 BC3F2 plants narrowed the GW2 locus to an 8.2-kb region. Only one predicted ORF was considered a viable candidate for GW2 in this region [47]. Homology search in databases showed that GW2 encodes a previously unknown RING-type protein with E3 ubiquitin ligase activity, which is known to function in the degradation by the ubiquitin–proteasome pathway. Comparison of the nucleotide sequences of the FAZ1 and WY3 alleles of GW2 uncovered a 1-bp deletion resulting in a premature stop codon in exon 4 of the WY3 allele. The premature stop codon led to truncation of 310 amino acid residues; the remaining portion of the protein consisted of a 115-residue polypeptide of ~13 kDa. Sequence analysis of GW2 from Oochikara, another rice variety that has a wider grain width, similar to WY3, showed a nucleotide sequence identical to the WY3 allele. These data indicate that reduction or loss of function of GW2 results in increased grain width. Rice transformation was used to produce transgenic plants expressing different levels of GW2. The transgenic plants with antisense strands of GW2 and with reduced levels of endogenous expression had a significantly wider grain width than plants containing the vector control. Transgenic plants overexpressing GW2 cDNA under the control of the 35S promoter, which produced high levels of expression, showed reduced grain width [47].

Disease Resistance

Disease resistance loci (R genes) conferring resistance to specific races of the pathogen, carrying corresponding avirulence (Avr) genes, have been studied in light of the gene-for-gene hypothesis proposed by Flor [48]. Plants that contain such race-specific R-genes with resistance to specific pathogen races, offer an interesting system to study plant pathogen interactions, although the genes are very rarely useful in the crop as they are quickly overcome by the pathogen gaining virulence.

In addition, there are non-race-specific disease resistance loci which provide plant resistance to a wider range or races of the pathogens. The first plant resistance gene cloned was *Hm1* from maize, which belonged to this class of resistance genes, was isolated by transposon tagging [49]. Two RFLP probes were mapped to 5 centimorgan (cM) proximal and distal to the *Hm1* locus, respectively, using progeny from the cross K61/Pr1 × K61 [50]. These two probes were used to classify segregating progeny of transposon insertion *hm1* mutants to determine which *HM1* alleles they had inherited. A transposable element probe was then used to identify a restriction fragment that cosegregated with *hm1* mutant allele. This fragment was isolated and sequenced, and the DNA flanking the transposon insertion was used as probe in northern blot analysis. A 1.3-kb RNA band was detected in polyadenylated [poly(A)$^+$] RNA from the resistant inbred strain Pr1 (*Hm1-Pr1*), while the susceptible strain, K61 (*hm1-2*), and the mutants either had no detectable hybridizing mRNA, or an mRNA of aberrant size. Difference at the transcriptional level between resistant and susceptible genotypes makes it unlikely that susceptible genotypes possess an alternative form of HM1 with specificity for a substrate other than HC toxin. A 1.6-kb cDNA clone was isolated by homology with the probe and sequenced. DNA sequence databases revealed homology between the *HM1* cDNA and the NADPH-dependent dihydroflavonol-4-reductase (DFR) genes of maize, petunia, and snapdragon. This homology supports the prediction that *HM1* encodes HC toxin reductase (HCTR) [49].

Among the race-specific R-genes, the tomato-leaf mold interaction has been well studied and yielded the first examples of cloned R-genes. The avirulence gene *Avr9* in the leaf mold fungus *Cladosporium fulvum* was shown to specify a 28-amino acid secreted peptide that elicits a necrotic response when injected into tomato plants carrying the *Cf-9* resistance gene [51]. The *Cf-9* gene was introgressed into cultivated tomato from wild species *L. pimpinellifolium* accession PI126915 [52]. The *Cf*-9 locus was mapped to the short arm of chromosome 1 [53], and a targeted transposon tagging strategy was employed to isolate the *Cf-9* gene [54]. A tomato line homozygous for *Cf*-9 was crossed to a transgenic tomato line carrying a *Ds* element located 3 cM from the *Cf*-9 locus, and additionally to a transgenic tomato plant containing a stabilized *Ac* (s*Ac*) element. The F1 plants were crossed and the progenies selected to produce tagging parents heterozygous for *Ds* and s*Ac* and homozygous for *Cf-9*. To tag *Cf*-9 the

tagging lines were crossed as female parents to a tomato line homozygous for the *Avr9* transgene and lacking *Cf*-9. The progeny of this cross, which were heterozygous for *Cf*-9 and *Avr9*, became necrotic and died shortly after seed germination, but the mutants for *Cf*-9 survived. Approximately 160,000 progeny were germinated and 118 survivors were recovered. A total of at least 37 independent *Ds* insertions into *Cf-9* were identified. Specific *Cf-9* primers were used in conjunction with *Ds* primers to map the *Ds* insertions on the basis of PCR product size. Twenty-eight *Ds* insertions were mapped to the same 3-kb region of the tomato genome. All stable mutants tested were susceptible to race 5 of *Cladosporium fulvum*, which indicates concordance between the loss of response to the *Avr9* transgene and loss of resistance to a race of the fungus carrying *Avr9*. Isolation via plasmid rescue and sequencing of the flanking genomic sequence showed that *Cf-9* encodes a putative membrane-anchored extracytoplasmic glycoprotein with homology to the leucine-rich repeat family of proteins [54].

Late blight caused by the oomycete pathogen *Phytophthora infestans*, and responsible for the Irish Potato Famine of the mid-nineteenth century that induced vast migration, is still one of the most devastating of plant diseases causing more than $3 billion loss annually [55]. The *P. infestans* pathogen easily overcomes the R-genes crossed in from various wild relatives of potato. Diploid *Solanum bulbocastanum* from Mexico, which was not easily crossable to potato (*Solanum tuberosum*) cultivars, had been characterized to have high resistance to *P. infestans* and was used to characterize the resistance gene loci. The *RB* (for "Resistance from *S. bulbocastanum*") locus was mapped to chromosome 8 of *S. bulbocastanum* [56]. BAC walking by the reiterative screening of a BAC library using probes derived from the ends of previously identified BAC clones, was initiated using linked RFLP markers and the contig subsequently used to develop PCR-based markers to enhance map resolution in the *RB* region. High-resolution mapping using 542 BC2, 1,060 BC3, and 206 BC4 genotypes delimited the *RB* region to approximately 55 kb [57]. This region contained one truncated and four complete CC–NBS–LRR (coiled coil–nucleotide binding site–Leu-rich repeat)-class R-gene analogs (RGAs). Each of the four complete RGAs was amplified from *S. bulbocastanum* using Long Range-PCR and cloned into a binary vector for complementation studies. Katahdin, a late blight susceptible potato variety was transformed with the four complete R-gene analogs. Only the transgenic plants containing *RGA2*-PCR construct displayed resistance to all six isolates of *P. infestans*, including 126C18, a "super race" that overcomes all 11 major R genes identified in *Solanum demissum*, the complementation demonstrating that *RGA2* represents the functional *RB* gene [58].

In a simultaneous effort to the analysis of the RB locus, the *Rpi-blb1* resistance locus was characterized in intraspecific crosses between *S. bulbocastanum* accessions segregating for resistance, and mapped closely to marker CT88 on chromosome 8 [59]. A BAC contig was isolated across the *Rpi-blb1* locus, and sequence analysis revealed 4 RGAs of the CC–NBS–LRR class. One of the genes termed *Rpi-blb1* complemented potato and tomato cultivars to confer resistance to complex races of the *P. infestans* pathogen. In subsequent analysis of resistant complex interspecific hybrids designated ABPT derived from *S. bulbocastanum*, the *Rpi-blb2* locus was identified and mapped to a position on chromosome 6 at a similar position as the tomato *Mi* locus (see below) conferring resistance to nematode, aphids, and white flies. The *Rpi-blb2* locus harbored 15 Mi-1 gene homologues, one of which conferred *P. infestans* resistance in tomato and potato. The Rpi-blb2 protein shares 82% sequence identity to the tomato Mi-1 protein and exemplifies the evolution of a resistance gene cluster to confer diverse resistance specificity to a wide range of organisms such as nematodes, insects, and oomycetes [60].

The barley recessive *mlo* locus conferring resistance against the fungal pathogen *Blumeria graminis* f. sp. *hordei* (Bgh) has been mapped on chromosome 4 [61]. High-resolution mapping using 2,022 F2 segregants identified a DNA marker cosegregating with *mlo* and two flanking markers at a distance of 0.24 and 0.4 cM, respectively. Screening of a large insert yeast artificial chromosome (YAC) library identified three YAC clones containing the cosegregating marker and two flanking markers. Subcloning experiments of one YAC clone into bacterial artificial chromosome (BAC) vector and further mapping and sequencing delimited the *mlo* region to 30 kb. Sequence analysis identified one sequence contig of 5.8 kb, including the cosegregating

marker, revealing an extensive region of high coding probability. Reverse transcriptase-PCR using a series of primers deduced from this region and sequencing revealed a single extensive open reading frame (ORF) of 1,599 bp. The deduced 60 kDa protein was predicted to be membrane-anchored by at least six membrane-spanning helices. Genomic PCR-based sequencing of 11 mutagen-induced *mlo* resistance alleles and their corresponding wild-type DNAs identified nucleotide alterations (point mutations or deletions) in all tested mutant alleles that at the amino acid level result either in single amino acid substitutions or truncated versions of the predicted wild-type protein. A comparison among the wild-type gene sequences of seven tested barley cultivars indicated not a single amino acid difference. Inter-mutant crosses were performed using lines containing different mutant alleles. F2 seedlings were screened for rare disease–susceptible individuals after inoculation with an isolate of powdery mildew which is virulent on each of the parental *Mlo* wild-type cultivars. Homozygous susceptible F3 progeny were isolated and used for molecular analysis using RFLP markers tightly linked (<4 cM) on each side of the *Mlo* locus. Seven susceptible individuals exhibited flanking molecular marker exchange, indicating reciprocal crossover (CO) events. Genomic PCR-based sequencing demonstrated that all seven CO type recombinants restored wild-type sequences [62].

Root-knot nematodes (*Meloidogyne* spp.) are endoparasites of thousands of crop species and are important pests of tomato worldwide [63]. The root-knot nematode resistance gene (*Mi*) was introduced into cultivated tomato, *S. lycopersicum*, from its wild relative *Lycopersicon peruvianum* in the early 1940s [64], and today it provides the only form of genetic resistance against this pathogen. The *Mi* locus was localized to the short arm of chromosome 6 [65] and multiple markers linked to Mi were identified [66–69]. In these studies, it was found that the nematode-resistant tomato line Motelle, and related lines, retained only a small introgressed region (650 kb) from *L. peruvianum*. Efforts to localize the *Mi* gene were hampered for many years because of the severe repression of recombination near this gene in *Lycopersicon esculentum* lines carrying the introgressed *L. peruvianum* DNA [67, 68, 70]. This handicap was circumvented by screening large populations of tomato for recombinants and by identifying recombinants within *L. peruvianum* populations [71]. After data from *S. lycopersicum* and *L. peruvianum* recombinant analyses were combined, *Mi* could be localized to a genomic region of <65 kb. Subcloning experiments of one YAC clone containing the *Mi* locus identified four overlapping BACs hybridizing to the *Mi*-flanking DNA probes. Large-scale sequencing of two BACs identified six open reading frames; three of these are homologous to each other and to previously identified R genes of the nucleotide binding–LRR class. Two of them, *Mi-1.1* and *Mi-1.2*, appear to be intact genes; the third is a pseudogene predicted to not encode a functional product. Complementation studies were performed by transforming a nematode-susceptible tomato line using constructs containing the *Mi-1.1* and *Mi-1.2* genes. Eighty-seven percent of transformants carrying *Mi-1.2* were resistant to the root-knot nematode, whereas all transformants carrying *Mi-1.1* were completely susceptible, confirming *Mi-1.2* as the functional *Mi-1* gene [72].

In cultivated tomato, tomato mosaic virus (ToMV) infections are controlled by the introgressed *Tm-1*, *Tm-2*, and *Tm-2*^{2} resistance (*R*) genes [73, 74]. Among these resistances the *Tm-2*^{2} resistance was shown to be remarkably durable and, therefore, of ongoing practical importance. The *Tm-2*^{2} locus has been localized to tomato chromosome 9, but map-based cloning of this gene has been shown to be difficult, especially due to the lack of recombination in the centromeric region [75]. The two-component Ac/Ds transposon system was utilized to isolate the tomato *Tm-2*^{2} gene. A tomato line (homozygous *Tm-2*^{2}) was crossed with another line (homozygous for *sAc*). One F2 plant from this cross (homozygous for both *sAc* and *Tm-2*^{2}) was subsequently used in a cross with a tomato line having *Ds* transposon on chromosome 9 with distance 2 cM from the resistance gene. About 100 independent plants with the genotype *Ds*/–; *sAc*/–; *Tm-2*^{2}/*Tm-2*^{2} were selected and used as males and females in a cross with a transgenic tomato line which is homozygous for the ToMV *MP* (Movement Protein) gene for a large-scale tagging experiment. Previous observation had shown a lethal combination of the *Tm-2*^{2} gene and the *ToMV MP* transgene in the seedling stage of tomato plants. From about 30,000 seeds obtained from these crosses, five putative mutants were obtained. All contained the *Ds* element and the *MP* gene and only

one mutant plant still contained *sAc*. To test whether these five putative mutants were really mutants in the *Tm-2²* gene, cuttings of these plants were inoculated with ToMV. All the five putative mutants were susceptible to ToMV infection. Plasmid rescue was used to obtain transposon-flanking plant DNA from the mutant plants. Sequencing of 9.8 kb of rescued plant DNA identified an open reading frame (ORF) of 2,586 bp, which corresponds to a polypeptide of 861 amino acids. *In silico* analysis of the ORF did not predict the presence of introns. The polypeptide encoded by the 2,586-bp ORF shows the characteristics of the CC–NBS–LRR class of R proteins, and the 5′- and 3′-RACE experiments confirmed the prediction of the gene structure. Functional complementation was performed by introducing either *Tm-2²* gene under the control of its own promoter and polyadenylation signal or *Tm-2²* gene under the control of the CaMV 35S promoter and the NOS polyadenylation signal into a susceptible tomato line. The transgenic plants of the two constructs were resistant to ToMV [76].

Plant and Inflorescence Architecture

Maize Domestication Traits Most crop plants differ considerably from their wild ancestors in major morphological phenotypes that have probably resulted from visual selection of desired plant type. This is most dramatic in maize, where the major difference between maize and its probable wild ancestor teosinte is that teosinte typically has long branches with tassels at their tips whereas maize possesses short branches tipped by ears [77]. Most of the variation for the dramatic differences in inflorescence morphology between maize and teosinte is explained by five quantitative trait loci (QTLs) [78]. Complementation tests indicated that one of these QTLs, which is on chromosome arm 1L, is the locus for the maize mutant *teosinte branched l* (*tbl*) [79]. To isolate the *tb1* gene, homozygous *tb1-ref* (a spontaneous mutant in a maize population) plants were crossed to an active *Mu* transposon line, and 26,000 F1 plants were grown. Among these, three new *tb1* mutants (*tb1-mum1*, *tb1-mum2*, *tb1-mum3*) were observed. Each new mutant was crossed to maize inbred A532, and 14 progeny from each of these crosses were used for Southern blot analysis with marker loci that closely flank *tb1* to discriminate progeny that possessed the *tb1-ref* versus the new *Mu* alleles. The progeny were also screened by Southern blot analysis with *Mu* element probes, and a *Mu3* element that cosegregated with *tb1-mum*1 was identified. Overlapping genomic restriction fragments carrying the cosegregating *Mu3* element were cloned into λ vectors. Subclones of these were used for Southern blot analyses of the 20 progeny of each of the three mutant plants, showing that each of these mutants has an insertion into this region of the genome that did not exist in their sibs and the progenitor stocks. These observations provided the crucial evidence that the *tb1* has been newly tagged. Portions of the λ clones were sequenced and oligonucleotide primers were designed to be used in conjunction with a *Mu*-specific primer to amplify the insertion fragments for each of the three *Mu* alleles. DNA sequence analysis of these fragments identified the *Mu* insertion point of each allele. A 0.8-kb restriction fragment flanking the *Mu* element was used to screen a cDNA library derived from immature ear. A single clone with a 1,360-bp insert, excluding the poly(A)tail was obtained. The cDNA sequence is fully collinear with the genomic sequence of maize inbred A619, without any evidence for introns. BLAST analysis with the sequence showed that tb1 shares two short regions of homology with the snapdragon *cycloidea* gene. The pattern of *tb1* expression and the morphology of *tb1* mutant plants suggest that *tb1* acts both to repress the growth of axillary organs and to enable the formation of female inflorescence. The maize allele of *tb1* is expressed at twice the level of the teosinte allele, suggesting that gene regulatory changes underlie the evolutionary divergence of maize from teosinte [80]. Analysis of nucleotide polymorphism in *tb1* gene of maize and teosinte populations showed that during maize domestication the effects of selection were limited to the gene's regulatory region and could not be detected in the protein-coding region [81]. Fine mapping showed that the intergenic sequences approximately 58–69 kb 5′ to the *tb1* cDNA confer pleiotropic effects on *Zea mays* morphology. Moreover, an allele-specific expression assay showed that sequences >41 kb upstream of *tb1* act in *cis* to alter *tb1* transcription [82].

The most critical step in maize (*Zea mays* ssp. *mays*) domestication was the liberation of the kernel from the hardened, protective casing that envelops the kernel in

the maize progenitor, teosinte [83]. This evolutionary step exposed the kernel on the surface of the ear, such that it could readily be used by humans as a food source. A large effect QTL for glume induration (husk hardening) was mapped to chromosome 4 using two F2 populations derived from crosses between maize race Reventador (Nay 15) and Balsas teosinte, *Z. mays* ssp. *parviglumis* or between maize race Chapalote and *Z. mays* ssp. *mexicana* [84]. This large-effect QTL segregates as a single Mendelian locus in an isogenic background, and has been designated *tga1* [83]. Maize Mo17 bacterial artificial chromosome (BAC) libraries were screened using a marker on maize chromosome 4, which is tightly linked to *tga1* and a BAC contig near *tga1* was identified. The BAC end and other sequences from this contig were used to BLAST search the rice genome sequence, and a region on rice chromosome 8 that is collinear with the region near *tga1* on maize chromosome 4 was identified. Subsequent BLAST searches using the collinear rice sequence identified a second maize Mo17 contig near *tga1*. Marker analysis showed that these 2 contigs flank *tga1*. Maize B73 contigs that correspond to the two Mo17 contigs were subsequently identified. DNA sequence analysis revealed that the two B73 contigs overlap, and could be merged to a single supercontig of ~1.5 megabases. Fine mapping using 3,106 F2 plants delimited *tga1* to a 6-kb region. Further BLAST searches using the 6-kb region at *tga1* revealed that it has homology to SBP (squamosa-promoter binding protein) transcriptional regulators. DNA sequence analysis of the SPB gene for the *tga1-ems1* stock, another *tga1* allele generated by ethyl methanesulfonate mutagenesis of maize line W22 that matched the phenotype of the teosinte allele in homozygous state, revealed that it differs from its parental (W22) allele by a nonconservative amino acid substitution of a phenylalanine for a leucine at position 5. This mutation in the *tga1-ems1* allele confirmed the conclusion from positional cloning that *tga1* is the SBP gene, and demonstrated that a single amino acid substitution was sufficient to confer the difference between the maize and teosinte phenotypes. Northern blots, real-time PCR, and *in situ* hybridization did not show any quantitative or qualitative differences in *tga1* expression between the isogenic lines (W22 and W22: *tga1*) or between the maize inbred W22 and teosinte itself, suggesting that differences between the maize and teosinte proteins may be critical to phenotype. Further mapping using seven additional recombination events within the 6-kb region narrowed the location of the causative site for the functional difference between maize and teosinte phenotypes to a 1,042-bp segment. DNA sequence analysis of this 1,042-bp segment using 16 diverse maize and 12 teosinte individuals identified seven fixed differences between maize and teosinte. Six of these seven are single base-pair polymorphisms that lie just 5′ of the coding sequence and potentially affect *tga1* expression. The seventh difference encodes an amino acid substitution of lysine (K) in teosinte to asparagine (N) in maize at position 6. Western blot analysis showed that *tga1* protein encoded by the teosinte allele is more abundant than the one encoded by the maize allele over a range of developmental stages that might underlie the phenotypic differences. The K → N substitution might alter protein stability, or it might affect translation efficiency or protein function [85].

Wheat Inflorescence In wheat, the *Q* allele confers the square-headed phenotype and free-threshing character, and is possessed by most of the cultivated wheats, but most wild wheats have the *q* allele and, therefore, speltoid spikes that are not free threshing [86]. Early experiments involving the cytogenetic analysis of aneuploid (abnormal chromosome number) plants located the *Q* gene on the long arm of chromosome 5A [87, 88]. Using chromosome deletion lines, Endo and Gill (1996) physically mapped the *Q* gene to a submicroscopic deletion interval on the long arm of chromosome 5A [89]. Comparative mapping of anonymous RFLP clones, AFLP (amplified fragment length polymorphism) markers, and mRNA differential display analysis of lines which have deletion break points that flank the *Q* locus identified 18 markers within the *Q* gene deletion interval. These markers were used to construct a genetic linkage map of the region in F2 populations derived from chromosome 5A disomic (homologous chromosome pair) substitution lines. The genetic map corresponding to the deletion segment was 20-cM long, and markers as close as 0.7 cM to the *Q* gene were identified [90]. The closest marker to the *Q* gene was used to screen four high-density *Triticum monococcum* BAC filters resulting in

the identification of one BAC clone. Chromosome walking involved the reiterative screening of the BAC library using probes derived from the ends of previously identified BAC clones. A BAC contig was then constructed that spans a physical distance of 300 kb corresponding to a genetic distance of 0.9 cM. The physical map of *T. monococcum* had perfect colinearity with the genetic map of wheat chromosome arm 5AL. Analysis of fast neutron *q* mutants using markers derived from the BAC contig at the *Q* locus confirmed that the *T. monococcum* BAC contig spanned the *Q* locus and narrowed the region for prospective *Q* gene candidates to a 100-kb segment, which contains an APETALA2 (AP2)-like gene that cosegregates with *Q* [91]. Sequence analysis showed that this AP2 gene consists of ten exons and nine introns. The M2 generation of a population of *Triticum aestivum* cv.Chinese Spring (CS) EMS mutants was screened for the speltoid phenotype to identify putative knockouts. Three speltoid mutants harboring point mutations within the AP2 gene were identified. One mutant had a single base substitution in an AP2 DNA binding domain (exon 5) that resulted in the change of a cysteine to a tyrosine. Two other mutants had point mutations in the donor site of intron 2 and the acceptor site of intron 7, respectively, resulting in alternate splicing in these mutants. The sequence analysis of the mutants validated that the AP2 gene is the *Q* locus. Comparison of the genomic sequences of the AP2 gene revealed six conserved differences between *Q*- and *q*-containing genotypes. Four of these differences, including a variable microsatellite (DNA sequence repeat), were present in introns and one was in the 3′ UTR. One conserved nucleotide difference changed a predicted amino acid where, at position 329, all *Q*-containing genotypes possessed an isoleucine while all *q*-containing genotypes possessed a valine. Yeast two-hybrid analysis showed that the full-length Q protein has the ability to form a homodimer, whereas the point mutation of q greatly reduced homodimer formation of the full-length q protein. Rachis fragility, glume shape, and glume tenacity mimicked the q phenotype in transgenic plants exhibiting posttranscriptional silencing of the transgene and the endogenous *Q* gene. Variation in spike compactness and plant height were associated with the level of transgene transcription due to the dosage effects of *Q* [92].

Barley Inflorescence Barley spikes have a unique structure consisting of three one-flowered spikelets at each spike node. Cultivated barley, *Hordeum vulgare* subsp. *vulgare*, can be divided into two forms according to the morphology of the spikelets: two-rowed and six-rowed. The two-rowed condition is believed to be primitive, because wild barley, *Hordeum vulgare* subsp. *spontaneum*, is two-rowed. Row type is controlled by multiple alleles at the *vrs1* locus (formerly *v* for *vulgare*) on chromosome 2H [93, 94]. A recessive mutation from *Vrs1* to *vrs1* changes two-rowed barley to six-rowed barley. High-resolution mapping using 373 BC7F1 plants and 278 BC6F2 plants identified four RFLP-derived STS markers closely linked to the *vrs1* locus [95]. The orders of four marker loci and *vrs1* locus were the same in six different mapping populations developed from nine different barley cultivars (*H. vulgare* subsp. *vulgare*) or mutant and wild barley (*H. vulgare* subsp. *spontaneum*) [96]. Five AFLP markers within the 0.9-cM region associated with the *vrs1* locus were subsequently developed using well-characterized near-isogenic lines as plant materials [97]. Recombinants within the 0.9-cM region were analyzed further using STS markers generated from ESTs (expressed sequence tags) and BAC DNA sequences. PCR screening of BAC clones of cv. Morex using an STS marker located 0.01 cM proximal to the *vrs1* locus isolated one BAC clone. Chromosome walking identified six bacterial artificial chromosome (BAC) clones covering completely the candidate genomic region. The contig containing *vrs1* is composed of 518,343 bp. Annotation showed three predicted genes: *HvHox1* and *HvEP2* appeared to be intact genes, whereas *HvEP1* is highly degenerated and interrupted by several insertions of transposable elements. *HvHox1* is the only gene that lies between two markers that define the break points and is thus a likely candidate for *Vrs1*. The ORF of two-rowed barley encoded a polypeptide composed of 222 amino acid residues, including a homeodomain-leucine zipper (HD-ZIP) motif. Expression of *Vrs1* was strictly localized in the lateral-spikelet primordia of immature spikes, suggesting that the VRS1 protein suppresses development of the lateral rows. Loss of function of *Vrs1* resulted in complete conversion of the rudimentary lateral spikelets in two-rowed barley into fully developed fertile spikelets in the six-rowed phenotype [98].

The wild progenitor of barley, *H. vulgare* subsp. *spontaneum*, has covered (hulled) caryopses in which the hull (outer lemma and inner palea) is firmly adherent to the pericarp epidermis at maturity. In cultivated barley, the hulled or naked caryopsis is one of the most important agronomic traits because of the direct link to its use. Most cultivars have the hulled caryopsis and are mainly used for animal feed and brewing malts. In contrast, naked barley has a caryopsis with easily separable husks upon threshing and is suitable for edible purposes. The naked caryopsis is considered a key domestication character in barley because extensive pearling to remove the hull is unnecessary [99]. The covered/naked caryopsis in barley is controlled by a single locus (*nud*, for nudum) located on chromosome arm 7HL [100]; the covered caryopsis allele (*Nud*) is dominant over the naked one (*nud*). Bulked segregant analysis on an F2 population derived from a cross between Kobinkatagi (naked type) and Triumph (hulled type) was performed using 1,894 primer combinations, and 12 AFLP markers were selected. Among them, five closely linked and two cosegregating AFLP markers were mapped around the *nud* locus using 151 F2 individuals [101]. High-resolution mapping using 2,380 segregants derived from five cross-combinations identified AFLP-derived markers flanking the *nud* locus at the 0.6-cM proximal and the 0.06-cM distal side, respectively [102]. Further mapping using 2,828 progeny segregating for the trait from two cross-combinations delimited the *nud* locus to a 0.64 cM interval. Integration of the flanking markers into a high-density barley expressed sequence tag (EST) map selected two barley ESTs flanking the *nud* locus. BLASTN analysis identified their respective homologous rice ESTs 370 kb apart on rice chromosome arm 6L. Two rice genes within the collinear region were used as vehicles to develop closer barley markers. A BAC library of the covered barley cultivar Haruna Nijo was screened using the closest marker to the *nud* locus. Seven rounds of chromosome walks selected 20 BAC clones and a 500 kb-contig spanning the *nud* locus was constructed. In the physical map, the *nud* locus was covered completely with four overlapping BAC clones. An ethylene response factor (ERF) family transcription factor was the only gene that lies in the region delimited by the genetic and physical mapping and, therefore, is considered as a *Nud* candidate gene [103]. Sequencing of the *nud* region obtained from two naked lines [Kobinkatagi (a Japanese landrace) and *nud*-Bowman (an isogenic line carrying the *nud* allele in the genetic background of the covered cultivar Bowman)] revealed a deletion of 16,680 bp relative to the corresponding region of the Haruna Nijo BAC contig sequence. The 16,680-bp deletion included the entire ERF gene. Thus, the gene structure analysis of naked cultivars supports the candidacy of the ERF gene. Sequence analysis of the candidate gene in two X-ray-induced naked mutants showed that each of the two mutants carried a different single base mutation in the putative functional motif of the ERF gene, but their wild-type varieties (Haisa's and Ackermann's Donaria, respectively) had a nucleotide sequence that is identical to that of Haruna Nijo. RNA in situ hybridization using the antisense probe revealed that, in Bowman, *Nud* was expressed strictly in the testa where adherence occurs, while no signal above background was detected in *nud*-Bowman. The *Nud* gene has homology to the Arabidopsis *WIN1*/*SHN1* transcription factor gene, whose deduced function is control of cuticular wax and cutin-related lipid biosynthesis pathways [104–106]. Staining with a lipophilic dye (Sudan black B) detected a lipid layer on the pericarp epidermis only in covered barley [103].

Rice Plant Architecture Tillering in rice (*Oryza sativa* L.) is an important agronomic trait for grain production. Screening of a collection derived from spontaneous mutations identified *monoculm 1* (*moc1*) mutant. The *moc1* plants nearly completely lose their tillering ability, producing only one main culm, in contrast to the multiple tillers in wild-type plants. Genetic analysis with reciprocal crosses between *moc1* and wild-type plants revealed that *moc1* is a single locus mutation. The *MOC1* locus was mapped to the long arm of chromosome 6 of *O. sativa* using 280 F2 mutant plants generated from the crosses between *moc1* and Minghui 63. Fine mapping using 2,010 F2 mutant plants and newly developed molecular markers delimited the *MOC1* locus to a 20-kb region. Annotation of the 20-kb sequence identified an ORF that encodes a protein highly homologous (44% identity) to the tomato *LATERAL SUPPRESSOR* (*LS*). The corresponding ORF from *moc1* and wild-type plants

were amplified by PCR and sequenced. DNA sequence comparison revealed a 1.9-kb retrotransposon inserted in this ORF in the *moc1* mutant. Transformation of a binary plasmid carrying a 3.2-kb wild-type genomic fragment containing the entire ORF plus a 1.5-kb upstream sequence and the 316-bp downstream sequence, but not the one carrying a 3′ truncated *MOC1* gene, was able to rescue the monoculm phenotype of the *moc1* mutant [107].

Typical common wild rice (*Oryza rufipogon*) tends to have a prostrate growth habit during the vegetative phase and develop erect panicle-bearing stalks during the reproductive phase. Cultivated rice has an erect-growth habit throughout the entire growth phase, which may increase plant density, enhance photosynthesis efficiency, and improve grain yield. A set of introgression lines was constructed using an accession of Yuanjiang common wild rice (YJCWR, *Oryza rufipogon*) with prostrate growth habit as a donor, and an elite indica cultivar Teqing (*O. sativa*) with erect-growth habit as a recipient [108]. One introgression line (YIL18) displaying prostrate growth was obtained, which harbored two YJCWR chromosomal segments on the long arm of chromosome 3 and the short arm of chromosome 7. The tiller angle of YIL18 was larger than that of Teqing. The grain number on the main panicle (GNP) in YIL18 was only 57.6% of that in the recipient Teqing, a result of the lesser number of primary branches and secondary branches on the main panicle. Genetic linkage analysis within 246 F2 individuals derived from the cross between YIL18 and Teqing showed that prostrate growth was completely associated with a marked decrease of GNP and controlled by a single semidominant gene, *PROG1* (*PROSTATE GROWTH 1*), located on short arm of chromosome 7. High-resolution mapping using 3,600 recessive homozygote plants with erect growth from the F2 population delimited *prog1* within an 8.8-kb region. Only one ORF was identified within this region that encodes a putative single Cys2-His2 zinc-finger protein. Transformation of a binary plasmid carrying the entire *O. rufipogon PROG1* with 596-bp or 2,914-bp 5′-flanking regions, but not the one carrying only the 2,914-bp 5′-flanking region, showed complementation of the prostrate growth phenotype. Comparison of the coding sequences of *PROG1* in YJCWR and *prog1* in Teqing showed 15 SNPs and 6 insertion/deletions (indels) that encoded 23 amino acid changes between *PROG1* and *prog1*. Sequencing of the *prog1* coding regions of 182 erect-growth varieties of cultivated rice, including 87 indica and 95 japonica cultivars from 17 countries showed that all the cultivars contained identical mutations as *prog1* in Teqing, including 15 SNPs and 6 indels [109].

Cereal Seed Quality and Color

Cereal grain is the staple food of most of the world, representing the major carbohydrate energy source in the diet. One of the most important crop traits is grain quality, which determines the price and variety of food that can be prepared from the cereal crop. Thus the major cereal crops such as rice, wheat, maize, barley, and oats have undergone selection from the onset of the crop domestication process. In comparison to other crop traits, seed quality and color are primarily biochemical traits and have been studied at the protein, gene, and metabolite or grain component level.

Maize Lysine Content The protein nutritional quality of maize is improved by increase in the essential amino acid lysine in the *opaque-2* (*o2*) mutant locus, which was localized on the short arm of chromosome 7 [110]. To isolate the gene by transposon tagging, normal maize strains (*O2*/*O2*) carrying a mutable allele of *C1* containing an autonomous *Spm* (*c1-m5*) or a mutable allele of *Wx* containing a nonautonomous, defective derivative of *Spm* (*dSpm*) (*wx-m8*) were used as pollen donors for opaque plants (*o2*/*o2*). Three opaque-mutable *o2*/*o2-m* kernels were selected from approximately 530,000 F1 seeds. These kernels were grown to maturity and self-pollinated. DNAs prepared from leaf samples of F2 individual plants were used for Southern blot analysis using a *Spm*-specific probe. The opaque-mutable plants contained a novel 8.4-kb band absent in both parents and missing from at least some of the individuals that had been classified as opaque. Those kernels that had been classified as opaque but possessed the fragment were evidently not mutable because of failure of the *Spm* to transpose, or else transposition occurred so late in endosperm development that revertant sectors were undetectable, giving the kernels an opaque rather than an obvious opaque-mutable phenotype. The fragment was cloned and

restriction enzyme mapping of the clone revealed the presence of a full-length, autonomous 8.3 kb *Spm* and an adjacent sequence of about 150 bp. This non-*Spm* region was sequenced and used as a probe on Southern blot using a BC1 population derived from a cross between opaque (*o2*/*o2*) and normal (*O2*/*O2*) plants. All the plants derived from opaque (*o2*/*o2*) seeds showed a single 6.5-kb fragment, whereas plants from seeds with a normal phenotype (*o2*/*O2*) had a 10-kb fragment in addition to the 6.5-kb fragment. This demonstrates that the 150-bp *Spm*-fanking sequence derived from the opaque-mutable plant represents a single-copy sequence that cosegregates with alleles of the *o2* locus. This fragment was subsequently used as a probe to clone a 17-kb *Hin*dIII fragment of the wild-type *O2* allele from the *c1-m5* parent [111]. A cDNA library prepared from wild-type endosperms was screened with probes derived from the *O2* genomic clone. Analysis of *O2* cDNA sequence showed that the deduced *02* protein sequence contains a "leucine-zipper" domain characteristic of some mammalian and fungal transcription activation factors. DNA binding assays demonstrated that the O2 protein or only a fragment specifying the leucine-zipper domain bound to two specific regions on the 5′ side of the coding sequence in a zein genomic clone [112].

Cereal Amylose Content The maize *Waxy* locus determines starch quality by the level of amylose and amylopectin. Isolation of the gene was carried out by a combination of identification of the protein and the gene associated with the kernel mutant phenotype. The mutant *wx-m6* and *wx-m9* alleles have *Ds* transposon inserts at the *Wx* locus, while the *wx-m8* allele is attributable to insertion of a defective transposon of the *Spm* transposon family. Starch granules from immature endosperm tissue of kernels carrying the *wx-m6*, *wx-m9*, and *wx-m8* alleles were isolated and the starch granule-bound proteins were analyzed on SDS-polyacrylamide gels [113]. When the kernels did not contain an autonomous controlling element (either *Ac* or *Spm*) and showed no somatic reversion of the *wx-m6*, *wx-m9*, or *wx-m8* mutations, the 58 kd protein was either not detectable or present at a very low level. When the *Spm* element was present with the *wx-m8* allele, sectors of endosperm tissue showed the *Wx* phenotype and a 58 kd protein was present in isolated starch granules. By contrast, when *Ac* was present together with the *wx-m6* allele, endosperm tissue showed sectors that were intermediate between the *Wx* and the *wx* phenotypes and a novel 60 kd protein was detectable on starch granules. Hence, reversion of the *wx-m6* mutation gives a protein that is structurally abnormal and appears to have an altered enzymatic activity. Only the 58 kd protein is present in starch granules from kernels that are heterozygous for the *wx-m6* and *wx-m8* alleles, but contain only the *Spm* element. Therefore, the structurally altered protein detected upon reversion of the *wx-m6* allele in the presence of *Ac* is not detected in the presence of *Spm*. Were the *Wx* locus a regulatory locus, both the 58 kd and the 60 kd proteins would be present in a kernel that is heterozygous for the *wx-m6* and *wx-m8* mutations and contains the *Spm* element. It was concluded that the *Wx* locus is the structural locus for the 58 kd protein [113].

Poly(A)$^+$ RNA purified from *Wx* and *wx* endosperms was translated in a rabbit reticulocyte in vitro translation system, and an antigenically related polypeptide was identified using antiserum raised against the *Wx* protein. No major 58 kd polypeptide was evident among the translation products, but a major 65 kd polypeptide was translated from *Wx*, and not from wx poly(A)$^+$ RNA. The 65 kd in vitro translation product may be a precursor that is synthesized, but not processed in the rabbit reticulocyte extract. Poly(A)$^+$ mRNA fractions substantially enriched for *Wx* mRNA were used to construct cDNA clones. Screening of cDNA clones was performed by selective hybridization to the mRNA sequence encoding the immunoprecipitable 65 kd *Wx* polypeptide. Further testing showed that the *Wx* cDNA hybridized to an abundant 2.3-kb poly(A) + RNA present in immature endosperm of plants homozygous for the *Wx* allele, but absent from immature endosperm tissue of a plant homozygous for a stable recessive *wx* allele. Finally, the *Wx* cDNA was shown to hybridize to a unique sequence in maize genomic DNA. That the unique sequence corresponds to the *Wx* locus was deduced from the results of hybridization analyses of DNA isolated from maize strains with and without a *Ds* insertion mutation at the *Wx* locus. The presence of the insertion at the *Wx* locus was correlated with the presence of a 2.4-kb insertion in the sequence homologous to the cloned *Wx* cDNA,

unequivocally establishing the homology of the cDNA to the *Wx* locus [113]. Sequence analysis of genomic and cDNA clones of the wild-type *Wx* gene showed that the coding region comprises 3,718 bp and is composed of 14 exons and 13 small introns. N-terminal sequencing of the mature Wx protein led to the identification of a maize amyloplast-specific transit peptide of 72 amino acid residues [114].

The *Wx* gene homologues were isolated from a number of species by isolating homologous clones or identification of DNA/protein sequences homologous to the maize *Wx* gene/protein. A rice genomic library was constructed in λEMBL-3 vector and screened using the maize *Waxy* gene DNA as probe. Two overlapping genomic clones containing the *Wx* gene sequence were identified. Sequencing and alignment with *Wx* gene of maize and barley revealed that the *Wx* gene in rice contains 13 introns and 14 exons. The full-length of rice *waxy* preprotein is 609 amino acid residues [115]. An analysis of Wx transcripts, Wx protein, and amylose content of 31 rice cultivars revealed that endosperm amylose and Wx protein contents are correlated with the ability of the cultivar to excise intron I from the leader sequence of the Wx transcript [116]. Sticky or glutinous rice quality is related to the low amount of amylose due to the mutant wx allele.

Sweet Corn The maize *sh2* mutant used in "supersweet" corn has negligible starch. The *Sh2* gene was predicted to be involved in carbohydrate metabolism, since the mutant *sh2* endosperm contains reduced starch. Candidate *Sh2* clones were identified from a cDNA library prepared from endosperm by differential screening with labeled cDNA of near-isogenic W64 *Sh2* and W64 *sh2* mRNA. The 1.3-kb cDNA clone pES6-66 hybridized to wild-type cDNA and not *sh2* cDNA. Clone pES6-66 was subsequently shown to hybridize to a restriction fragment length polymorphism (RFLP) associated with normal *Sh2* function in F2 segregants of *Sh2* × *sh2* crosses in three backgrounds [117]. To determine whether this clone was indeed *Sh2* or simply a closely linked gene, a series of maize stocks that differed only at the *Sh2* locus were used, including a wild-type *Sh2* allele (progenitor), *sh2-m1* (mutable) allele containing *Ds* at the *sh2* locus, and *Sh2* revertants [118]. Southern blot analysis using 500-bp fragment derived from pES6-66 as probe showed that *sh2-m1* yielded a fragment that is approximately 1,600 bp larger than that seen in the progenitor or revertant. The data are compatible with *sh2-m1* containing a 1,600-bp insertion. RNA gel blot analysis indicated that the size of the wild-type transcript is approximately 2.0–2.2 kb. A cDNA library constructed from poly A^+ RNA of *shrunken-1*, *bronze-mutable4* (*sh1 bz-m4*) was screened using probes derived from pES6-66. Eight of the 14 clones isolated had the 1.95-kb insert and were almost full length. The 900-bp and 1,050-bp *Eco*RI fragments from one of these clones were subcloned and sequenced. Examination of the 1.95-kb cDNA sequence identified only one open reading frame (ORF) coding for 542 amino acids and making up a polypeptide of 59,845 Da. Homology search identified the *Escherichia coli* glucose-l-phosphate adenyltransferase (ADP-glucose pyrophosphorylase or ADP-glucose synthetase, EC 2.7.7.27) was the only protein having significant similarity to the amino acid sequence of the *Sh2* cDNA [118].

The maize *sugary1* (*su1*) mutant accumulates twice as much sugar as sweet corn, having reduced starch. Mutations at the *su1* locus were generated by crossing active *Mutator* (*Mu*) plants with standard lines. Mutant alleles were identified following the self-pollinations of the F1 plants. Normal and sugary sibling kernels from a population segregating 1:1 for the nonmutant allele *Su1* and *su1-R4582*::*Mul* were germinated, and genomic DNA used for Southern blot analysis using the *Mu1* fragment as probe. A 4.0-kb *Eco*RI fragment containing sequences homologous to the transposon *Mu1* that cosegregated with *su1-R4582*::*Mu1* was identified. This 4.0-kb *Eco*RI fragment was subsequently cloned by screening a BAC library, based on hybridization to a probe internal to *Mu1*, and the genomic DNA insert was subcloned as part of plasmid pMJ60. The nucleotide sequence of both termini of the transposon were determined and found to match the known sequence of *Mul*. A 1.0-kb *Bam*HI-*Eco*RI genomic fragment flanking *Mu1* in the cloned DNA was purified and used as a hybridization probe (termed BE1000). This genomic probe detected a 4.0-kb *Eco*RI fragment that was present in all *su1-R4582*::*Mu1/su1-Ref* plants but was missing from all *Su1/su1-Ref* plants. Thus, the cloned *Mu* transposon is the same element that is within or tightly linked to the *su1* gene locus. Probe

BE1000 identified a second *Eco*RI fragment of 2.6 kb that was present in all plants examined, and representative of the nonmutant progenitor allele. Presumably, insertion of the 1.4-kb transposon *Mul* within this 2.6-kb region resulted in the 4.0-kb *Eco*RI fragment that cosegregated with *su1-R4582::Mu1*. Mutations of the *su1* gene locus other than *su1-R4582::Mu1* were analyzed to determine whether they also cosegregated with physical alterations in the cloned region of the genome. Populations segregating for the nonmutant allele *Su1* and either *su1-R2412*, *su1-R7110*, or *su1-R3162* was digested with *Eco*RI and probed in DNA gel blot analysis with genomic fragment BE1000. In the *su1-R7110* and *su1-R3162* families, the 4.0-kb *Eco*RI fragment was present in all the seedlings grown from sugary kernels but was not observed in any seedlings grown from nonmutant sibling kernels. A different *Eco*RI fragment, 4.6 kb in length, was found to cosegregate with *su1-R2412*. The mutation *su1-R2412* is likely to have occurred via insertion of a 2.0-kb element into this same region. Approximately 200,000 lambda clones from a maize endosperm cDNA library were screened with genomic probe BE1000. DNA from eight different hybridizing clones was digested with EcoRI; the largest cDNA insert was 2.4 kb in length. Rapid amplification of cDNA ends (RACE) protocol was used to obtain the 5′ end of the *sul* cDNA. Sequencing of *su1* cDNA and BLAST search of the deducted amino acid sequence showed that *su1* cDNA specified a polypeptide of at least 742 amino acids, which is highly similar in amino acid sequence to bacterial enzymes that hydrolyze α-(1 → 6) glucosyl linkages of starch [119].

Rice Seed Color Red pericarp is ubiquitous among the wild ancestors of cultivated rice (*Oryza rufipogon*), in which it is closely associated with seed shattering and dormancy. On the other hand, most rice (*Oryza sativa*) that is grown and consumed throughout the world has white pericarp, showing that white pericarp is associated with domestication and remains under strong selection in most rice breeding programs today. A QTL associated with red pericarp, *rg7.1*, was mapped on chromosome 7 using two independent BC2 populations derived from crosses between an accession of *O. rufipogon* (IRGC-105,491) from Malaysia and, in one case, a US tropical japonica cultivar, Jefferson, and in the other case, a widely planted tropical indica cultivar, IR64. The log of the odds scores associated with the *rg7.1* QTL peaks in these two populations were 99 and 33, respectively [120, 121]. The peak of both QTLs corresponded to the previously mapped position of the mutant locus, brown pericarp, *Rc* [122]. Fine mapping using 1,410 BC2F3 plants and high-resolution mapping using 4,000 BC2F6 plants narrowed the rg7.1 QTL to an 18.5-kb region. Two genes encoding CACTA type, *En/Spm* subclass, transposon proteins, and one gene encoding bHLH protein were detected within the 18.5-kb target region. Sequence comparison of bHLH locus between cv Jefferson and H75, an *Rc* mutant stock belonging to the japonica subspecies, carrying a functional allele, but much more closely related to cv Jefferson than to *O. rufipogon*, showed that the coding sequence of the bHLH allele in H75 was identical to the cv Jefferson sequence except for a 14-bp indel in exon 6. This 14-bp sequence was present in the H75 stock as well as in *O. rufipogon*, but was deleted in cv Jefferson and cv Nipponbare. The deletion induces a frame shift in the sequence, resulting in two premature stop codons before the end of exon 6. The stop codons truncate the protein before the bHLH domain. Sequence analysis of bHLH of Surjamkuhi, an indica line that carries a third allele, *Rc-s*, conditioning light red seed pigmentation, showed that the sequence of this line differed from the *O. rufipogon* allele at only four sites (positions 96, 660, 1353, and 1833–1844). The first two changes proved to be synonymous substitutions. The change at position 1353 consisted of a C-to-A change in exon 6. This single-nucleotide polymorphism (SNP) was independent of any change seen in previous comparisons and represented a premature stop codon before the bHLH domain, truncating the protein and rendering the effect of the remaining indel immaterial. Conventional reverse transcriptase-PCR showed no expression of *Rc* in leaf tissue; however, expression was seen in panicles before fertilization, pericarp from grains in the milk or dough stage of filling, and pericarp from mature seeds. Similar expression levels of Rc were detected in cv Jefferson and *O. rufipogon* [121].

Seed Shattering

Wild plant species shatter their seed from the mature infloresence and thus disperse seed for the next

generation to be seeded, while during domestication humans have selected for non-shattering plant types from which the seed can be harvested or gathered from the crop. Study of shattering has been very fruitful in Arabidopsis, setting up the genetic models for shattering phenotypes [123] that have been also revealed in other crops like the cereals.

In rice, a major QTL for seed shattering, *sh4*, has been mapped on chromosome 4 using segregating populations derived from crosses between *O. sativa* ssp. indica and the wild perennial species *O. rufipogon* [124, 125], between *O. sativa* ssp. japonica and *O. rufipogon* and two other closely related wild species *O. glumaepetula* and *O. meridionalis* [126, 127], and between *O. sativa* ssp. indica and the wild annual species *Oryza nivara* [128]. High-resolution mapping using 12,000 F2 (*O. sativa* ssp. indica x *O. nivara*) delimited *sh4* to a 1.7-kb region of a gene with a previously unknown function. The comparison of the 1.7-kb sequences between the mapping parents revealed seven mutations located in the intron, exon, or 5′ upstream of the start codon. Sequencing of this 1.7-kb region from an additional 14 rice cultivars of *O. sativa*, 21 accessions of *O. nivara*, 6 accessions of *O. rufipogon*, and 1 accession of each of the four remaining wild A-genome species and their association with shattering phenotypes showed that a nucleotide substitution of G for T or an amino acid substitution of asparagine for lysine in the first exon was selected for the development of non-shattering cultivars during rice domestication. Annotation of the sh4 protein identified a Myb3 DNA binding domain and a nuclear localization signal, suggesting that *sh4* is a transcription factor. Transformation of a binary plasmid carrying *sh4-GFP* fusion under the control of *Ubi* promoter into rice cv. Taipei 309 determined the nuclear localization of GFP-tagged *sh4*. Transformation into rice cv. Taipei 309 of a binary plasmid carrying *O. nivara* sequence from the 3′ nontranslated region to the inclusion of the G-T mutation site and *O. sativa* sequence for the rest sequence until the 5′ regulatory region, but not the one carrying a shorter *O. nivara* sequence excluding the G-T mutation site, showed significantly reduced strength of grain attachment to pedicel [128].

A major QTL for seed shattering, *qSH1*, was first mapped on chromosome 1 using 182 F2 plants of a cross between Kasalath (a shattering-type *indica* cultivar) and Nipponbare (a non-shattering-type *japonica* cultivar). A near-isogenic line (NIL) was developed that contained a short chromosomal segment from Kasalath at the *qSH1* region in a Nipponbare genetic background. The NIL had a stronger seed-shattering phenotype than either Kasalath or Nipponbare. High-resolution mapping using 10,388 plants from the BC4F2 and BC3F2 populations segregating at the *qSH1* region succeeded in mapping the functional natural variation in 612 bp and only one single-nucleotide polymorphism (SNP) was found within this region. No distinct open reading frame (ORF) was identified in the SNP region based on gene prediction for the *qSH1* region in both Nipponbare and Kasalath genome sequences. However, one ORF for a rice ortholog of the Arabidopsis *REPLUMLESS* (*RPL*) gene was found 12 kb away from the SNP. The *RPL* gene encodes a BEL1-type homeobox and is involved in the formation of a dehiscence zone (or abscission layer) alongside the valve in the *Arabidopsis* fruit (silique). Transformation of 26-kb Kasalath genomic fragments scanning the predicted ORF and the SNP regions into the non-shattering Nipponbare cultivar showed complementation of the complete seed-shattering phenotype. The other fragments were not able to complement the phenotype, even if they contained the entire ORF region or the SNP region. These results indicated that both the ORF and the SNP regions were required for full shattering function. *In situ* hybridization analysis revealed that in the NIL the ORF was expressed at the inflorescence meristem in the stage of rachis meristem establishment [inflorescence stage 1 (In1)]. It was also expressed at both the anther region and the provisional abscission layer at the base of the spikelet in the stage of floral organ differentiation (In7) and in the stage of rapid elongation of the rachis and branches (In8). On the other hand, in Nipponbare, the ORF was expressed in the same way as in the NIL, except that it was not expressed at the provisional abscission layer in either In7 or In8. These results, together with the complementation results, indicated that this *RPL* ortholog was the *qSH1* gene and that the identified SNP affected only the spatial mRNA expression pattern of *qSH1* at the abscission layer [129].

A shattering mutant line of rice, Hsh, was derived from a non-shattering japonica variety, Hwacheong, by

N-methyl-N-nitrosourea (MNU) treatment. Optical microscopy revealed that Hsh had a well-developed abscission layer similar to the wild rice *Oryza nivara*, while Hwacheong did not produce an abscission layer. Genetic analysis showed that the easy shattering of Hsh was controlled by the single recessive gene *sh-h*. Using an F2 population consisting of 240 individuals derived from the Hsh/Blue&Gundil cross the *sh-h* was mapped on chromosome 7 [130]. Fine mapping using six newly developed SSR markers delimited the *sh-h* locus to a 150-kb region. Further mapping using three newly developed SNP markers on five F3 recombinant lines derived from F2 heterozygous plants narrowed the *sh-h* locus to a 34-kb region. Analysis of genomic sequences from Hwacheong, Hsh, and Blue&Gundil lines identified eight genes in this region. Among these, a gene which encodes a protein containing a conserved carboxy-terminal domain (CTD) phosphatase domain was considered to be a strong candidate for the *sh-h* locus because of the presence of a point mutation at the 3′ end splice site of its seventh intron. Sequencing of the RT-PCR products of Hwacheong and Hsh revealed that, compared with Hwacheong, a 15-bp deletion was induced by altered splicing in the mRNA isolated from Hsh. Because the consensus sequence for splicing at this "AG" site was changed to "TG," the next "AG" sequence, 15-bp downstream, was used as a new splice site. The 15-bp deletion in the Hsh mRNA resulted in the deletion of five amino acids in the C-terminal region of the CTD-like phosphatase domain (CPDc), which are situated within the phosphatase active site. Two transferred DNA (T-DNA) insertion mutants and one point mutant exhibited the enhanced shattering phenotype, confirming that this CTD phosphatase-like gene is indeed the *sh-h* gene. RNA interference (RNAi) transgenic lines with suppressed expression of this gene exhibited greater shattering [131].

Tolerance to Abiotic Stresses

Wild species are adapted to the environment where they are found; survive over long periods of time by creating diversity where environmental selection pressures allow the fit to reproduce and survive. The environmental stresses include abiotic stresses such as heat, cold, drought, salt, light, radiation, heavy metals, soil pH, and others. Plant response to these stresses has been studied and shows common and unique responses [132].

Salinity Stress Tolerance Salinity tolerance has been amenable to study for phenotypic variation and the genetic dissection into quantitative traits. In rice, a major QTL for shoot K + content under salt stress, *SKC1*, was mapped to chromosome 1 using 133 lines of an F2 and an equivalent F3 population derived from a cross between a salt-tolerant indica variety Nona Bokra, and a susceptible elite japonica variety Koshihikari [133]. Fine mapping was performed using 192 BC2F2 plants, and a high-resolution map was subsequently generated with 2,973 BC3F2 plants using markers newly developed on the basis of the PAC clone sequences. Progeny testing of fixed recombinant plants (BC3F4) delimited the *SKC1* locus to a 7.4-kb region, and only one predicted open reading frame (ORF) was identified in the region. The *SKC1* promoter region (2,554-bp upstream of the initial codon) and the coding region of the cDNA clone (1,665 bp) were ligated and subcloned into a plant binary vector. This construct was transferred into the japonica variety Zhonghua 11, which contained the same *SKC1* allele as Koshihikari and is salt susceptible. Shoot K + concentrations were substantially higher in all six T1 progeny containing the Nona Bokra *SKC1* transgene compared with plants containing the vector control under salt stress but not under normal condition. Database searches showed substantial similarity between SKC1 and the HKT-type transporters found in plants, bacteria, and fungi. Comparison of nucleotide sequences between Nona Bokra and Koshihikari alleles showed six nucleotide substitutions in the coding region that lead to four amino acid changes, which may be responsible for the functional difference between the two alleles. RT-PCR analysis in both Koshihikari and NIL (*SKC1*) showed that *SKC1* transcript levels were upregulated by salt stress in the root but not in the shoot. Under normal condition, K^+ and Na^+ contents in NIL (*SKC1*) shoots were not substantially different from those in Koshihikari. But under salt stress, NIL (*SKC1*) shoots had a higher K^+ content and lower Na^+ content than Koshihikari shoots. No substantial differences in K^+ and Na^+ contents were observed in the roots under either normal or stress condition. Consistent with the results of shoot analysis, K^+ and Na^+ contents in xylem sap were not different between NIL (*SKC1*) and Koshihikari; under salt stress, however, the xylem sap of NIL (*SKC1*) contained more

K^+ and less Na^+ than that of Koshihikari. These results indicated that *SKC1* is involved in regulating K^+/Na^+ homeostasis in the shoots [134].

Submergence Tolerance A major QTL for submergence tolerance, *Sub1*, was mapped to chromosome 9 using 169 F2 plants and the resulting F3 families of a cross between a tolerant indica rice line, IR40931-26 (a descendant of FR13A), and a susceptible japonica line, PI543851 [135]. High-resolution mapping using 2,950 F2 individuals and the resulting F3 families of a cross between DX18-121 (a tolerant F3 plant from the first population) and M-202 (a submergence-susceptible japonica cultivar that is widely used in California) identified 2 AFLP markers localized within 0.2 cM of *Sub1* [136]. The *Sub1* locus was delimited to an interval of 0.06 centimorgan using 4,022 F2 individuals from the cross between DX18-121 and M-202. Physical mapping with five overlapping bacterial artificial chromosome (BAC) clones derived from submergence-intolerant indica rice varieties and a nearly complete contig of 13 binary clones from IR40931-26 showed that *Sub1* region physically spans over 182 kilobases (kb). This interval encodes three genes containing ethylene response factor (ERF) domains and designated *Sub1A*, *Sub1B*, and *Sub1C*, ten non-ERF genes including four transcribed and six hypothetical protein-coding genes, and >50% retrotransposon-related sequences. The corresponding region of the japonica genome represented by the sequenced variety Nipponbare spans 142 kb and is considerably rearranged. Notably, *Sub1A* is absent from the rice reference Nipponbare genome. Accumulation of *Sub1A* and *Sub1C* messenger RNAs was strongly but transiently promoted by submergence in seedling leaves of tolerant FR13A, while *Sub1B* transcripts increased only slightly during submergence. The ten non-ERF genes in the indica *Sub1* region showed no evidence of expression in seedling leaves before or during submergence in IR40931-26 or the intolerant variety M-202. A survey of the *Sub1* locus haplotypes in 17 indica and 4 japonica varieties identified 2 *Sub1A*, 9 *Sub1B*, and 7*Sub1C* alleles on the basis of variation in amino acid sequence. The *Sub1A-1* and *Sub1C-1* alleles are limited to all six submergence-tolerant accessions. There was no *Sub1B* allele identified as being specific to submergence tolerance. In the tolerant *Sub1A-1* allele, a single-nucleotide polymorphism at position 556 is responsible for a Pro 186 (intolerant) to Ser 186 (tolerant) substitution in a MAPK site. Conversely, the *Sub1C-1* allele of tolerant lines lacks a MAPK phosphorylation site present in the alleles of the intolerant accessions. A *Sub1A-1* full-length cDNA under the control of the maize Ubiquitin1 promoter was transformed into an intolerant japonica variety Liaogeng. A screen of seedlings after 11 days of submergence identified four T1 families, derived from independent T0 Ubi:Sub1A + lines, with submergence-tolerant transgenic individuals, and progeny from two families were examined in detail. T1 families one and three showed a correlation between high expression of the *Sub1A-1* transgene and submergence tolerance. As observed in the FR13A descendant IR40931-26, tolerant Sub1A-1+ plants showed a significant impairment of shoot elongation under submergence compared with the intolerant parent Liaogeng and non-transgenic siblings [137].

Aluminum Tolerance In wheat (*Triticum aestivum* L.) mechanisms that minimize the harmful effects of Al ions have been investigated using near-isogenic lines [ET8 (Al-tolerant) and ES8 (Al-sensitive)] that differ in Al tolerance at a single dominant locus designated as *Alt1*. The *Alt1* locus cosegregates with an Al-activated malate efflux from root apices [138, 139]. One cDNA clone more highly expressed in the root apices of ET8 compared to that of ES8 was isolated by subtractive hybridization. The full-length cDNA was obtained by rapid amplification of cDNA ends (RACE)-PCR. The predicted protein is hydrophobic having six to eight putative transmembrane regions. Heterologous expression of this gene in *Xenopus oocytes* indicated that this gene encodes an Al-activated transporter that facilitates the efflux of malate but not citrate. Based on this result, the gene was named *ALMT1* (aluminum-activated malate transporter). Sequencing the *ALMT1* cDNAs derived from ET8 and ES8 showed that the sequences differed at six nucleotides that resulted in the deduced proteins differing at two amino acid residues. Sequence of the *ALMT1* coding region in Atlas66 (Al-tolerant cultivar) was identical to that of the ET8 allele, while the sequence in Scout66 (Al-sensitive cultivar) was identical to that of ES8. As found for the ET8 and ES8 lines, the expression of *ALMT1* in the root apices was

greater in Atlas66 compared to Scout66. Expression analysis of *ALMT1* in the 57 F2 individuals derived from a cross between ET7 and ES5, the near-isogenic progenitor lines of ET8 and ES8, showed that all the Al-sensitive seedlings expressed the ES8 allele only, whereas Al-tolerant seedlings expressed either ET8 allele only or both ET8 and ES8 alleles. Thus, the ET8 allele was expressed only in the Al-tolerant seedlings, indicating that, in this population, the Al-tolerant phenotype was correlated with the higher expression of this allele. Evaluation of Al tolerance and genotype analyses using an RFLP marker and a PCR-based assay on 204 F3 families derived from a cross between ET8 and ES8 showed that the ET8 allele of *ALMT1* completely cosegregated with the Al-tolerant phenotypes and the Al-tolerance locus, *Alt1*. Heterologous expression of *ALMT1* in cultured tobacco cells increased the tolerance of tobacco cells to Al treatment [140].

Future Directions

The present era of plant sciences is distinguished by an integration of multiple disciplines toward a transdisciplinary/interdisciplinary approach of doing science, utilizing the technology and knowledge from computer science and bioinformatics, genomics, dedicated instrumentation for high-throughput automation of laboratory and field tasks, which can all together provide integrated models and technologies for the improvement of crop traits. The paradigm shift in genetics, due to the reduced cost in genome sequencing, is to be able to generate the genome sequence of any plant genotype of interest that will totally revolutionize the approach to use this information. With a few genetic crosses followed by whole genome sequencing of all genotypes, it would be possible to identify genes and useful alleles that can be incorporated into plant improvement programs. This would make the traditional ways of isolating or identifying gene sequences for crop traits obsolete. The lag will be in phenotyping individual genotypes, which will remain the challenge for the biologist. The association of genes to a trait will still require that traits be characterized, dissected into components and parameters that can be measured, and the candidate gene(s) validated by some reverse genetics strategies using methods such as mutant analysis, transformation, and complementation, at least till the function of every gene/allele is known and how these genes and their alleles interact together to determine the phenotype of a crop trait.

Bibliography

Primary Literature

1. Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. Cell 127:1309–1321
2. Vaughan DA, Balázs E, Heslop-Harrison JS (2007) From crop domestication to super-domestication. Ann Bot 100:893–901
3. Meinke DW, Cherry JM, Dean C, Rounsley SD, Koornneef M (1998) *Arabidopsis thaliana*: a model plant for genome analysis. Science 282:662–682
4. Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815
5. Ossowski S, Schneeberger K, Clark RM, Lanz C, Warthmann N, Weigel D (2008) Sequencing of natural strains of *Arabidopsis thaliana* with short reads. Genome Res 18:2024–2033
6. Ossowski S, Schneeberger K, Lucas-Lledó JI, Warthmann N, Clark RM, Shaw RG, Weigel D, Lynch M (2010) The rate and molecular spectrum of spontaneous mutations in *Arabidopsis thaliana*. Science 327:92–94
7. Verma DP, Nash DT, Schulman HM (1974) Isolation and in vitro translation of soybean leghaemoglobin mRNA. Nature 251:74–77
8. Baulcombe D, Verma DP (1978) Preparation of a complementary DNA for leghaemoglobin and direct demonstration that leghaemoglobin is encoded by the soybean genome. Nucleic Acids Res 5:4141–4155
9. Goldberg RB, Hoschek G, Kamalay JC, Timberlake WE (1978) Sequence complexity of nuclear and polysomal RNA in leaves of the tobacco plant. Cell 14:123–131
10. Kamalay JC, Goldberg RB (1980) Regulation of structural gene expression in tobacco. Cell 19:935–946
11. Varsanyi-Breiner A, Gusella JF, Keys C, Housman DE, Sullivan D, Brisson N, Verma DP (1979) The organization of a nuclear DNA sequence from a higher plant: molecular cloning and characterization of soybean ribosomal DNA. Gene 7:317–334
12. Baulcombe DC, Key JL (1980) Polyadenylated RNA sequences which are reduced in concentration following auxin treatment of soybean hypocotyls. J Biol Chem 255:88907–88913
13. Link G, Coen DM, Bogorad L (1978) Differential expression of the gene for the large subunit of ribulose bisphosphate carboxylase in maize leaf cell types. Cell 15:725–731
14. Schell J, Van Montagu M (1977) Transfer, maintenance, and expression of bacterial Ti-plasmid DNA in plant cells transformed with *A. tumefaciens*. Brookhaven Symp Biol 29:36–49
15. Joos H, Timmerman B, Montagu MV, Schell J (1983) Genetic analysis of transfer and stabilization of Agrobacterium DNA in plant cells. EMBO J 2:2151–2160

16. Herrera-Estrella L, Block MD, Messens E, Hernalsteens JP, Montagu MV, Schell J (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J 2:987–995
17. Caplan A, Herrera-Estrella L, Inzé D, Van Haute E, Van Montagu M, Schell J, Zambryski P (1983) Introduction of genetic material into plant cells. Science 222:815–821
18. Barton KA, Binns AN, Matzke AJ, Chilton MD (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32:1033–1043
19. Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803–4807
20. Paszkowski J, Shillito RD, Saul M, Mandák V, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722
21. Fromm ME, Taylor LP, Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. Nature 319:791–793
22. Klein TM, Kornstein L, Sanford JC, Fromm ME (1989) Genetic transformation of maize cells by particle bombardment. Plant Physiol 91:440–444
23. Jain HK (2010) The green revolution: history, impact and future, 1st edn. Studium Press, Houston, ISBN 1933699639
24. Khush GS (1999) Green revolution: preparing for the 21st century. Genome 42:646–655
25. Brown LR (1970) Nobel peace prize: developer of high-yield wheat receives award (Norman Ernest Borlaug). Science 170:518–519
26. Hedden P (2003) Constructing dwarf rice. Nat Biotechnol 21:873–874
27. Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) "Green revolution" genes encode mutant gibberellin response modulators. Nature 400:256–261
28. Peng J, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP (1997) The Arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev 11:3194–3205
29. Maeda H, Ishii T, Mori H, Kuroda J, Horimoto M, Takamure I, Kinoshita T, Kamijima O (1997) High density molecular map of semidwarfing gene, sd-1, in rice (*Oryza sativa* L.). Breed Sci 47:317–320
30. Sasaki A, Ashikari M, Ueguchi-Tanaka M, Itoh H, Nishimura A, Swapan D, Ishiyama K, Saito T, Kobayashi M, Khush GS, Kitano H, Matsuoka M (2002) Green revolution: a mutant gibberellin-synthesis gene in rice. Nature 416:701–702
31. Spielmeyer W, Ellis MH, Chandler PM (2002) Semidwarf (sd-1), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. Proc Natl Acad Sci USA 99:9043–9048
32. Monna L, Kitazawa N, Yoshino R, Suzuki J, Masuda H, Maehara Y, Tanji M, Sato M, Nasu S, Minobe Y (2002) Positional cloning of rice semidwarfing gene, sd-1: rice "green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. DNA Res 9:11–17
33. Takahashi Y, Shomura A, Sasaki T, Yano M (2001) Hd6, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the alpha subunit of protein kinase CK2. Proc Natl Acad Sci USA 98:7922–7927
34. Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T (2000) Hd1, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene CONSTANS. Plant Cell 12:2473–2484
35. Yamamoto T, Lin H, Sasaki T, Yano M (2000) Identification of heading date quantitative trait locus Hd6 and characterization of its epistatic interactions with Hd2 in rice using advanced backcross progeny. Genetics 154:885–891
36. Monna L, Lin HX, Kojima S, Sasaki T, Yano M (2002) Genetic dissection of a genomic region for quantitative trait locus, Hd3, into two loci, Hd3a and Hd3b, controlling heading date in rice. Theor Appl Genet 104:772–778
37. Kojima S, Takahashi Y, Kobayashi Y, Monna L, Sasaki T, Araki T, Yano M (2002) Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of Hd1 under short-day conditions. Plant Cell Physiol 43:1096–1105
38. Alpert KB, Grandillo S, Tanksley SD (1995) fw2.2: a major QTL controlling fruit weight is common to both red and green-fruited tomato species. Theor Appl Genet 91:994–1000
39. Alpert KB, Tanksley SD (1996) High-resolution mapping and isolation of a yeast artificial chromosome contig containing fw2.2: a major fruit weight quantitative trait locus in tomato. Proc Natl Acad Sci USA 93:15503–15507
40. Frary A, Nesbitt TC, Grandillo S, Knaap E, Cong B, Liu J, Meller J, Elber R, Alpert KB, Tanksley SD (2000) fw2.2: a quantitative trait locus key to the evolution of tomato fruit size. Science 289:85–88
41. Xing Y, Zhang Q (2010) Genetic and molecular bases of rice yield. Annu Rev Plant Biol 61:421–442
42. Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. Science 309:741–745
43. Li J, Thomson M, McCouch SR (2004) Fine mapping of a grain-weight quantitative trait locus in the pericentromeric region of rice chromosome 3. Genetics 168:2187–2195
44. Fan C, Xing Y, Mao H, Lu T, Han B, Xu C, Li X, Zhang Q (2006) GS3, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor Appl Genet 112:1164–1171
45. Lin HX et al (1995) RFLP mapping of QTLs for grain shape traits in indica rice (*Oryza sativa* L. subsp. *indica*). Scientia Agricultura Sinica 28:1–7
46. Yoon DB, Kang KH, Kim HJ, Ju HG, Kwon SJ, Suh JP, Jeong OY, Ahn SN (2006) Mapping quantitative trait loci for yield components and morphological traits in an advanced backcross population between *Oryza grandiglumis* and the *O. sativa* japonica cultivar Hwaseongbyeo. Theor Appl Genet 112:1052–1062

47. Song XJ, Huang W, Shi M, Zhu MZ, Lin HX (2007) A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat Genet 39:623–630
48. Flor HH (1955) Host-parasite interaction in flax rust – its genetics and other implications. Phytopathology 45:680–685
49. Johal GS, Briggs SP (1992) Reductase activity encoded by the HM1 disease resistance gene in maize. Science 258: 985–987
50. Johal GS, Briggs SP (1990) Placement of hm1 on the Pioneer RFLP map. Maize Genet Coop Newsl 64:37
51. Van den Ackerveken GF, Van Kan JA, De Wit PJ (1992) Molecular analysis of the avirulence gene avr9 of the fungal tomato pathogen Cladosporium fulvum fully supports the gene-for-gene hypothesis. Plant J 2:359–366
52. Stevens MA, Rick CM (1988) Genetics and breeding. In: Atherton JG, Rudich J (eds) The tomato crop. Chapman & Hall, London, pp 35–109
53. Jones DA, Dickinson MJ, Balint-Kurti PJ, Dixon MS, Jones JDG (1993) Two complex resistance loci revealed in tomato by classical and RFLP mapping of the Cf-2, Cf-4, Cf-5 and Cf-9 genes for resistance to *Cladosporium fulvum*. Mol Plant Microbe Interact 6:348–357
54. Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JD (1994) Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789–793
55. Duncan JM (1999) Phytophthora – an abiding threat to our crops. Microbiol Today 26:114–116
56. Naess SK, Bradeen JM, Wielgus SM, Haberlach GT, McGrath JM, Helgeson JP (2000) Resistance to late blight in *Solanum bulbocastanum* is mapped to Chromosome 8. Theor Appl Genet 101:697–704
57. Bradeen JM, Naess SK, Song J, Haberlach GT, Wielgus SM, Buell CR, Jiang J, Helgeson JP (2003) Concomitant reiterative BAC walking and fine genetic mapping enable physical map development for the broad-spectrum late blight resistance region, RB. Mol Genet Genomics 269:603–611
58. Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, Jiang J (2003) Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci USA 100:9128–9133
59. van der Vossen E, Sikkema A, Hekkert BL, Gros J, Stevens P, Muskens M, Wouters D, Pereira A, Stiekema W, Allefs S (2003) An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant J 36:867–882
60. van der Vossen EA, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, Pereira A, Allefs S (2005) The Rpi-blb2 gene from *Solanum bulbocastanum* is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 44:208–222
61. Hinze K, Thompson RD, Ritter E, Salamini F, Schulze-Lefert P (1991) Restriction fragment length polymorphism-mediated targeting of the mlo resistance locus in barley (*Hordeum vulgare*). Proc Natl Acad Sci USA 88:3691–3695
62. Büschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, Töpsch S, Vos P, Salamini F, Schulze-Lefert P (1997) The barley Mlo gene: a novel control element of plant pathogen resistance. Cell 88:695–705
63. Williamson VM, Hussey RS (1996) Nematode pathogenesis and resistance in plants. Plant Cell 8:1735–1745
64. Smith PG (1944) Embryo culture of a tomato species hybrid. Proc Am Soc Hort Sci 44:413–416
65. Gilbert JC (1958) Some linkage studies with the Mi gene for resistance to root- knot nematodes. Rep Tomato Genet Coop 8:15–17
66. Klein-Lankhorst R, Rietveld P, Machiels B, Verkerk R, Weide R, Gebhardt C, Koornneef M, Zabel P (1991) RFLP markers linked to the root knot nematode resistance gene Mi in tomato. Theor Appl Genet 81:661–667
67. Messeguer R, Ganal M, Devicente MC, Young ND, Bolkan H, Tanksley SD (1991) High resolution RFLP map around the root knot nematode resistance gene (Mi) in tomato. Theor Appl Genet 82:529–536
68. Ho J-Y, Weide R, Ma HM, Wordragen MF, Lambert KN, Koornneef M, Zabel P, Williamson VM (1992) The root-knot nematode resistance gene (Mi) in tomato: construction of a molecular linkage map and identification of dominant cDNA markers in resistant genotypes. Plant J 2:971–982
69. Williamson VM, Ho J-Y, Wu FF, Miller N, Kaloshian I (1994) A PCR-based marker tightly linked to the nematode resistance gene, Mi, in tomato. Theor Appl Genet 87:757–763
70. Liharska TB, Koornneef M, Van Wordragen M, Van Kammen A, Zabel P (1996) Tomato chromosome 6: effect of alien chromosomal segments on recombinant frequencies. Genome 39:485–491
71. Kaloshian I, Yaghoobi J, Liharska T, Hontelez J, Hanson D, Hogan P, Jesse T, Wijbrandi J, Simons G, Vos P, Zabel P, Williamson VM (1998) Genetic and physical localization of the root-knot nematode resistance locus Mi in tomato. Mol Gen Genet 257:376–385
72. Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson VM (1998) The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307–1319
73. Pelham J (1966) Resistance in tomato to tobacco mosaic virus. Euphytica 15:258–267
74. Hall TJ (1980) Resistance at the Tm-2 locus in the tomato to tomato mosaic virus. Euphytica 29:189–197
75. Motoyoshi F, Ohmori T, Murata M (1996) Molecular characterization of heterochromatic regions around the Tm-2 locus in chromosome 9 of tomato. Symp Soc Exp Biol 50:65–70
76. Lanfermeijer FC, Dijkhuis J, Sturre MJ, de Haan P, Hille J (2003) Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-2(2) from *Lycopersicon esculentum*. Plant Mol Biol 52:1037–1049

77. Iltis H (1983) From teosinte to maize: the catastrophic sexual transmutation. Science 222:886–894
78. Doebley J, Stec A (1991) Genetic analysis of the morphological differences between maize and teosinte. Genetics 129:285–295
79. Doebley J, Stec A, Gustus C (1995) Teosinte branched1 and the origin of maize: evidence for epistasis and the evolution of dominance. Genetics 141:333–346
80. Doebley J, Stec A, Hubbard L (1997) The evolution of apical dominance in maize. Nature 386:485–488
81. Wang R-L, Stec A, Hey J, Lukens L, Doebley J (1999) The limits of selection during maize domestication. Nature 398:236–239
82. Clark RM, Wagler TN, Quijada P, Doebley J (2006) A distant upstream enhancer at the maize domestication gene tb1 has pleiotropic effects on plant and inflorescent architecture. Nat Genet 38:594–597
83. Dorweiler J, Stec A, Kermicle J, Doebley J (1993) Teosinte glume architecture1: a genetic locus controlling a key step in maize evolution. Science 262:233–235
84. Doebley J, Stec A (1993) Inheritance of the morphological differences between maize and teosinte: comparison of results for two F2 populations. Genetics 134:559–570
85. Wang H, Nussbaum-Wagler T, Li B, Zhao Q, Vigouroux Y, Faller M, Bomblies K, Lukens L, Doebley JF (2005) The origin of the naked grains of maize. Nature 436:714–719
86. Muramatsu M (1986) The vulgare super gene, Q: its universality in durum wheat and its phenotypic effects in tetraploid and hexaploid wheats. Can J Genet Cytol 28:30–41
87. Unrau J, Smith WE, McGinnis RC (1950) Spike density, speltoidy and compactoidy in hexaploid wheat. Can J Res Sect C Bot Sci 28:273–276
88. MacKey J (1954) Neutron and X-ray experiments in wheat and a revision of the speltoid problem. Hereditas 40:65–180
89. Endo TR, Gill BS (1996) The deletion stocks of common wheat. J Hered 87:295–307
90. Faris JD, Gill BS (2002) Genomic targeting and high-resolution mapping of the domestication gene Q in wheat. Genome 45:706–718
91. Faris JD, Fellers JP, Brooks SA, Gill BS (2003) A bacterial artificial chromosome contig spanning the major domestication locus Q in wheat and identification of a candidate gene. Genetics 164:311–321
92. Simons KJ, Fellers JP, Trick HN, Zhang Z, Tai YS, Gill BS, Faris JD (2006) Molecular characterization of the major wheat domestication gene Q. Genetics 172:547–555
93. von Ubisch G (1916) Beitrag zu einer Faktorenanalyse von Gerste. Z Indukt Abstam Vererbungs 17:120–152
94. Robertson DW, Wiebe GA, Shands RG, Hagberg A (1965) A summary of linkage studies in cultivated barley, Hordeum species. (Suppl. III, 1954–1963). Crop Sci 5:33–43
95. Komatsuda T, Li W, Takaiwa F, Oka S (1999) High resolution map around the vrs1 locus controlling two- and six-rowed spike in barley, *Hordeum vulgare*. Genome 42:248–253
96. Komatsuda T, Tanno K (2004) Comparative high resolution map of the six-rowed spike locus 1 (vrs1) in several populations of barley, *Hordeum vulgare* L. Hereditas 141:68–73
97. He C, Sayed-Tabatabaei BE, Komatsuda T (2004) AFLP targeting of the 1-cM region conferring the vrs1 gene for six-rowed spike in barley, *Hordeum vulgare* L. Genome 47:1122–1129
98. Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci USA 104:1424–1429
99. Staudt G (1961) The origin of cultivated barleys: a discussion. Economic Bot 15:203–212
100. Franckowiack JD, Konishi T (1997) Naked caryopsis. Barley Genet Newsl 26:51–52
101. Kikuchi S, Taketa S, Ichii M, Kawasaki S (2003) Efficient fine mapping of the naked caryopsis gene (nud) by HEGS (high-efficiency genome scanning)/AFLP in barley. Theor Appl Genet 108:73–78
102. Taketa S, Awayama T, Amano S, Sakurai Y, Ichii M (2006) High-resolution mapping of the nud locus controlling the naked caryopsis in barley. Plant Breed 125:337–342
103. Taketa S, Amano S, Tsujino Y, Sato T, Saisho D, Kakeda K, Nomura M, Suzuki T, Matsumoto T, Sato K, Kanamori H, Kawasaki S, Takeda K (2008) Barley grain with adhering hulls is controlled by an ERF family transcription factor gene regulating a lipid biosynthesis pathway. Proc Natl Acad Sci USA 105:4062–4067
104. Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activate wax biosynthesis, alter cuticle properties and confer drought tolerance when overexpressed in Arabidopsis. Plant Cell 16:2463–2480
105. Broun P, Poindexter P, Osborne E, Jiang CZ, Riechmann JL (2004) WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis. Proc Natl Acad Sci USA 101:4706–4711
106. Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Höfte H, Pauly M, Riechmann JL, Broun P (2007) The transcription factor WIN1/SHN1 regulates Cutin biosynthesis in *Arabidopsis thaliana*. Plant Cell 19:1278–1294
107. Li X, Qian Q, Fu Z, Wang Y, Xiong G, Zeng D, Wang X, Liu X, Teng S, Hiroshi F, Yuan M, Luo D, Han B, Li J (2003) Control of tillering in rice. Nature 422:618–621
108. Tan L, Liu F, Xue W, Wang G, Ye S, Zhu Z, Fu Y, Wang X, Sun C (2007) Development of *Oryza rufipogon* and *Oryza sativa* introgression lines and assessment for yield-related quantitative trait loci. J Integr Plant Biol 49:871–884
109. Tan L, Li X, Liu F, Sun X, Li C, Zhu Z, Fu Y, Cai H, Wang X, Xie D, Sun C (2008) Control of a key transition from prostrate to erect growth in rice domestication. Nat Genet 40:1360–1364
110. Coe EH Jr, Neuffer MG (1976) The genetics of corn. In: Corn and corn improvement, vol 18. American Society of Agronomy, Madison, pp 111–223

111. Schmidt RJ, Burr FA, Burr B (1987) Transposon tagging and molecular analysis of the maize regulatory locus opaque-2. Science 238:960–963
112. Schmidt RJ, Burr FA, Aukerman MJ, Burr B (1990) Maize regulatory gene opaque-2 encodes a protein with a "leucine-zipper" motif that binds to zein DNA. Proc Natl Acad Sci USA 87:46–50
113. Shure M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the Waxy locus in maize. Cell 35:225–233
114. Klosgen RB, Gierl A, Schwarz-Sommer Z, Saedler H (1986) Molecular analysis of the waxy locus of *Zea mays*. Mol Gen Genet 203:237–244
115. Wang ZY, Wu ZL, Xing YY, Zheng FG, Guo XL, Zhang WG, Hong MM (1990) Nucleotide sequence of rice waxy gene. Nucleic Acids Res 18:5898
116. Wang ZY, Zheng FQ, Shen GZ, Gao JP, Snustad DP, Li MG, Zhang JL, Hong MM (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the waxy gene. Plant J 7:613–622
117. Barton C, Yang L, Galvin M, Sengupta-Gopalan C, Borelli T (1986) Isolation of the Shrunken-2 and Brittle-2 genes from maize. In: Shannon JC, Knievel DP, Boyer CD (eds) Regulation of Carbon and Nitrogen reduction and utilization in maize. American Society of Plant Physiologists, Rockville, pp 363–365
118. Bhave MR, Lawrence S, Barton C, Hannah LC (1990) Identification and molecular characterization of shrunken-2 cDNA clones of maize. Plant Cell 2:581–588
119. James MG, Robertson DS, Myers AM (1995) Characterization of the maize gene sugary1, a determinant of starch composition in kernels. Plant Cell 7:417–429
120. Septiningsih EM, Trijatmiko KR, Moeljopawiro S, McCouch SR (2003) Identification of quantitative trait loci for grain quality in an advanced backcross population derived from the *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*. Theor Appl Genet 107:1433–1441
121. Sweeney MT, Thomson MJ, Pfeil BE, McCouch S (2006) Caught red-handed: Rc encodes a basic helix-loop-helix protein conditioning red pericarp in rice. Plant Cell 18:283–294
122. Kinoshita T (1998) Linkage mapping using mutant genes in rice. Rice Genet Newsl 15:13–74
123. Dinneny JR, Yanofsky MF (2005) Drawing lines and borders: how the dehiscent fruit of Arabidopsis is patterned. Bioessays 27:42–49
124. Xiong L, Liu K, Dai X, Xu C, Zhang Q (1999) Identification of genetic factors controlling domestication-related traits of rice using an F2 population of a cross between *Oryza sativa* and *O. rufipogon*. Theor Appl Genet 98:243–251
125. Cai HW, Morishima H (2000) Genomic regions affecting seed shattering and seed dormancy in rice. Theor Appl Genet 100:840–846
126. Sobrizal IK, Sanchez PL, Yoshimura A (1999) RFLP mapping of a seed shattering gene on chromosome 4 in rice. Rice Genet Newsl 16:74–75
127. Nagai YS, Sobrizal SPL, Kurakazu T, Doi K, Yoshimura A (2002) Sh3, a gene for seed shattering commonly found in wild rices. Rice Genet Newsl 19:74–75
128. Li C, Zhou A, Sang T (2006) Rice domestication by reducing shattering. Science 311:1936–1939
129. Konishi S, Izawa T, Lin SY, Ebana K, Fukuta Y, Sasaki T, Yano M (2006) An SNP caused loss of seed shattering during rice domestication. Science 312:1392–1396
130. Ji HS, Chu SH, Jiang W, Cho YI, Hahn JH, Eun MY, McCouch SR, Koh HJ (2006) Characterization and mapping of a shattering mutant in rice that corresponds to a block of domestication genes. Genetics 173:995–1005
131. Ji H, Kim SR, Kim YH, Kim H, Eun MY, Jin ID, Cha YS, Yun DW, Ahn BO, Lee MC, Lee GS, Yoon UH, Lee JS, Lee YH, Suh SC, Jiang W, Yang JI, Jin P, McCouch SR, An G, Koh HJ (2010) Inactivation of the CTD phosphatase-like gene OsCPL1 enhances the development of the abscission layer and seed shattering in rice. Plant J 61:96–106
132. Krishnan A, Ambavaram MMR, Harb A, Batlang U, Wittich PE, Pereira A (2009) Genetic networks underlying plant abiotic stress responses. In: Jenks MA, Wood AJ (eds) Genes for plant abiotic stress. Wiley, Ames
133. Lin HX, Zhu MZ, Yano M, Gao JP, Liang ZW, Su WA, Hu XH, Ren ZH, Chao DY (2004) QTLs for Na + and K + uptake of the shoots and roots controlling rice salt tolerance. Theor Appl Genet 108:253–260
134. Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37:1141–1146
135. Xu K, Mackill DJ (1996) A major locus for submergence tolerance mapped on rice chromosome 9. Mol Breed 2:219–224
136. Xu K, Xu X, Ronald PC, Mackill DJ (2000) A high-resolution linkage map of the vicinity of the rice submergence tolerance locus Sub1. Mol Gen Genet 263:681–689
137. Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ (2006) Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 442: 705–708
138. Delhaize E, Craig S, Beaton CD, Bennet RJ, Jagadish VC, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.). Part I. Uptake and distribution of aluminum in root apices. Plant Physiol 103:685–693
139. Delhaize E, Ryan PR, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.). Part II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol 103:695–702
140. Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J 37: 645–653
141. Bishop GJ, Harrison K, Jones JD (1996) The tomato Dwarf gene isolated by heterologous transposon tagging

encodes the first member of a new cytochrome P450 family. Plant Cell 8:959–969
142. Doi K, Izawa T, Fuse T, Yamanouchi U, Kubo T, Shimatani Z, Yano M, Yoshimura A (2004) Ehd1, a B-type response regulator in rice, confers short-day promotion of flowering and controls FT-like gene expression independently of Hd1. Genes Dev 8:926–936
143. Salvi S, Sponza G, Morgante M, Tomes D, Niu X, Fengler KA, Meeley R, Ananiev EV, Svitashev S, Bruggemann E, Li B, Hainey CF, Radovic S, Zaina G, Rafalski JA, Tingey SV, Miao GH, Phillips RL, Tuberosa R (2007) Conserved noncoding genomic sequences associated with a flowering-time quantitative trait locus in maize. Proc Natl Acad Sci USA 104:11376–11381
144. Watanabe S, Hideshima R, Xia Z, Tsubokura Y, Sato S, Nakamoto Y, Yamanaka N, Takahashi R, Ishimoto M, Anai T, Tabata S, Harada K (2009) Map-based cloning of the gene associated with the soybean maturity locus E3. Genetics 182:1251–1262
145. Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene VRN1. Proc Natl Acad Sci USA 100:6263–6268
146. Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1643
147. Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J (2002) A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science 296:343–346
148. Liu J, Van Eck J, Cong B, Tanksley SD (2002) A new class of regulatory genes underlying the cause of pear-shaped tomato fruit. Proc Natl Acad Sci USA 99:13302–13306
149. Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432–1436
150. Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science 270:1804–1806
151. Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JD (1996) The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84:451–459
152. Dinesh-Kumar SP, Whitham S, Choi D, Hehl R, Corr C, Baker B (1995) Transposon tagging of tobacco mosaic virus resistance gene N: its possible role in the TMV-N-mediated signal transduction pathway. Proc Natl Acad Sci USA 92:4175–4180
153. Cai D, Kleine M, Kifle S, Harloff HJ, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EM, Lange W, Stiekema WJ, Wyss U, Grundler FM, Jung C (1997) Positional cloning of a gene for nematode resistance in sugar beet. Science 275:832–834
154. Ori N, Eshed Y, Paran I, Presting G, Aviv D, Tanksley S, Zamir D, Fluhr R (1997) The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521–532
155. Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ, Fischer R, Prufer D (2001) Tomato Ve disease resistance genes encode cell surface-like receptors. Proc Natl Acad Sci USA 98:6511–6515
156. Ballvora A, Ercolano MR, Weiss J, Meksem K, Bormann CA, Oberhagemann P, Salamini F, Gebhardt C (2002) The R1 gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J 30:361–371
157. Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A (2002) The barley stem rust-resistance gene Rpg1 is a novel disease-resistance gene with homology to receptor kinases. Proc Natl Acad Sci USA 99:9328–9333
158. Ernst K, Kumar A, Kriseleit D, Kloos DU, Phillips MS, Ganal MW (2002) The broad-spectrum potato cyst nematode resistance gene (Hero) from tomato is the only member of a large gene family of NBS-LRR genes with an unusual amino acid repeat in the LRR region. Plant J 31:127–136
159. Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B (2003) Map-based isolation of the leaf rust disease resistance gene Lr10 from the hexaploid wheat (*Triticum aestivum* L.) genome. Proc Natl Acad Sci USA 100:15253–15258
160. Huang L, Brooks SA, Li W, Fellers JP, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene Lr21 from the large and polyploid genome of bread wheat. Genetics 164:655–664
161. Yahiaoui N, Srichumpa P, Dudler R, Keller B (2004) Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene Pm3b from hexaploid wheat. Plant J 37:528–538
162. Qu S, Liu G, Zhou B, Bellizzi M, Zeng L, Dai L, Han B, Wang GL (2006) The broad-spectrum blast resistance gene Pi9 encodes a nucleotide-binding site-leucine-rich repeat protein and is a member of a multigene family in rice. Genetics 172: 1901–1914
163. Brueggeman R, Druka A, Nirmala J, Cavileer T, Drader T, Rostoks N, Mirlohi A, Bennypaul H, Gill U, Kudrna D, Whitelaw C, Kilian A, Han F, Sun Y, Gill K, Steffenson B, Kleinhofs A (2008) The stem rust resistance gene Rpg5 encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains. Proc Natl Acad Sci USA 105: 14970–14975
164. Fu D, Uauy C, Distelfeld A, Blechl A, Epstein L, Chen X, Sela H, Fahima T, Dubcovsky J (2009) A kinase-START gene confers temperature-dependent resistance to wheat stripe rust. Science 323:1357–1360
165. Bulgarelli D, Biselli C, Collins NC, Consonni G, Stanca AM, Schulze-Lefert P, Valè G (2010) The

CC-NB-LRR-type Rdg2a resistance gene confers immunity to the seed-borne barley leaf stripe pathogen in the absence of hypersensitive cell death. PLoS One 5(9):pii: e12599
166. Paz-Ares J, Wienand U, Peterson PA, Saedler H (1986) Molecular cloning of the c locus of *Zea mays*: a locus regulating the anthocyanin pathway. EMBO J 5:829–833
167. Buckner B, Kelson TL, Robertson DS (1990) Cloning of the y1 locus of maize, a gene involved in the biosynthesis of carotenoids. Plant Cell 2:867–876
168. Bhattacharyya M, Smith AM, Noel Ellis TH, Hedley C, Martin C (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell 60:115–122
169. Fridman E, Pleban T, Zamir D (2000) A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. Proc Natl Acad Sci USA 97:4718–4723
170. Mao L, Begum D, Chuang H, Budiman MA, Szymkowiak EJ, Irish EE, Wing RA (2000) JOINTLESS is a MADS-box gene controlling tomato flower abscission zone development. Nature 406:910–913
171. Hattori Y, Nagai K, Furukawa S, Song XJ, Kawano R, Sakakibara H, Wu J, Matsumoto T, Yoshimura A, Kitano H, Matsuoka M, Mori H, Ashikari M (2009) The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. Nature 460:1026–1030

Books and Reviews

Charlesworth D, Willis JH (2009) The genetics of inbreeding depression. Nat Rev Genet 10:783–796

Doust A (2007) Architectural evolution and its implications for domestication in grasses. Ann Bot 100:941–950

Gibson S, Somerville C (1993) Isolating plant genes. Trends Biotechnol 11:306–313

Paterson AH (1995) Molecular dissection of quantitative traits: progress and prospects. Genome Res 5:321–333

Pereira A (2001) Transposon tagging methods in heterologous plants. In: Gilmartin P, Bowler C (eds), Molecular plant biology, vol I. Oxford University Press, Oxford, 294 p. ISBN: 0-19-963876-4

Salvi S, Tuberosa R (2005) To clone or not to clone plant QTLs: present and future challenges. Trends Plant Sci 10: 297–304

Sang T (2009) Genes and mutations underlying domestication transitions in grasses. Plant Physiol 149:63–70

Tanksley SD (1993) Mapping polygenes. Annu Rev Genet 27: 205–233

Tanksley SD, Ganal MW, Martin GB (1995) Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11:63–68

Wei H, Lin H-X (2010) Toward understanding genetic mechanisms of complex traits in rice. J Genet Genomics 37:653–664

Crop Yields Around the World: Closing the Gap and Raising the Potential

R. A. (Tony) Fischer
CSIRO Plant Industry, Canberra City, Australia

Article Outline

Glossary

Allocative efficiency Efficiency with which given variable input(s) is used.

Attainable yield Yield reached by farmers with economically optimum management and reasonable risk aversion.

Ecotilling Seeking related alleles across germplasm collections based on DNA homology.

Farm yield Average grain yield across farms in a region.

Harvest index Ratio of grain dry weight to total above-ground dry weight at maturity.

Molecular markers Short sequences of DNA that are homologous with sequences close to or within important genes.

Potential yield Grain yield in the absence of manageable abiotic and biotic stresses.

Potential yield water limited Grain yield in the absence of manageable abiotic and biotic stresses apart from that imposed by water supply through inadequate rainfall.

Radiation use efficiency Above ground biomass produced per unit of total solar radiation intercepted by green crop tissue.

Technical efficiency Practices, timing, and technical skills adopted by farmer in using inputs.

Yield gap Difference between farm yield and potential yield.

Definition of Subject

The entry assumes that yield increase will continue to play a dominant role in world food security, as it has over the last 60 years. It is restricted to annual grain crops, since these dominate the world's arable landscape (>70%) and humankind's food supply (>70%), including grain used as livestock feed. Crop yield is the weight of grain, at some agreed standard moisture content, harvested per unit of land area per crop (note, there can be two or even three annual crops per year in some favored environments, meaning a cropping intensity of 200% or 300%, respectively). The starting point for yield is usually the field, district, regional, or national average yield in kg or t per hectare, as reported in surveys or local or national statistics. Here this is referred to as farm yield (FY, t/ha). This and many related cropping statistics are collated annually for all countries by FAO (http://faostat.fao.org/site/567/default.aspx#ancor). FY is usually expressed relative to harvested land area (note: this can fall well below planted area in some situations). Although FY is quoted and used widely, it may not be as accurate as it appears due to uncertain grain admixtures and/or poor collection of the statistics. With survey data sampling error can also arise.

At the highest end of the yield scale it is useful to define *potential yield* (PY), which is the yield to be expected with the best adapted cultivar, the best management of agronomic inputs, and in the absence of manageable abiotic and biotic stresses [1]. Many complications are hidden within this apparently simple definition. PY is usually determined in plots, with of course sampling error. In order to be relevant to the surrounding district, the natural resource base of the plot (climate, soil type, topography) needs to be comparable, including any long-term management investments to improve this aspect of the site (e.g., liming, tile drainage). Water supply must be adequate for PY determination as defined, and this can come from well-distributed rainfall close or equal to crop potential evapotranspiration (crop water use from sowing to harvest without water limitation) or from full or supplemental irrigation; in addition, pests, weeds, and diseases must be held at negligible levels through the use of biocides if necessary. Finally, crops experiencing

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

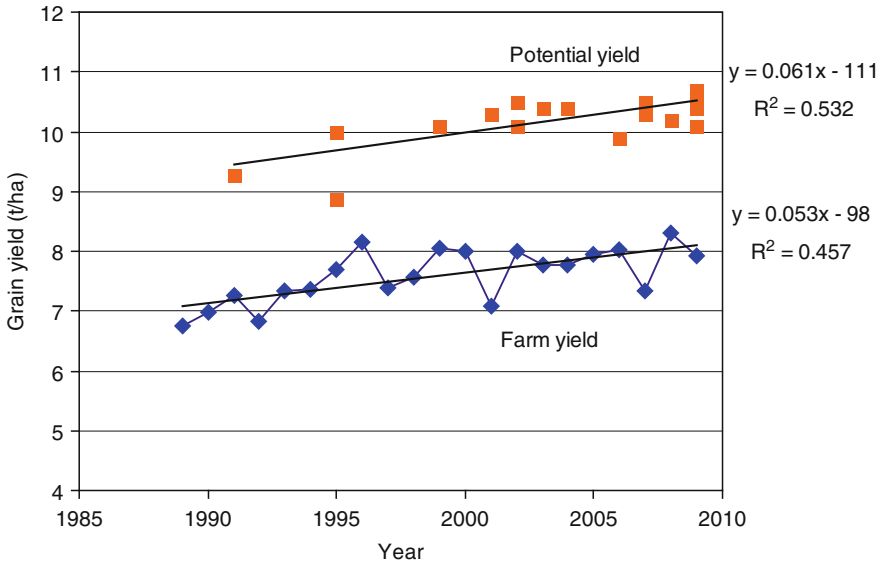
Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

relatively rare weather damage such as crop lodging or unseasonal frosting are excluded from PY measurement. Taken overall, it would seem PY might be impossibly difficult to measure, but it is reported often in the crop science literature, although not always with proper attention to the above caveats.

Since PY is usually measured in plots, edge effects arising from extra solar radiation or soil moisture or nutrients reaching outside plants must be avoided by discarding the plot edges. Two types of PY plots are commonest. Often they are well-managed yield trials to compare new varieties or advanced lines against older ones, or even historic ones, to give measures of breeding progress by plotting variety yield against year of release. The most useful such trials, for example those for wheat conducted by the UK Home Grown Cereals Authority (HGCA http://www.hgca.com/content.template/0/0/Home/Home/Home.mspx), measure yields with and without fungicide protection. Only the former yields are a measure of true PY, but fungicide protection is still not very common in such yield trials around the world, although visible disease levels are usually reported and can be negligible. The second source of PY data comes from experiments conducted by crop physiologists to calibrate and/or validate crop simulation models: the models, driven largely by various aspects of solar radiation and temperature, can then be used to predict PY in other environments (e.g., sowing dates, years, locations). For such purposes modeling accuracy has steadily improved, but models need to be updated with the latest varieties every few years, since breeders are steadily altering varieties (e.g., phasic development, improving PY). Sometimes, crop contest yields or crop record yields are considered to be synonymous with PY. But they need to be treated with caution, because they usually exceed PY measured as defined here, probably because they refer to very favorable circumstances (e.g., soils, weather, management), relative to the district average; nevertheless, we can learn from such yields if all site and management variables are quantified. For example, properly verified world record yields, invariably higher than PY values, not only extend our simulation models but also reveal that there is no anatomical limitation to very high yields, the main limitation evident with such yields being the stem strength needed to support them.

Since much of the world's grain crops are grown in rainfed situations where water supply from precipitation plus starting soil storage falls well below the potential evapotranspiration, it is also useful to define a *water-limited potential yield* (PYw, t/ha): it is the yield obtained with no other manageable limitation to the crop apart from the water supply. Obviously, it will depend on the amount of water, so PYw is usually plotted relative to water supply (or use), the slope being the crop water use efficiency or water productivity, commonly reported in kilograms per hectare per millimeter. Complications can arise due to variation in rainfall distribution with respect to crop development stages, but PYw, defined as a linear function of the water supply, is a very useful simple benchmark, as argued in a recent in-depth review by Passioura and Angus [2], while simulation modeling has been especially useful in dealing with expected deviations due to variation in the distribution of water supply.

In any given region, between FY and PY (or PYw), there is another useful yield notion, namely *attainable yield* (AY, t/ha), which is defined here as the yield attained by a farmer with average natural resources adopting economically optimal practices and levels of inputs. Since risk of financial loss is almost always part of a farmer's decision to invest in increased inputs, the AY definition must temper "optimum level" with "prudent attention to risk"; as an example this could mean input investments must be expected to return at least 50% in net profit at the margin. Of course, AY will reflect the economic circumstances of the crop and region, in particular grain prices relative to input ones, all measured at the farm gate. Thus, it is not easy to know AY, but general experience suggests that it lies around 20–30% below PY in situations where world prices and reasonable transport costs operate. FY for wheat in the UK probably meets these conditions (Fig. 1). Where this does not occur, for example, in much of sub-Saharan Africa where infrastructure and institutions are weak, the AY as defined above may be much lower; alternatively where inputs and grain prices are heavily subsidized, AY could approach closer to PY. Because of these uncertainties, it is easier to talk of the *yield gap* in terms of the FY to PY gap, bearing in mind that even in the most advanced cropping situations in developed economies, operating at close to world prices, FY will remain significantly below PY. Since it



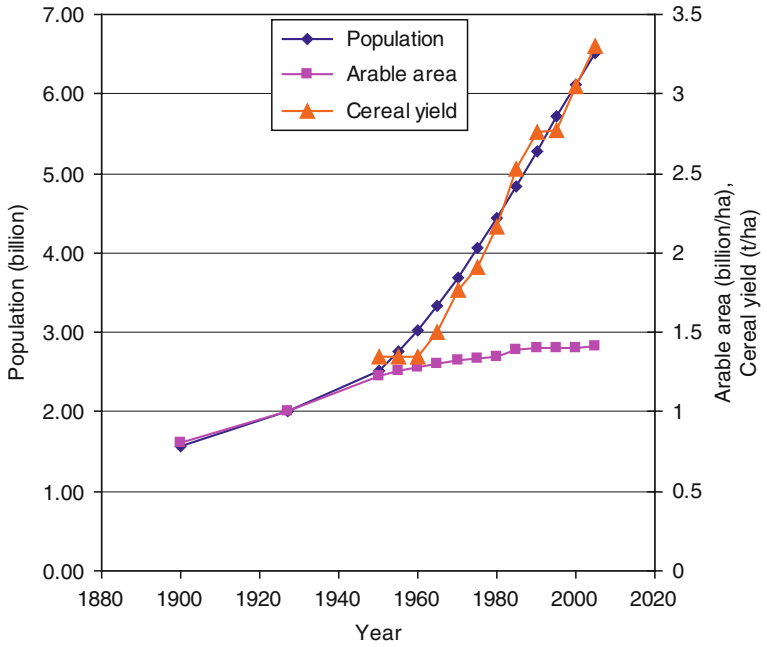
Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 1
Change in farm yield (FY) and potential yield (PY) with time for wheat in the UK. Source: FAOStat and HGCA. Note that in this and other figures (except Fig. 4), PY is plotted against year of variety release, for varieties are compared side by side in recent yield trials under modern management

is more appropriate to express this gap as a percentage of FY, it can be assumed from the literature that this minimal gap is reached when the PY–FY gap is about 30% (of FY). Any larger gap that this, which is usually the case, is often defined as an "economically exploitable yield gap," but bearing in mind that the expected exploitation can be as much a task for national and local government and agribusiness, as for the farmers.

Introduction

Crop yield has been the subject of attention since the days of Malthus and earlier. More recently, many have pointed out that the improved supply of food calories per capita globally, and reduced real costs of grain, over the last 60 years has been due almost entirely to global crop yields growing faster than the burgeoning world population (e.g., [4]), for there have been only small increases in cropped area (Fig. 2). The world population will grow to over nine billion by 2050, and grain demand is projected to increase around 60–70% (compared to 2000) [5], with others projecting even greater increases, and certainly more rapid increase coming early in this period. Seventy percent over 50 years amounts to an exponential increase of 1.1%; output growth must match this or real prices will increase (see also later). There is some potential new arable land of reasonable quality in South America and sub-Saharan Africa (and in northern latitudes of Russia), but increase in arable area demands heavy capital investment and brings negative environmental consequences; indeed, maintenance of arable area could be a challenge in densely populated fast-urbanizing regions like South and East Asia, and where there is continuing arable land degradation. Crop area can also increase through intensification of annual cropping but that usually depends on expansion in irrigation, which has in fact slowed markedly, especially in Asia where water availability for agriculture is now constrained. As a consequence, most argue that the projected increase in grain demand must continue to be met by yield increase.

Many grain crops have exceeded 1% exponential yield growth in the early decades of the modernization of agriculture last century, including the Green Revolution period for rice and wheat in Asia, but global yield growth trends for most crops over the last 20 years have slowed and have become linear, with current

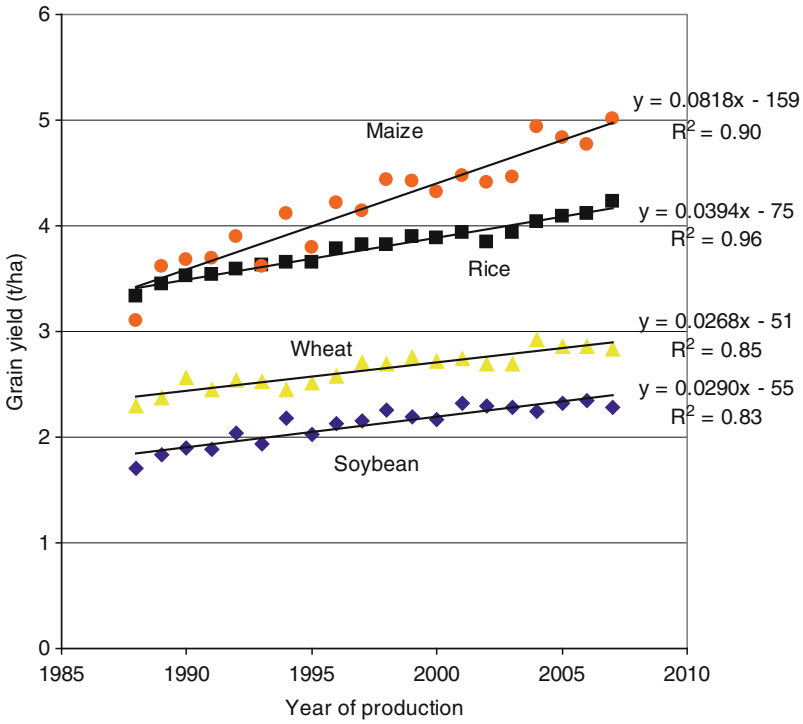


Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 2
Change in world population, global arable area, and cereal yield. Adapted with permission for Evans (1998)

growth rates at or below 1%; maize at 1.6% and soybeans at 1.1% are the only major crops exceptions to this (Fig. 3; note growth rate is expressed here by calculating the linear slope as a percentage of current predicted yield for each crop). Increasing the proportion of a crop which is irrigated can increase yield growth, as happened with wheat in Asia in the 1960s, but further increases in irrigation area are unlikely, due to cost and/or lack of water, except possibly in sub-Saharan Africa. This leaves the maintenance, or preferably boosting, of rates of yield increase in existing cropped situations as the major route for meeting growing demand for grain, and the major challenge facing agricultural science. And despite some media sentiment to the contrary, agricultural scientists are fully aware that this goal has to be met while sustaining, or better still, improving the natural resource base of cropping, and while confronting the uncertain and predominantly negative projected impacts of climate change.

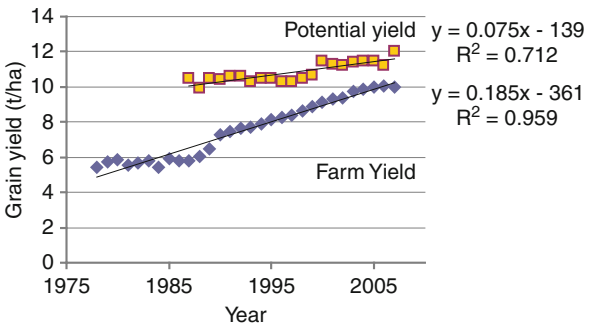
From the definition of the subject, it can be seen that there are two sources of yield increase: raising PY and closing the PY to FY gap (see also Figs. 1, 4 and 5). In most situations, both processes contribute simultaneously to FY increase, with farmers adopting new technologies (varieties, management techniques, input levels) that have been developed years or decades earlier by researchers at which time the associated rise in PY occurred. Recently, two trends in this process are becoming evident. Firstly, management or agronomic innovations for increasing PY, including their positive interactions with new varieties (e.g., the universal nitrogen by variety interaction in wheat and rice, or the plant density by hybrid variety interaction in maize), are becoming fewer, leaving a greater proportion of PY progress to breeding advances. This is a somewhat controversial observation but is clearly supported by analysis of the winter wheat yield changes in the UK [6]. Secondly, in some special situations, as mentioned earlier, the yield gap is approaching the minimal 30% or so dictated by current economics; examples include winter wheat in the UK (Fig. 1) and probably all of Western Europe), rice in Egypt (Fig. 4), and maize in the US corn belt (e.g., Iowa, see Fig. 6).

While it is not always easy to separate PY advance from gap closing, since this volume has given more



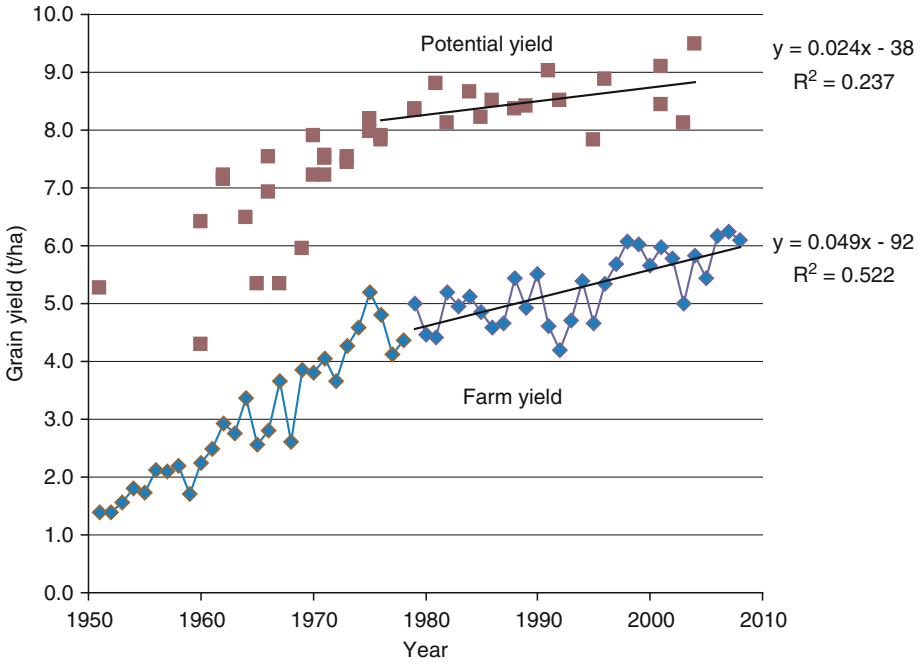
Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 3
World average yield versus time for wheat, rice, maize, and soybeans over last 20 years. Source: FAOStat

attention to the former, in particular the possibilities arising with new molecular biological techniques becoming available to plant breeders, this entry will give more attention to closing the yield gap than to raising PY. Both issues have been more fully discussed in Fischer et al. [5]. Another reason for concentrating here on closing the yield gap is that, notwithstanding the evidence of small yield gaps in some situations, it is likely that yield gap closing offers better prospects globally for quickly lifting FY progress, as is needed, than does boosting PY progress. The entry continues by looking at the size of the yield gap in various key situations around the world. Passing then to gap closure, traditionally this is considered to arise from farmer adoption of yield-enhancing technologies, the area of agricultural extension or technology transfer. While there is a lively body of socioeconomic research on this process, which will be considered briefly, the way the yield gap is defined here, it is hard to see the new products of breeding and agronomic research reducing the yield gap. This is because their initial

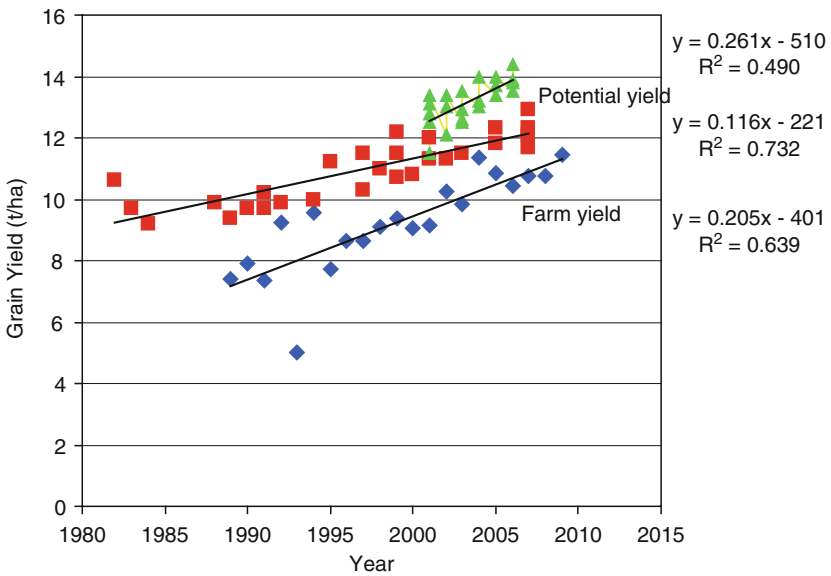


Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 4
Change in PY and in FY for rice in Egypt. Source: Badawi [3]; A. E. Draz, 2009, personal communication

impact should be on PY as defined, actually increasing the gap, followed by their gradual adoption which, if complete, might be expected to reduce the gap to the same relative value as before, other things being equal.



Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 5
Wheat PY and FY changes with time, Yaqui Valley, Mexico. Source: [5]



Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 6
Change in maize FY in Iowa compared to that in PY as estimated by Pioneer (Hammer et al. ([7], *red squares*) and de Kalb (Edgerton [8], *green triangles*) hybrid yields plotted against year of hybrid release

Needless to say the real situation is more complex and examples will be given of how such research, including progress in molecular biology, can help directly reduce the gap (e.g., more adoptable technologies, less costly ones, and better biotic stress resistance). The entry finishes by discussing briefly the current situation with progress in PY and PYw themselves. What will not be discussed further is the likelihood that atmospheric CO_2 increase will continue to improve all grain yields, other things remaining equal, but the rate of increase is currently around only 0.2% in a C3 crop like wheat [9], and less for C4 crops.

Estimates of Yield Gaps

Fischer et al. [5] attempted to estimate yield gaps and their rate of change for a number of key representative cereal-producing regions in the world. The most reliable estimates are summarized in Table 1, supplemented with a few new crops and numbers.

The yield gap estimates for wheat are remarkably consistent at around 50% except for the UK, where as mentioned before the 25% gap probably implies that FY is approaching AY, an AY largely determined by ruling world prices in the UK. Figure 5 illustrates the Yaqui Valley data, where there was substantial yield gap closing as the modern semidwarf varieties took over in the 1960s; lately, however, the yield gap has remained fairly steady at 50%. Lobell et al. [12] recently presented 12 published estimations of the yield gap for wheat in developing countries, the gap ranged from 5% to 150% but the average was close to that in Table 1, namely 55%.

For rice, Table 1 shows gap values around 100% or more for most developing country situations except for Central Luzon in the wet season (65%), and a remarkably low 15% for Egypt (see also Fig. 4). The value of 55% for Japan reflects the heavy emphasis on producing high-quality grain, acting as brake on FY progress, because food-quality rice varieties and agronomy deliver lower yields. From the 41 rice estimates from developing countries in [12], the average gap was 65%, with modeling evidence that the gap was 120% in northwest India (cf. 110% in the Indian Punjab in Table 1).

Table 1 has only two cases for maize: Iowa at 25% contrasts with Kenya at 200+%; sub-Saharan Africa as a whole is similar to Kenya. Tittonell et al. [13] in a detailed survey of farm yields in three districts in the favored Kenyan Highlands confirms the figure shown in Table 1, recording a mean FY of 1.5 t/ha when PY was 6–7 t/ha. Lobell et al. [12] give nine examples of rainfed maize in tropical and subtropical developing countries (average gap 200%) and examples of irrigated and rainfed maize in Nebraska based on simulation of PY and PYw which suggest gaps of 35% and 55%, respectively, both larger than that for rainfed maize in the adjacent wetter state of Iowa (Table 1). While there is no doubt about the huge yield gap with maize in Africa, some uncertainty exists in these critical estimates from the USA, where maize is probably the most intensively researched and promoted crop in the world. No doubt the gap is quite small in Iowa and Nebraska, where it has been closing lately, and FY may be close to AY (Fig. 6), although more PY progress data is needed for a clearer conclusion.

The soybean examples in Table 1 also show a large difference in the yield gap between a developed and developing country, but do not show developing countries Argentina and Brazil, where FY across more than 35 M ha with favorable rainfall is as high as the USA, and PY is unlikely to be any higher. Finally, millet, grown in India under very harsh conditions, shows a gap no worse that of soybeans in adjacent more favorable areas of India.

Closing Yield Gaps

Yield gaps are reduced by farmers adopting new technologies or practices (new at least for them) or adopting higher rates of inputs (e.g., many farmers in western Kenya use added nutrients, either fertilizer or organic ones, but the rate of nutrients applied may be very low relative to the need/optimum (e.g., [13])). Some of this is usefully illustrated in Fig. 7 derived from Byerlee [14].

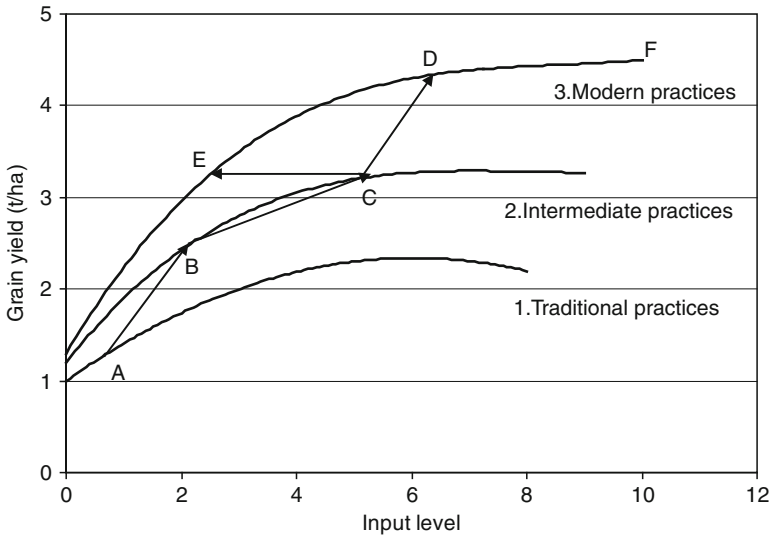
FY under traditional practices with low inputs is shown by point A on curve 1. Adopting new technologies such as an improved new variety and practices (e.g., line sowing and fertilizer) lifts technical efficiency and brings curve 2 into play. The farmer may move from point A to point B, as in the first years of the Green Revolution. Allocative efficiency could then rise further as fertilizer levels increase, following curve 2 to

Crop Yields Around the World: Closing the Gap and Raising the Potential. Table 1 Estimates of current yield gaps for key crops and regions (compiled from [5] and elsewhere as noted)

Region	Environment	Crop area (M ha)	Yield (t/ha)[a] and gap(%), 2007 or 2008		
			FY	PY	Gap
Wheat					
Yaqui V, Mexico	Irrigated, low latitude	0.16	6	9	50
Punjab, India	Irrigated, low latitude	3.9	4.3	6.25	45
Western Australia	Subhumid, low latitude	4.5	1.8	2.6[b]	45
N. Dakota	Subhumid, high latitude	3.4	2.5	3.7[b]	50
UK	High rain, winter wheat	1.8	8.2	10.4	25
Eastern China	Irrigated, winter wheat	16	4.7	7	50
Kansas	Subhumid, winter wheat	3.6	2.6	3.9[b]	45
Rice					
Central Luzon	Wet season + irrigation	0.8	3.8	6	60
Punjab, India	Wet season + irrigation	2.4	3.8	8	110
Japan	Wet season + irrigation	3	6.5	10	55
South Asia	Wet season, rainfed	28.5	1.8	3.6	100
Central Luzon	Dry season, irrigated	0.4	4.5	9	100
Egypt	Dry season, irrigated	0.7	10.1	11.6	15
Maize					
Iowa	Temperate, high rainfall	5.3	10.8	15.5[c]	45
Kenya	All altitudes, moderate rain	1.75	1.8	6[b]	200+
Soybeans					
USA[d]	Temperate, high rainfall	30	2.8	3.6	30
India[e]	Subtropical, tropical, moderate rainfall	9	1	2.2[b]	120
Millet					
India[f]	Subtropical, rainfed	11	0.9	1.8[b]	100

[a]Predicted from linear trends
[b]Actually PYw
[c]Reestimated in Fischer and Edmeades [9]
[d]Estimate by R. A. Fischer, 2010 unpublished
[e]Bhatia et al. [10]
[f]Murty et al. [11]

point C, approaching some economic optimum. An even newer variety and the best available practices (e.g., herbicides, conservation tillage) which together define current PY (point F) could lift the technical frontier further (the uppermost curve 3). Farmers might choose to further increase technical efficiency by moving to the uppermost curve, maximizing allocative efficiency at around point D, or sacrificing allocative efficiency, but not yield, by reducing inputs (point E). Of course, curve 3 is not static: for any given



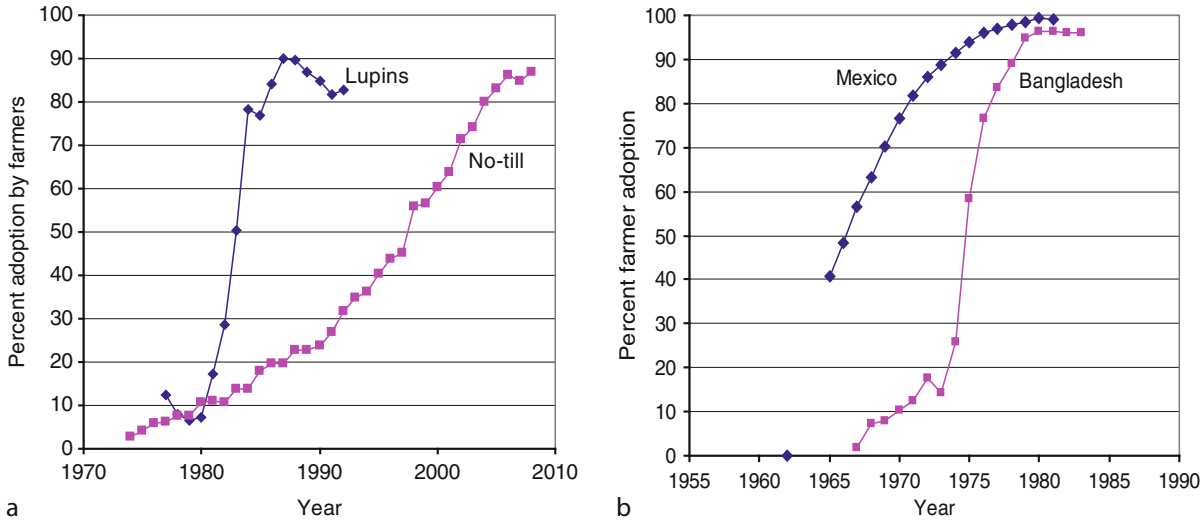
Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 7
Illustration of pathways from low FY to close the gap with PY; modified from Byerlee [14]

environment it may be moved further upward with new technologies, and it may also shift downward due to problems of resource degradation. This figure also serves to illustrate that the yield gap is not the same as an efficiency gap, gaps between curves are technical efficiency ones, gaps along curves are allocative ones, both combine to make up the FY to AY gap.

Adoption of New Technology

There is a rich agricultural economic literature on the adoption of new technologies by farmers. Examples for the adoption of two new technologies in the rainfed cropping in Australia are shown in Fig. 8a (a new crop, lupins, and a new practice, no till). The new crop was adopted faster than the new agronomic technique. Developing countries have proved equally fast in adopting new varieties (Fig. 8b) when the conditions are favorable. The well-recognized patterns of lag, rate of adoption, and ceiling adoption level clearly differ between technologies and farmers (regions); in addition, the infrastructural and institutional context is important. For the technology itself, key elements are its perceived relative advantage (return over cost, risk of loss, convenience, etc.) over that which it replaces, and its trialability, meaning the ease with which a new technology can be tested on a small scale. Farmer characteristics influencing adoption are very diverse and can also interact with the nature of the innovation: education, age, health, exposure to extension and related media and demonstrations, group pressure, and support can be important. But there is little doubt that farmers around the world, large and especially the small, women and men, aspire to increase monetary return and given the means to do so, will adopt more profitable practices.

It is in the area of rural infrastructure and institutions that developed and developing country farmer circumstances differ most. One obvious example is the effect of poor roads on increasing input costs and reducing prices for surplus farm output. For example, the price ratio of N fertilizer to grain is on average double that in other regions of the world and higher still in inland landlocked regions of sub-Saharan Africa [18]. The high cost of credit, uncertainties surrounding contracts (including land tenure), the risk of poor-quality inputs, theft or unrest, and the prevalence of unfavorable and uncertain tax and pricing policies, all make adoption less attractive in many developing situations. Sometimes, price subsidy on inputs and outputs can compensate for these brakes on adoption, such as in the recent situation where a well-crafted fertilizer supply



Crop Yields Around the World: Closing the Gap and Raising the Potential. Figure 8
Adoption of new technologies by (**a**) farmers in regions of Australia (lupins in Wongan Hills district [15] and no till in Loddon district [16]), and (**b**) semidwarf wheat varieties in Mexico and in Bangladesh [17]

and subsidy policy permitted Malawi, a nation of small holders, to produce an unprecedented surplus of maize and to do so without a maize price collapse. But in general and especially in sub-Saharan Africa the lack of public investment in infrastructure, institutions and agricultural extension, and the lack of sound policy are the major contributors to the yield gap.

Looking at farm-level technical constraints that contribute to the yield gap, in any situation there are usually multiple constraints, and the challenge is to determine which constrains should take priority, while recognizing that interventions often interact positively and are thus more effective when adopted together [19, 20]. This can only be answered by on-farm survey and experimentation. Such work started many years ago with farming system research, farm management clubs, and rapid rural appraisal. It continues in many guises in the developed world, especially influenced by the privatization of agricultural extension, the use of remote sensing and ICT advances, and the entry of input suppliers, in particular seed companies, into agricultural extension.

In the developing world the more traditional approaches remain, although with growing emphasis on farmer participation [21]. Lobell et al. [12] recount how IRRI conducted on-farm rice experiments in Asia in the 1970s to test high inputs, learning that farmer yields varied greatly between fields, as did responses to inputs especially fertilizer and insecticide, which were often uneconomic. This pointed to the importance of field-to-field variability, and the need to adjust inputs accordingly and as the season unfolds, whether by site-specific nutrient management, which reached maturity some 20 years later [22], or via field-level pest monitoring as part of IPM packages. Titonell et al. [13] recount a similar picture of substantial variability in soil fertility, resource use intensity, and yields among small farmer maize fields in western Kenya. Another lesson is surely that this is scientist-intensive expensive research, usually taken over by the farmer and his advisers in the industrial world, and explaining why large yield gaps often persist in the developing one, where circumstances demand innovative approaches in order to reach the billion small farmers (e.g., [21]).

Very recently IRRI again looked at rice yield gaps, this time using expert knowledge to assess constraints and possibilities for irrigated rice in South Asia [23]. For this crop, FY is currently 5.1 t/ha over 34.3 M ha; it was estimated that on average yield was constrained 1.9 t/ha (37%) by yield-limiting factors, which included individual constraints from nutrient insufficiency (10%), diseases (7%), weeds (7%),

water shortage (5%), and rats (4%). The exercise was repeated for the 28.5 M ha of rainfed lowland and upland rice in South Asia with a current FY of 1.8 t/ha: yield-limiting factors amounted to 68% of FY, including nutrients (23%), disease (15%), and weeds (12%). The IRRI paper predicted that with a substantial research, development and extension effort, the adoption of existing technology and ongoing breeding over the next 15 years could reduce these losses by one third (irrigated rice) or one quarter (rainfed rice) adding about 1% to FY growth rate.

With wheat in the Yaqui Valley we have a recent concerted effort to understand the yield gap, PY–FY (currently 50%, Fig. 5, Table 1), this time using the latest high-resolution satellite imagery to estimate field-level yields for all fields in the Valley [24] and supplement a long history of farm surveys. Despite the moderate size and wealth of farms relative to India or Kenya, again field-to-field variability in yield was substantial. It was estimated from images over several years that wheat yields were constrained by late planting, delays in the first post-plant irrigation, and summer fallow weeds [25]. Improved institutions and farm management decisions could largely eliminate these constraints, which averaged over years totaled about 10–15% of FY, and would bridge about half of the gap to estimated *AY* in the Valley. These authors [26] used inter alia classification and regression trees to relate yield to constraints in their complex data sets. The same technique was used by Tittonell et al. [13] to explore management and soil constraints to 150 field-level maize yields in highland Kenya: the rate of added nutrients was the strongest explanatory variable, followed by date of planting and plant density, as yield ranged from 1.2 (low nutrients, late plant) to 4.2 t/ha (high nutrients, normal plant, high density).

Two recent studies illustrate for rainfed cropping the power of simulation modeling and water productivity boundary functions in understanding yield gaps in surveyed farmer fields and in particular removing that part of the apparent gap which is actually due to non-manageable weather (mostly rainfall distribution). Grassini et al. [27] found these distribution effects quite important for sunflower yield variation among fields in the western pampas of central Argentina. Hochman et al. [28] looking at farm wheat yields across Australia, found less influence of rainfall distribution, and more scope for farmers to lift water productivity via improved management (e.g., earlier planting, higher seeding density, higher N input). A weakness of both these studies is that they refer to better farmers rather than to a random sample of fields, for which the opportunities of gap-closing interventions are likely to be greater.

The persistence of large yield gaps in the developing world especially draws attention to situations where these gaps have been closed. Rice in Egyptian is an obvious example where strong R, D, and E engaged a small geographically focused industry of small holders under a sound price policy (Fig. 4). A second example of dramatic technology adoption, albeit with lesser immediate implications for FY than for sustainability of the whole cropping system, relates to the uptake of conservation tillage for wheat, maize, and soybeans in southern South America (Argentina, Brazil, and Paraguay), rising from nothing in 1970 to 24 M ha in 2000. This was very much driven by farmer groups and the farmers themselves faced with the threat of serious soil degradation and by the opportunity provided by knock-down herbicides and knowledge spillover from the North (e.g., [29]). This revolution has yet to reach other developing continents (but is beginning in northwest South Asia). A third success story among small poor farmers has recently appeared with the introduction and expansion of winter maize in northeastern India and Bangladesh.

Despite individual success stories like rice in Egypt, yield gaps in general appear to be quite persistent and close only slowly; this happens even when gaps are well above that to be expected from economics and risk aversion and even when PY progress has slowed such that catch-up through eliminating excessive lags in varietal adoption is not a big issue. The problem is that gap closing on the large scale needed requires massive investments in rural infrastructure and institutions as well as technology transfer, and these are not forthcoming, as maize in sub-Saharan Africa exemplifies. Elsewhere public sector agencies, in particular reaching the billion small farmers in Asia [21], aided by the private sector, in particular in Latin America, have made some inroads on the yield gap; they should continue to do so largely in proportion to the investments made, but there is also scope for innovation, for example, based on modern ICT technologies.

The employment of agronomists by private seed companies is a pattern that is bound to be followed in the developing world as its seed industry grows in strength and competitiveness. With gap closing, there are no spill-ins as there are in the case of PY advance through R and D, innovations need to be adapted locally, but it can be argued that the Internet and mobile phones are relevant spill-in technologies for delivering information to farmers small and large, a role which could greatly expand.

Innovative Adoption-Friendly Crop Management and New Varieties

Research can facilitate the adoption of technologies to lessen those constraints which contribute to the FY to PY gaps. Table 2 lists common constraints, separating research targeting agronomic solutions from that involving breeding, while institutional and infrastructural solutions already mentioned are shown in the last column. Some agronomic technologies are still under development (e.g., improved seasonal weather forecasts), but much of the necessary agronomic research is adaptive, generally fitting technologies from more advanced farming systems. For example, the widespread adoption of direct seeding of wheat after rice in northwest India, which by saving on land preparation time has meant that sowing dates are less likely to be late and yield expectations are hence improved, required research and development on appropriate small-scale drills for this direct seeding operation. Sometimes, the agronomic technology comes from a "less-advanced" field cropping situations or from farmers themselves: for example, the use of plastic film mulches, quite common in northern China with wheat, maize, and oilseeds. Such mulches give substantial yield benefit through retaining soil moisture and aiding soil warming in the spring, and challenge agronomic researchers to adapt them in a sustainable fashion elsewhere, including developed countries.

It is with breeding that there are greater gap-closing opportunities. Considering that actual losses from diseases and insects globally exceed 20% of yield with wheat, rice, and maize [30], any improvement in genetic resistance has an immediate benefit for FY when the improved varieties are adopted. Conventional host plant resistance breeding continues to make progress on this front, while genetic engineering has brought exciting new opportunities. Although engineered resistance does not lift PY, it lifts FY wherever farmers cannot control pests and diseases with traditional means. The experience with Bt cotton in India shows how important this can be: small holder yields are at least 30% better simply because before the advent of Bt cotton they were unable to eliminate damage no matter how much insecticide was used [31]. It is likely that the advent of GM corn resistant to root worm is boosting farm yields in the USA because root worm damage went unnoticed beforehand (and is very difficult to treat with chemicals). Fungal diseases have yet to succumb to genetically engineered host plant resistance, but it is reasonable to expect varietal releases in this area in the next decade; engineered viral resistance has already been deployed in some crops [31]. Actual yield losses due to weeds are estimated globally at about 10% by Oerke [30], to which should be added the costs of current control measures. More competitive cultivars can help, but again genetic engineering has brought revolutionary advances in ease, cost, and effectiveness of weed control. Of course, with any chemical-based susceptibility of biotic stress agents, there will be the risk of resistance evolving in the target organism, but integrated management, albeit requiring greater farmer skills, can prevent that. Given proper R, D, and extension, the potential impact of herbicide-tolerant cultivars on FY in labor-limited African cropping is likely substantial. Of course, all breeding solutions to aid gap closing presuppose an effective seed production and distribution system. Systems are gradually improving in developing countries and most commercial farmers grow improved varieties although the rate of turnover of varieties is often too slow. There is little doubt that wherever plant breeding is privatized, competition drives quicker variety turnover; in advanced systems, it also drives significant agronomic extension by the breeding firms keen to maximize variety by management interactions and to retain clients.

Lifting Potential Yield

Although separated by a yield gap and a time lag, many situations show a close relationship between progress in PY and that in FY, as new technologies eventually

Crop Yields Around the World: Closing the Gap and Raising the Potential. Table 2 Constraining factors contributing to the farm yield–potential yield gap and their alleviation so that farm yield can approach the attainable yield corresponding to the current potential yield with realistic economics

Constraint	Resolution		
	Research		
	Agronomic	Breeding	Institutional/infrastructural
General farmer constraints			
Lack of farmer awareness or conviction or skill	On-farm demonstration	On-farm testing and selection	Education, media campaigns, extension
Risk aversion by farmer	Forecasts, tactical decision making (e.g., for N top dress)	Tolerance of extreme weather events, like drought, flooding, hail, frost, wind	Insurance schemes, favorable credit terms, price stability
Inadequate labor supply	Mechanization, reduced tillage, herbicides	Select for uniform maturity to favor mechanical harvesting	Facilitate labor migration; credit for mechanization
Technical constraints			
Lacking major long-term soil amelioration	Drainage, land leveling, liming, deep tillage, gypsum	Waterlogging, acidity, and salt tolerance	Long-term credit for major soil amelioration, secure tenure
Excess tillage and loss of moisture, soil compaction	Conservation tillage options and suitable machinery, controlled traffic	Suitable varieties: disease and herbicide tolerance	Credit for new machinery
Manageable topsoil soil toxicities	Ameliorate (e.g., lime for acidity)	Acidity, aluminum tolerance	Input suppliers, credit for lime
Suboptimal nutrient supply	Diagnostics, application of nutrients, slow release forms, tactics to match supply with need	Some scope for improved N, P, and Zn uptake and utilization efficiency	Infrastructure, input suppliers, credit, quality control
Soil variation within and between adjacent fields	Diagnostics to aid adjustment of application rates	Greater tolerance of soil stresses	
Growing old varieties, or use of poor seed	Better on-farm seed management and storage	F1 hybrids, licensed traits, royalties to encourage strong seed industry	Strong seed industry and proper regulation, credit
Incorrect time of sowing	Mechanize, reduced tillage to speed sowing; treatments to warm soil for spring sowing	Make available varieties with range of maturities; herbicide and cold tolerant varieties	Policy to favor mechanization, contract seeding
Poor plant population	Better drilling procedures and machines, quality seed storage	More robust varieties (e.g., long coleoptile in wheat, more tillering)	Seed testing and regulations
Diseases and pests, above and below ground	Biocides, sanitation, crop rotation, IPM	Host plant resistance	Input suppliers, pesticide quality control

Crop Yields Around the World: Closing the Gap and Raising the Potential. Table 2 (Continued)

Constraint	Resolution		
	Research		
	Agronomic	Breeding	Institutional/infrastructural
Weeds	Herbicides, cultivation, sanitation, crop rotation	Enhance crop plant competitiveness, herbicide tolerance	Herbicide quality control, release regulation
Poor water management in irrigated systems	Improve water application techniques and skills, land levelling	Greater tolerance water shortage and excess	Efficient supply systems to farm
Long-term soil degradation	Crop rotation, fertilizer, green manuring, farm yard manure, conservation tillage, amelioration	Varieties adapted to biotic and abiotic stresses of high plant residue levels, and with good residue production	Tenure regulations ensuring land ownership by farmer

find their way to farmers. Often the process is facilitated by new varieties linked to agronomic advances, for new varieties are fairly quickly adopted in most commercial farming. Sometimes, the PY advances owe more to innovative farmers than to researchers, as for example, when farmers moved to earlier planting permitted by direct seeding in southern Australia. In all cases, increase in PY is an important component driving increased FY, inevitably so where the yield gap is approaching the economic minimum (e.g., wheat in the UK), but less so where it is large (e.g., paddy or rainfed rice in India), and even less when the gap is huge (e.g., maize in sub-Saharan Africa).

Current rates of PY progress were summarized for the key cereals in [5]. These numbers come from breeders' and researchers' trials containing varieties of different vintage grown under modern agronomic management with little or no biotic stress; thus, the rates capture as well the positive interactions between new varieties and modern management. Linear regression is used to calculate the absolute rate of progress which is then expressed as a percentage of most recent predicted PY in the series, hopefully a recently released variety (>2006) but such data is not always available. For wheat, progress ranged from 0.3% to 1% p.a. with an average of 0.6% (n = 6) and with little difference between water-unlimited and water-limited PY progress (see also Figs. 1 and 5). For rice, the range was 0.2–0.7% (average of 0.4%, n = 4). Progress in PY at the International Rice Research Institute (IRRI) is disappointing, at close to zero; however, there is a one-off yield jump of 10–15% through the recent exploitation of F1 hybrids feeding into the tropical rice regions, and following upon on a similar gain in China over the last 20 years as hybrids moved to >50% of their acreage. For maize, there is only one estimate in [5], coming from Iowa and showing 1%. Data of Luque et al. [32] in a somewhat similar environment in Argentina give a rather similar number for hybrids released up to 1997 (1.3%), but since then PY progress may have slowed (M. Otegui, 2009, personal communication). A subsequent report from Iowa with hybrids of a rival company showed progress at 1.7% but the breeding period sampled was only 2001–2006 [8]. In an irrigated favorable Mediterranean environment, data presented by Campos et al. [33] indicate PY progress in the Iowa Pioneer hybrids of 1% (but interestingly progress in PYw of 1.5% under artificial severe mid-season water stress). PY progress in cereals is discussed in more detail in [9]. Potential yield for soybeans in the USA appears to be progressing at 0.7% p.a. (R. A. Fischer, 2010, unpublished).

From the above, it is apparent that even in advanced situations with substantial breeding investment, potential yield progress from this source is currently at or well below 1%, with maize showing more progress than other major crops. Such PY progress is unlikely alone to drive FY progress at the rates needed. Prospects of lifting this rate of PY progress were now discussed briefly.

New Yield-Positive Agronomic Techniques

Improved agronomy has played a large if not dominant role in past yield increase (e.g., increased fertilizer use, better weed control, better seeding techniques for earlier seeding and more reliable crop stands, and water conserving reduced tillage), and most improvements have interacted positively with variety improvement (e.g., [20]). While many authors have consistently failed to anticipate agronomic advances in the past [34], currently it is hard to see any new ones raising PY or PYw (as distinct from advances specifically targeting improved input efficiency). One example might be improved seasonal forecasts which would permit the farmer to better tailor management and variety to expected weather, something currently limited by the low skill of forecasts. Another, which would clearly lift PYw, would be the reduction of soil evaporation with inexpensive plastic films (e.g., spray-applied nanofilms), copying a common practice seen in northwest China field crops, but designed so as not to contaminate the environment. Also it is possible advances can still come from better management of soil–root–microbe interactions, a complex and neglected area of research: poorly explained observations of "yield decline" and "break crop effects" point in this direction, as do reports of positive effects of increased soil organic matter (independent of nutrition), controlled traffic and reduced soil compaction.

Greater Investment in Current Conventional Breeding Efforts for Yield

Yield improvement through plant breeding is generally regarded as delivering benefits far in excess of the costs [35, 36]. Nevertheless, it has been suggested that returns from the investment in breeding personnel are diminishing, for example in maize, and that yield gains are getting harder to achieve [37]. This is occurring despite greater efficiency through computerization, advanced biometrics, mechanization, robotics, and techniques for rapid generation advance, all part of any conventional breeding today. What is quite unclear however is the marginal return on investment in conventional breeding; for example, what would happen if the number of breeders, and of crosses, selections, and yield trials, were increased say 50% in any currently substantial program? In crops where breeders are devoting many resources to maintaining disease resistance (e.g., wheat) or eating quality (e.g., wheat, rice), and thus unable to give full attention to yield, PY progress is likely to increase, although probably less proportionally that the proportional investment increase. A related question is whether any extra funds would be better spent on new breeding tools.

New Tools to Increase Progress in Conventional Breeding

Genetic variation is the basis of yield progress. It is unlikely that all or even the major part of the existing genetic variation has been utilized in any crop. Sampling germplasm collections is a formidable task, but new tools are becoming available for seeking out new and useful variation, such as the Focused Identification of Germplasm Strategy (FIGS, [39]) and Ecotilling [38]. Because of the low chance of success, this work tends to be in the area of publically funded prebreeding, with only medium to long-term impacts on breeding, but it is worth noting that the steady reduction in genome sequencing costs is also opening up new ways of allele searching [40].

Both parental and especially early generation selection can benefit from the use of low cost performance-related markers. For the last 50 years or so, physiologists have attempted to identify such markers or traits, physiological or morphological, or even whole ideotypes, which would lead to greater yield. There have been some successes, for example, the first high-yielding semidwarf tropical rice variety, IR8, resulted from the pursuit of a specific ideotype [41], and breeding for resistance to some toxicities is usually based on early generation physiological screening (e.g., aluminum tolerance). However, indirect selection for both PYw and especially for PY has proved challenging, and much physiology has been restricted to the retrospective identification and understanding of traits which have changed as direct selection has increased yield. A good example are the many traits that have been found to change as maize yields increased in North America under direct selection for yield, lodging resistance, dry down, and disease resistance alone [37, 42]. As understanding of yield determination improves, including the recognition that seemingly useful traits can carry trade-offs, and as measurements of many

traits become cheaper, especially through the use of remote sensing in the field, the possibilities for physiological traits have improved. At CIMMYT, maize breeding for drought (PYw) has definitely benefitted from the inclusion of physiological traits such as anthesis to silking interval in managed drought environments [43]. More recently, with wheat there has been testing of canopy temperature as an early generation selection criterion, a surrogate for stomatal conductance and possibly photosynthetic rate under irrigation, or possibly for rooting depth under drought; it shows promise under both conditions [44], as does multispectral canopy reflectance under irrigation [45]. In CSIRO, Australia, ideotrait selection for greater PYw has been under way for several decades, with focus on traits such as water use efficiency via carbon isotope discrimination, coleoptiles length, low tillering, and high stem carbohydrate content at flowering, with moderate success [46]. Very recent research points to genetic differences in wheat for grain set under drought and ways for screening this trait which is likely to be a significant bottleneck in PYw determination [47].

While physiology may be beginning to look more useful in early generation yield selection, it is now being challenged by the rapidly growing use of molecular markers for traits, initially qualitative ones but lately also quantitative traits, including yield itself. The huge decline in cost of detecting molecular markers, and their increasing density across crop genomes is driving this. Heffner et al. [48] review the possibilities of such marker-based genome selection in crops. Earthington et al. [49] describe in some detail the extent to which molecular markers are being incorporated into the Monsanto crop breeding programs: rates of progress in multiple trait indices (e.g., including yield, grain moisture, standability, and test weight for maize) can be doubled; indeed, for these authors marker-aided selection is the "new" conventional breeding. These approaches presume accurate phenotyping of genotypes for yield and its interactions with target environments, a challenge in all breeding. However, it is interesting that physiology hardly features in this new scenario, although other groups are busily mapping putatively useful physiological traits at the molecular level (e.g., [50, 51]). It remains to be seen whether physiological traits, either observed directly or identified by accurate markers, will have a place in marker-aided yield breeding, but it is possible some combination of markers and early generation trait phenotyping using smart remote sensing will prove an even more efficient strategy for PY advance. In addition physiology, just like plant pathology, will continue to identify individual traits whose incorporation into leading varieties may lift PY or PYw, a process which could benefit from the use of molecular trait markers, just as is becoming the case with new hard-to-screen host plant disease resistance genes.

Multilocation multiple-year yield testing remains the essential final step in all plant breeding for yield; testing under different management regimes (plant density, planting date, soil stored water) is also recognized as increasingly important. Crop simulation models are now good enough to help breeders understand the environmental and sometimes the genetic bases of the interactions encountered, and thus deploy the testing more efficiently, and more efficiently indentify the genotypes best suited to major environment × management combinations. An excellent example is the analyses of multiple environment rainfed trials of sorghum in Queeensland [52].

Genetic Engineering for Yield

There are many claims of engineered traits that may increase crop yield, but very few of these have been backed up by yield measurements in the field. Crop physiologists have regularly pointed out how different is the performance of a genotype growing in a pot in the glass house from that of one at normal plant density in the field environment [53, 54]. For this reason, they remain skeptical of the value of these claims and await with much interest the results of field tests, for, to date, it appears that only a handful of papers describe performance in proper field tests. In an excellent example, wheat transformed with a more active ADP glucose pyrophosphorylase in the grain to increase grain sink strength and grain size, was thoroughly field tested, but there was no yield benefit [55]. More recently, Monsanto has reported increased PYw in field plots with maize transformed with two separate events [56, 57]; results in the latter case point to modest PYw increases and no change in PY, but the nature of the drought and of the crop physiological responses are not well described. More experimental data is needed before much confidence can be placed in this event,

or in engineering for yield in general, for both genetic and physiological understanding of yield determination indicates that many processes are involved, and that the impact of any single transformation is likely to be small even if key regulatory genes or bottleneck processes are targeted. Indeed, it is now being recognized that if functional genomics is to have a significant impact on yield through engineering yield processes, it must pay more attention to the relevant plant and crop physiology connecting gene action to field performance [58]; even the current fashion for automated phenotyping in controlled facilities must be balanced by substantial investment in phenotyping in field plots in managed environments.

Some Tentative Conclusions on Future Potential Yield Progress

As PY increases, relative rates of PY progress are decreasing; this is probably more than the consequence of a linear rate of progress relative to a rising denominator, and may, in situations where the breeding investment is large (e.g., maize in North America, wheat in the UK and the rest of Western Europe), also reflect diminishing returns as biological limits are approached. Theoretical limits to harvest index are already being reached, but those to radiation use efficiency appear well above current measured values [59]. There have been a couple of attempts to project PY into the future. For example, Sylvester-Bradley et al. [60] propose that a longer period of light capture and a higher radiation use efficiency could lift PY of winter wheat in the UK to 17.4 t/ha by 2050, a linear rate of progress of 167 kg/ha/year, or two and half times the current rate of progress seen in Fig. 1. Taking a different tack, Monsanto has predicted a doubling of US maize yield between 2000 and 2030, based on equal contributions from conventional breeding, marker-aided selection, and genetic engineering, and amounting to a linear annual rate of progress which is 3.3% of today's yields. Physiological theory and commercial optimism both, however, have their limitations. Looking more cautiously at the ways of impacting PY progress outlined above, it is concluded that breeding progress will dominate in the future, that conventional breeding, including the exploitation of heterosis, will continue to deliver small but declining gains, while new approaches may boost gains enough to maintain current rates of linear PY progress at between 0.5% and 1%, other things such as climate remaining equal (note that given the way PY is defined here, this progress is independent of any direct effect of atmospheric CO_2 increase). Observers expect the investment in the relevant R and D to remain large, as the private sector involvement increases to balance a declining public sector, and many argue that it should grow further, although the marginal rate of return is not well known.

Future Directions and Synthesis

The key issue for future food security is the real grain price needed to balance burgeoning demand against increasing supply. World food production demand and supply to 2050 have recently been projected by Hubert et al. [61] with the IFPRI Impact model, and by Tweetin and Thompson [62]. Thus, the baseline case of the former paper projects a 56% increase in cereal production over 2000, accompanied by substantial real price increases over the 2000 base, with negative effects on malnutrition. Demand growth is greater for maize and least for rice, such that the projected real price increases over 2000 are maize (97%), wheat (90%), and rice (60%). Clearly, a 56% increase in production is not enough. For Tweetin and Thompson [62], cereal supply rises somewhat more, by around 75% in 2050, but real prices still increase (on average 44%). The IFPRI projections allow for biofuel demand (peaking in the late 2020s at 16% of total maize consumption, after which second-generation biomass biofuel takes over), rising energy costs and likely restrictions on irrigation water supply. They also point to a much greater increase in grain demand in the first 25 years (1.4% pa) than in the second 25 years (0.4%), clearly placing more pressure on short- to medium-term yield progress. The IFPRI model contains crop area as a variable but, given the cost of developing new lands and its scarcity, this only increases significantly if prices more than double under a very low yield progress scenario.

In the IFPRI baseline projection, yield grows to meet demand at around 1% exponential projected over the whole period. In [62], it is expected to grow linearly at a rate equivalent to 1.4% of current yields. These rates compare to the current rates of world yield

growth, which are wheat and rice (0.9%), maize (1.6%), and soybeans (1.1%), but it must be recalled that the actual rates all appear to be linear, not exponential, and that the projected rates in neither of the models are enough to hold real prices steady at 2000 levels. In addition, the elasticity of demand is such that a supply shortfall due to below baseline yield growth would be disastrous (as we saw 2 years ago although some other factors added to the sharp doubling of prices then): the projections in [61] suggest a 40% fall in yield growth below baseline (to 0.6% exponential) would lift 2050 real cereal prices to more than 200% above 2000 real prices!! Alternatively, 1.4% yield growth would mean continuing declines in real grain prices.

These numbers highlight the importance of yield growth that has been the subject of this entry and reveal that current FY growth rates are inadequate to hold real prices down. Since PY growth can contribute to FY growth everywhere, and is the only source of growth in advanced agricultural systems where FY is approaching PY, it assumes great underlying importance, even as current rates of growth, except possibly for maize, seem to be well below 1% and gradually falling as a percentage of yield. Unfortunately, the brief examination here suggests that the prospects of boosting PY growth, largely now relying on plant breeding, are small, or at least rather uncertain, especially in the short to medium term when yield increases are most critical. Much will depend on whether the molecular marker-aided yield selection can boost rates, for example, double them, as claimed by some; genetic engineering seems unlikely to lift PY in the short to medium term, although the prospects to lift PYw may be somewhat better if water stress-related yield bottlenecks can be relieved. And none of these projections have factored in the possible negative effects of climate change.

As a consequence of the above, yield gap closing assumes great importance for future world food security, especially as there still appear to be large exploitable gaps (>50%) in many situations, and as the way forward is clearer. There are few uncertainties regarding what needs to change at the farm level, but less optimism regarding how to achieve this quickly enough and from where the necessary resources will come. The biggest gaps are associated with small farmers in the developing world, and there is little doubt that strategies to help these farmers will have the greatest benefits for alleviating poverty and food insecurity. And note that the association between large yield gaps and small farmers is not an inevitable association, as can be seen among small holders in Egypt or parts of eastern China. This entry has emphasized that improvements in rural infrastructure and institutions are critical, but that breeding and agronomic research also has a role to play in gap closing, as is illustrated by the impact of simple direct seeding machinery and earlier sowing on wheat yields in the Indo-Gangetic Plains or of Bt cotton with Indian small holders. It is a great pity, however, that the types of on-farm adaptive research and development needed for gap closing in such situations are often thwarted by policy inertia toward agriculture in general, and poor rewards by science funders in particular, although the situation may be gradually improving. In developed countries, new paradigms seem to be emerging in which private sector companies, especially breeding and seed companies, invest not only in developing better varieties but also in applied agronomic research linked to variety development and promotion, and this may account for some of the gap closing in the USA and Western Europe. In addition, and especially in Australia and the southern cone of South America, privately hired agronomists are assuming a critical role in yield gap closing and are rewarded accordingly. Taken overall such changes auger well for ongoing yield gap closing, even in the short to medium term, in the developed world, but their impact at the farm level in developing countries, and that of more traditional approaches, will remain limited by infrastructure and institutional constraints in the near to medium-term future. The cautious optimism expressed a decade ago by leading agricultural scientist Evans [4] regarding world food security may well be overly optimistic.

Bibliography

1. Evans LT, Fischer RA (1999) Yield potential: its definition, measurement and significance. Crop Sci 34:1544–1551
2. Passioura JB, Angus JF (2010) Improving productivity of crops in water limited environments. Adv Agron 106:37–75
3. Badawi AT (1998) Sustainability of rice production in Egypt, IRC 98/7-2. Cairo, Egypt
4. Evans LT (1998) Feeding the ten billion: plants and population growth. Cambridge University Press, Cambridge

5. Fischer RA, Byerlee D, Edmeades GO (2009) Can technology deliver on the yield change to 2050? Paper prepared for expert meeting on "How to Feed the World in 2050", FAO, Rome,24–26 June 2009. (www.fao.org/wsfs/forum2050/wsfs-background-documents/wsfs-expert-papers/en/)
6. Mackay I, Horwell S, Garner J, White J, McKee J, Philpott H (2010) Re-analysis of the historical series of UK variety trials to quantify the contributions of genetic and environmental factors to trends and variability in yield over time. Theor Appl Genet 121 (in press)
7. Hammer GL, Dong Z, McLean G, Doherty A, Messina C, Schussler J, Zinselmeier C, Paszkiewicz S, Cooper M (2009) Can changes in canopy and/or root system architecture explain historical maize yield trends in the U.S. Corn Belt? Crop Sci 49:299–312
8. Edgerton MD (2009) Increasing crop productivity to meet global needs for feed, food and fuel. Plant Physiol 149:7–13
9. Fischer RA, Edmeades GO (2010) Breeding and cereal yield progress. Crop Sci 50:S-85–S-98
10. Bhatia VS, Singh P, Wani SP, Chauhan GS, Rao AVRK, Misra AK, Sirinuas K (2008) Analysis of potential yields and yield gaps of rainfed soybean in India using CROPGRO-Soybean model. Agric Forest Meteorol 148:1252–1265
11. Murty MVR, Singh P, Wani SP, Khairwal IS, Srinivas K (2007) Yield gap analysis of sorghum and pearl millet in India using simulation modeling. J SAT Agric Res 5:1–85
12. Lobell DB, Cassman KG, Field CB (2009) Crop yield gaps: their importance, magnitudes, and causes. Annu Rev Environ Resour 34:4.1–4.26
13. Titonell P, Shepherd KD, Vanlauwe B, Giller KE (2008) Unravelling the effects of soil and crop management on maize productivity in smallholder agricultural systems of western Kenya – an application of classification and regression tree analysis. Agric Ecosyst Environ 123:137–150
14. Byerlee D (1992) Technical change, productivity, and sustainability in irrigated cropping systems of South Asia: Emerging issues in the post-green revolution Era. J Int Dev 4:477–496
15. Marsh SP, Pannell DJ, Lindner RK (2000) The impact of agricultural extension on adoption and diffusion of lupins as a new crop in western Australia. Aust J Exp Agric 40:571–583
16. Llewellyn RS, D'Emden FH (2009) Adoption of no-till cropping practices in Australian grain growing regions. Report to GRDC, Canberra
17. Byerlee D, Moya P (1993) Impacts of international wheat breeding research in the developing world, 1966–90. D.F. CIMMYT, Mexico
18. Morris M, Kelly VA, Kopicki RJ, Byerlee D (2007) Fertilizer use in African agriculture, Lessons learned and good practice guide. The World Bank, Washington
19. de Wit CT (1992) Resource use efficiency in agriculture. Agric Syst 40:125–131
20. Fischer RA (2009) Farming systems in Australia: exploiting the synergy between genetic improvement and agronomy. In: Sadras V, Calderini D (eds) Crop physiology: applications for genetic improvement and agronomy. Elsevier, Amsterdam, pp 23–54
21. Paroda RS (2004) Scaling up: how to reach a billion resource-poor farmers in developing countries. Plenary paper, 4th international crop science congress, Brisbane. (www.cropscience.org.au/icsc2004/plenary/4/223_paroda.htm)
22. Dobermann A, Witt C, Dawe D, Abdulrachman S, Gines HC, Nagarajan R, Satawathananont S, Son TT, Wang GH, Chien NV, Thoa VTK, Phung CV, Stalin P, Muthukrishnan P, Ravi V, Babu M, Chatuporn S, Sookthongsa J, Sun Q, Fu R, Simbahan GC, Adviento MAA (2002) Site-specific nutrient management for intensive rice cropping in Asia. Field Crops Res 74:37–66
23. Dobermann A, Mackill D (2008) Investment returns opportunities in rainfed/irrigated environments of South Asia. Unpublished discussion paper, IRRI, The Philippines, p 7
24. Lobell DB, Asner GP, Ortiz-Monasterio JI, Benning TL (2003) Remote sensing of regional crop production in the Yaqui Valley, Mexico: estimates and uncertainties. Agric Ecosyst Env 94:205–220
25. Ortiz-Monasterio JI, Lobell DB (2007) Remote sensing assessment of yield losses due to sub-optimal planting dates and fallow period weed management. Field Crops Res 101:80–87
26. Lobell DB, Ortiz-Monasterio JI, Asner GP, Naylor RL, Falcon WP (2005) Combining field surveys, remote sensing, and regression trees to understand yield variations in an irrigated wheat landscape. Agron J 97:241–249
27. Grassini P, Hall AJ, Mercau J (2009) Benchmarking sunflower water productivity in semiarid environments. Field Crops Res 110:251–262
28. Hochman Z, Holzworth D, Hunt JR (2009) Potential to improve on-farm wheat yield and WUE in Australia. Crop Pasture Sci 60:708–716
29. Ekboir J (ed) (2002) CIMMYT 2000–2001 World wheat overview and outlook: developing no-till packages for small-scale farmers. D.F. CIMMYT, Mexico
30. Oerke EC (2006) Crop losses to pests. J Agric Sci 144:31–43
31. James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA brief no. 39. ISAAA, Ithaca
32. Luque SF, Cirilo AG, Otegui ME (2006) Genetic gains in grain yield and related physiological attributes in Argentine maize hybrids. Field Crops Res 95:383–397
33. Campos H, Cooper M, Habben JE, Edmeades GO, Schussler JR (2004) Improving drought tolerance in maize: a view from industry. Field Crops Res 90:19–34
34. Evans LT (1993) Crop evolution, adaptation and yield. Cambridge University Press, Melbourne
35. Evenson RE, Gollin D (eds) (2003) Crop variety improvement and its effect on productivity: the impact of international agricultural research. CAB International, Wallingford
36. Byerlee D, Dubin HJ (2010) Crop improvement in the CGIAR as a global success story of open access and international collaboration. Int J Common 4:452–480
37. Duvick DN (2005) The contribution of breeding to yield advances in maize (*Zea mays* L.). Adv Agron 86:83–145

38. Comai L, Young K, Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Henikoff S (2004) Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. Plant J 37:778–786
39. Bhullar NK, Street K, Mackay M, Yahiaoui N, Keller B (2009) Unlocking wheat genetic resources for the molecular identification of previously undescribed functional alleles at the Pm3 resistance locus. Proc Natl Acad Sci USA 106: 9519–9524
40. Phillips R (2010) Mobilizing science to break yield barriers. Crop Sci 50:S-99–S-108
41. Jennings PR (1964) Plant type as a rice breeding objective. Crop Sci 4:13–15
42. Tollenaar M, Lee EA (2006) Dissection of physiological processes underlying grain yield in maize by examining genetic improvement and heterosis. Maydica 51:399–408
43. Edmeades GO, Bolaños J, Elings A, Ribaut J-M, Bänziger M, Westgate ME (2000) The role and regulation of the anthesis-silking interval in maize. In: Westgate ME, Boote KJ (eds) Physiology and modeling kernel set in Maize. Crop Science Society of America special publication no. 29. CSSA, Madison, pp 43–73
44. Brennan JP, Condon AG, Van Ginkel M, Reynolds MP (2007) An economic assessment of the use of physiological selection for stomatal aperture-related traits in the CIMMYT wheat breeding programme. J Agric Sci 145:187–194
45. Babar B, Reynolds MP, Van Ginkel M, Klatt AR, Raun WR, Stone ML (2006) Spectral reflectance to estimate genetic variation for in-season biomass, leaf chlorophyll, and canopy temperature in wheat. Crop Sci 46:1046–1057
46. Richards RA (2004) Physiological traits used in the breeding of new cultivars for water-scarce environments. In: Proceedings of the 4th international crop science congress, Brisbane. (www.cropscience.org.au/ICSC2004/symposia/1/3/1470_richardsr.htm)
47. Ji X, Shiran B, Wan J, Lewis DC, Jenkins CLD, Condon AG, Richards RA, Dolferus R (2010) Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. Plant Cell Environ 33(6):926–942
48. Heffner EL, Sorrells ME, Jannick J-L (2009) Genomic selection for crop improvement. Crop Sci 49:1–12
49. Eathington SR, Crosbie TM, Edwards MD, Reiter RS, Bull JK (2007) Molecular markers in a commercial breeding program. Crop Sci 47(S3):S154–S163
50. Rebetzke GJ, van Herwaarden AF, Jenkins C, Weiss M, Lewis D, Ruuska S, Tabe L, Fettell NA, Richards RA (2008) Quantitative trait loci for water-soluble carbohydrates and association with agronomic traits in wheat. Aust J Agric Res 59:891–905
51. Reynold M, Tuberosa R (2008) Translational research impacting on crop productivity in drought-prone environments. Curr Opin Plant Biol 11:171–179
52. Chapman SC, Cooper M, Hammer GL (2002) Using crop simulation to generate genotype by environment interaction effects for sorghum in water-limited environments. Aust J Agric Res 53:379–389
53. Sinclair TR, Purcell LC (2005) Is a physiological perspective relevant in a "genocentric" age? J Exp Bot 56:2777–2782
54. Fischer RA (2008) Improvements in wheat yield: Farrer, physiology and functional genomics. Agric Sci NS 1/08:6–18
55. Meyer FD, Talbert LE, Martin JM, Lanning SP, Greene TW, Giroux MJ (2007) Field evaluation of transgenic wheat expressing a modified ADP-glucose pyrophosphorylase large subunit. Crop Sci 47:336–347
56. Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstron DC, Benson RJ, Castiglioni PP, Donnarummo MG, Hinchley BS, Kumimoto RW, Maszle DR, Canales RD, Krolikowski KA, Dotson SB, Gutterson N, Ratcliffe OJ, Heard JE (2007) Plant nuclear factor Y(NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci USA 104:16450–16455
57. Castiglioni P, Warner D, Bensen RJ, Anstrom DC, Harrison J, Stoecker M, Abad M, Kumar G, Salvador S, D'Ordine R, Navarro S, Back S, Fernandes M, Targolli J, Dasgupta S, Bonin C, Luethy M, Heard JE (2008) Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol 147:446–455
58. Spiertz JHJ, Struik PC, van Laar HH (eds) (2007) Scale and complexity in plant systems research: gene-plant-crop relations. Proc Frontis Workshop, Wageningen, The Netherlands, April 24–26, 2006. Springer-Verlag, New York
59. Zhu X-G, Long SP, Ort DR (2008) What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? Curr Opin Biotech 19:153–159
60. Sylvester-Bradley R, Foulkes J, Reynolds M (2005) Future wheat yields: evidence, theory and conjecture. In Sylvester-Bradley R, Wiseman J (eds) Proc 61st Easter School in Agricultural Sciences. Yield of farmed species. Nottingham University Press, pp 233–260
61. Hubert B, Rosegrant MW, van Boekel MAJS, Ortiz R (2010) The future of food: scenarios for 2050. Crop Sci 50:S33–S50
62. Tweeten L, Thompson SR (2008) Long-term agricultural output supply-demand balance and real farm and food prices. Working paper AEDE-WP 0044-08, Ohio State University

Cropping Systems: Shaping Nature

Rafael J. López-Bellido, Luis López-Bellido
Departamento de Ciencias y Recursos Agrícolas y Forestales, University of Córdoba, Córdoba, Spain

Article Outline

Glossary

Agriculture Agriculture and farming are often considered to be the same concept. However, both concepts can vary in their territorial scope of application. Agriculture is the production of food and goods through farming and forestry. Agriculture encompasses a wide variety of specialties and techniques. For this reason, its definition has developed to become: The science, art, and business of cultivating soil, producing crops, and raising livestock. The major agricultural products can be broadly grouped into foods, fibers, and raw materials. As of late, agriculture also uses plants to produce biofuels, biopharmaceuticals, and bioplastics.

Agricultural practices Agricultural practices are a set of techniques applied to on-farm production and postproduction processes, resulting in food and nonfood agricultural products.

Agronomic crops Agronomic crops typically involve a crop that is grown for grain, feed, or for processing into oil, starch, protein, or flour. Major agronomic crops include corn (grown for feed, ethanol, or processing), soybeans, wheat, hay (alfalfa and legume and grass mixtures), rice, peanuts, and cotton. Hay is also considered forage [1].

Agroecosystem An agricultural system or agricultural ecosystem is the basic unit of study for an agroecologist. This term is somewhat arbitrarily defined as a spatially and functionally coherent unit of agricultural activity, and includes the living and nonliving components involved in the unit as well as their interactions. An agroecosystem can be viewed as a subset of a conventional ecosystem. As the name implies, at the core of an agroecosystem lies the human activity of agriculture. However, an agroecosystem is not restricted to the immediate site of agricultural activity (e.g., the farm), but rather includes the region that is impacted by this activity, usually by changes to the complexity of species assemblages and energy flows, as well as to the net nutrient balance.

Agronomy The science which establishes the theory and practice of crop production and soil management [1].

Ecosystem A functioning community of nature that includes fauna and flora together with the chemical and physical environment with which they interact. Ecosystems vary greatly in size and characteristics, and can be a mud puddle, a field, or orchard, or a forest. An ecosystem provides a unit of biological study and can be a unit of management [2].

Environment The totality of the surrounding external conditions (biological, chemical, and physical) within which an organism, community, or object exists. The environment can be defined at any scale. The term is not exclusive in that organisms can be and usually are part of another organism's environment. Thus, one can speak of the environment as that within which humankind lives (i.e., separate and external) or, of humankind as a component of the environment [2].

Definition of the Subject

Cropping system (CS) is a general term that describes how a producer or farmer might grow a crop [2]. Pragmatically, CSs effectively address the *what to grow*, *when to grow it*, and *how to grow it* considerations of crop production in the context of optimizing multiple goals [3]. A CS must bring together the biological, technical, economic, and sociological aspects of

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

the land area farmed [4]. Therefore, a CS is a set of agronomic or agricultural practices used in a crop under specific conditions. The CS generates an agroecosystem, agri-environment, or agricultural system. Common synonyms of CS include crop management system, crop production system, farming system, crop production practices, etc. For example, a farming system is defined as a population of individual farm systems that have broadly similar resource bases, enterprise patterns, household livelihoods, and constraints, and for which similar development strategies and interventions would be appropriate [5].

The classification of CSs has been based on the following criteria: available natural resource base, including water, land, grazing areas, and forest; climate, of which altitude is one important determinant; landscape, including slope; farm size, tenure and organization; dominant pattern of farm activities and household livelihoods, including field crops, livestock, trees, aquaculture, hunting and gathering, processing and off-farm activities; and taking into account the main technologies used, which determine the intensity of production and integration of crops, livestock, and other activities [5]. Multiple cropping, in which several crops are grown sequentially in 1 year, and intercropping, when several crops are grown at the same time, are other kinds of annual CSs known as polycultures [6].

In general, each and every agronomic practice used in a CS affects different aspects of the system, i.e., the tillage system prepares the seedbed but affects the availability of water and nutrients, carbon cycle, erosion, diseases, etc. The use of the term CS is often applied to a rotation (wheat-fallow CS), tillage system (no-tillage CS), method of fertilization (organic CS), or agroecosystem (semiarid CSs) to simplify the designation of the CSs. However, it is important to note that a CS involves much more than a rotation, tillage system, fertilization method, or climatic conditions.

Introduction

Since the dawn of settled agriculture about 10 millennia ago, human beings have integrated different agronomic practices for application to selected plants (crops), and as a result generated CSs that transformed the natural ecosystem into agroecosystems. Agricultural practices such as genotype selection, crop rotation, natural fertilizers, irrigation, etc., were developed long ago, but have made great strides in the past century. The history of agriculture has played a major role in human history as agricultural progress has been a crucial factor in worldwide socioeconomic change. When human beings began to produce food beyond their needs, others activities began to take place. It is this agricultural surplus that made the development of civilization possible. A crucial time in the history of agricultural began with the discovery of America. A global exchange of previously local crops and livestock breeds occurred. Key crops involved in this exchange from the New World to the Old included the tomato, maize, potato, manioc, cocoa bean, and tobacco, with several varieties of wheat, spices, coffee, and sugar cane going from the Old World to the New. With the rapid rise of mechanization in the early twentieth century, particularly in the form of the tractor, farming tasks could be accomplished with a speed and on a scale previously impossible. Also during this period, the Haber–Bosch method for synthesizing ammonium nitrate represented a major breakthrough. Synthetic nitrogen, along with mined rock phosphate, pesticides, and mechanization, allowed crop yields to overcome previous constraints. Furthermore, global yield increases were experienced later in the twentieth century when high yield varieties of wheat, corn, and rice were introduced as a part of the Green Revolution. The Green Revolution exported technologies (including pesticides and synthetic nitrogen) from the developed world to the developing world. However, concerns have been raised over the sustainability of intensive agriculture. Intensive agriculture has become associated with decreased soil quality, and there has been increased concern over the effects of fertilizers and pesticides on the environment, particularly as the population increases and food demand expands.

As a result, in the past few decades, a move toward sustainability in agriculture has also developed, integrating ideas of socioeconomic justice and conservation of resources and the environment within an agricultural system [7, 8]. Sustainable agriculture is defined as practices that meet current and future societal needs for food and fiber, for ecosystem services, and for healthy lives, and that do so by maximizing the

net benefit to society when all costs and benefits of the practices are considered. If society is to maximize the net benefits of agriculture, there must be a fuller accounting of both the costs and the benefits of alternative agricultural practices, and such an accounting must become the basis of policy, ethics, and action. Additionally, the development of sustainable agriculture must accompany advances in the sustainability of energy use, manufacturing, transportation, and other economic sectors that also have significant environmental impacts [9]. Sustainable agriculture has renewed research in alternative technologies such as no-tillage, integrated pest management, etc. Recent mainstream technological developments include genetically modified (GM) crops. Also, agriculture has needed – and still needs – subsidies from many governments to ensure an adequate food supply and its competitivity in global markets. However, at times, these subsidies, especially when instituted by developed countries, have been noted as protectionist, inefficient, and environmentally damaging [10].

This chapter is not an overview of a subject as complex and broad as worldwide CSs, where oversights are inevitable. It must first be pointed out that about 85% of the Earth's cultivated land is planted with annual crops [11], and thus the principle CSs are used in herbaceous plants. Of these, wheat, rice, and maize provide more than 60% of human dietary calories, either as cereals for direct human consumption or embodied in livestock products produced from animals fed with feed grains and their by-products [9, 12]. Moreover, it is likely that these same cereal crops will continue to account for the bulk of future human food supply because they produce greater yields of human-edible food, are easily grown, stored, and transported, and require less fuel and labor for processing and cooking than other food crops [12]. Given their importance, this chapter focuses primarily on these crops, but also provides a general view that can be applied to any CS. The chapter starts off with a brief description of CS components and factors that must be taken into consideration during their design (*Components and Design*). To provide a new focus on defining CSs, and as a result agroecosystems, an anthropological view of a CS is then provided (*Anthropological View*). Following, the most important effects of these "human ecosystems" on the environment are reviewed (*Agricultural Practices' Footprint*). At this point, the chapter goes on to analyze the achievements of agricultural research and their importance to man (*Praise of Agricultural Science*). From here, agricultural practices that were unsuccessful in CSs and which must be urgently eliminated are studied (*Eradication of Fallow, Tillage, and Monoculture*). Afterward, the basic principles on which current and future CSs should be inspired are discussed. In fact, the application of these principles to a certain degree involves returning to techniques from the past, based on monitoring technologies of the future (*Biological Synergism and Technical Synchronization*). The application of these principles logically leads to what are called *Dynamic Cropping Systems*, which shift away from the application of a series of rigid agricultural practices to the agroecosystem at each growth stage. Finally, the chapter tackles the *Future Challenges* for CSs in the twenty-first century.

Components and Design

All agroecosystems generated by a CS break away from "natural" ecosystems, and the main effects are the loss of biodiversity and the export of resources. This causes environmental imbalance which means more or less of a struggle, depending on the intensity of the CS, to stabilize it. The more distant it is from a "natural" stable ecosystem, the more inputs are required by the CS as the result of a greater imbalance. In fact, one of the greatest concerns caused by agriculture, which has been a problem from the beginning due to the selection of few species, is the lack of biodiversity in many CSs. Advocates of diversification argue that it provides greater income stability [2].

Many CSs throughout the world are characterized by variable climate and soils, resulting in a high-risk condition for agricultural producers due to extreme variability in precipitation and seasonal temperatures [13]. In other words, crop production occurs in an environment that is always changing. With every growing season, producers must attend to numerous factors that influence their management decisions. Cropping systems vary among farms depending on the available resources and constraints; geography and climate of the farm; government policy; economic, social, and political pressures; and the philosophy and culture of the

farmer [5, 6]. This is a daunting challenge, especially when one considers that producers' decisions are carried out in a financial environment of diminishing economic returns, where one wrong decision could mean financial hardship and potentially the end to a way of life [14].

A CS involves numerous interactive factors that may limit or facilitate crop production. The factors can be divided into the following groups [14]: (1) biophysical environment: weather/climate and soil; (2) socioeconomic externalities: market conditions and government programs; and (3) available technology. These three factors affect or guide the agricultural practices of the CS (crop election, rotation, tillage system, planting date, fertilization, crop protection, etc.), and are conceived on the basis of productive and economic objectives, as well as the social and environmental objectives that must be imposed by sociopolitical externalities.

Most developed countries' agriculture operates in a market-driven economy, although government policies can have an influence on what farmers produce and how they produce it. As with other businesses, agricultural producers respond to economic incentives and disincentives, and make decisions to maximize their welfare; usually measured as net income. Specifically, the farmer must consider the influence of technological advancements, income supports embodied in farm legislation, and changes in market structure and consumer demand. Many of the technological advancements have required large-scale production units to justify the investment. The influence of commodity support programs has been ambiguous. As farm legislation has evolved to decouple production decisions from program benefits, the incentives to specialize in program crops (crops that receive price and/or income benefits under governmental legislation) have diminished. However, wealth and risk effects, albeit small, may have promoted or inhibited the adoption of a more sustainable system. Changes in market structure, channels, and consumer demand in the past five decades have been dramatic with consolidation and specialization in both production and marketing sectors. However, the diversity of consumer demand has also created opportunities for more integrated farm operations. There is an increasing number of consumers who have become concerned about how and where their food has been produced (food security). While price and income supports may have been biased toward specialization (as these programs were targeted to specific commodities), the reduction in risk associated with the programs has enabled producers to expand the number and diversity of their production enterprises [15].

The rapid change in the agricultural industry driven by continuously arising challenges (climate change, market globalization, environmental concerns) requires the development of new methods of production in order to guarantee sustainable agriculture [16]. The design of a CS undergoes a study of the biophysical environment and socioeconomic externalities, and an inventory of available technologies. The first step in developing a CS is the definition of goals and constraints for the new CS. Constraints may result from soil and climate but also from environmental or economic concerns [17]. Afterward, a crop portfolio must be established, usually based on climate, and containing a diverse array of adaptable crop species, economic potential, crop production practices, and soil and water management considerations. The crop portfolio is used to screen adaptable crops for a region and includes the best management practices for the production of each adaptable crop [14]. In others words, it is the selection of crops compatible with the set of constraints. Next is the comparison and choice of the most satisfying crop and management options [16]. Designing new CSs is a long process and occurs in a rapidly changing environment. This is exemplified by climate but also by the economy (prices and policies) and the changing demands and functions that society assigns to agricultural systems. The design process must therefore integrate objectives of the resilience and flexibility of CSs in an unpredictable environment [18]. Obviously, the use of models for the design of CSs can be an important tool for the farmer, although there are some drawbacks [16]. Geographic information systems, qualitative decision-matrix analyses, a simple rule-based model using multi-criteria evaluations, and a machine learning-based land-transformation model can be used harmoniously to study complex socio-ecological systems. Models evaluate how each technique performs in the study of complex socio-ecological systems using a multi-tier framework, detailing how each method analyzes the resource system,

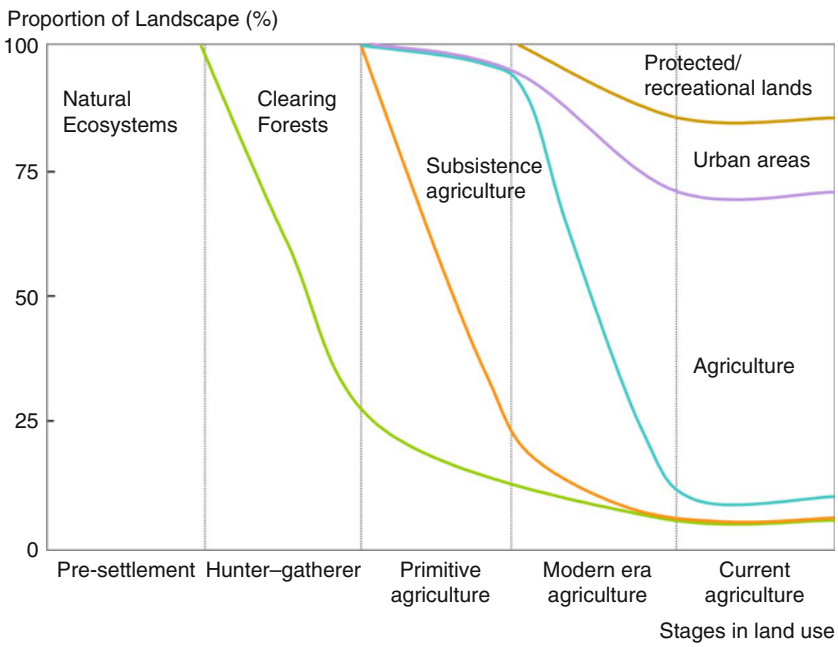
resource units, governance system, users and interactions, and outcomes in the system. Model use enhances our understanding of the land-use decision-making process [19].

Anthropological View

Beginning around 8,500 BC, the transition from the hunter–gatherer lifestyle to food production enabled people to settle down next to their permanent agricultural lands, instead of migrating to follow seasonal shifts in wild food supplies. Food production was accompanied by a human population explosion that has continued unabated to this day, resulting from two separate factors. First, the sedentary lifestyle permitted shorter birth intervals. Nomadic hunter–gatherers had previously spaced out birth intervals at 4 years or more because a mother shifting camp can carry only one infant or slow toddler. Second, plant and animal species that are edible to humans can be cultivated in much higher density in our agricultural land than in wild habitats [20].

Agriculture also led to an explosion of technology because sedentary living permitted the accumulation of heavy technology such as forges that nomadic hunter–gatherers could not carry and because the storable food surpluses resulting from agriculture could be used to feed full-time craftspeople and inventors. By also feeding full-time kings, bureaucrats, nobles, and soldiers, those food surpluses led to social stratification, political centralization, and standing armies. All of these overwhelming advantages are what enabled farmers to eventually displace hunter–gatherers [21].

Like all species, humans have exercised their impulse to perpetuate and propagate themselves. In doing so, humankind has "domesticated" ecosystems in ways that enhance its food supplies, reduce exposure to predators and natural dangers, and promote commerce. On average, the net benefits to humankind of "domesticated" nature have been positive. We have, of course, made mistakes, causing unforeseen changes in ecosystem attributes, while leaving few, if any, truly wild places on Earth [22]. Domestication of plants and animals may be the single most important feature of the human domination of our planet (Fig. 1). Domestication involves the selection of traits that fundamentally alter wild species to become more useful to us. Conservation has often been framed as the science



Cropping Systems: Shaping Nature. Figure 1

Stages in "domestication" of nature by human being in relation with the area occupied (Adapted from [23])

aimed at protecting nature, and especially protecting nature from people. However, there really is no such thing as nature untainted by people [24]. Facing this reality should change the scientific focus of environmental science. Instead of recounting doom-and-gloom statistics, it would be more fruitful to consider the domestication of nature as the selection of certain desirable ecosystem attributes, such as increased food production, with consequent alteration to other ecosystem attributes that may not be desirable. Under this paradigm, our challenge is to understand and thoughtfully manage the trade-offs among ecosystem services that result from the inescapable domestication of nature [22].

It is clear that cities are the main consumers of most ecosystem services. This is important because the desire and value for these services determines the traits that humans select for preservation or elimination. For example, if humans want to maximize food production, landscapes will be domesticated to accommodate a few high productivity species, plus the human-associated species able to survive in these modified landscapes. If people want more wildlife for recreational hunting, populations of predators of game species will be reduced, and the edge habitat that a few game species prefer will be increased. The choices and actions of urban dwellers influence nature far removed from cities, yet urban dwellers are increasingly unaware of these impacts [22].

Agricultural Practices' Footprint

The huge magnitude of human impacts in the environment is recent, but the presence of impacts such as purposeful wildfires goes back thousands of years. The reality of the human footprint renders discussions about what areas of the world to set aside as wild and protected areas as somewhat irrelevant; more germane is a discussion of what trade-offs we are willing to accept as a result of the domestication of nature [22].

The main environmental impacts attributed to agriculture come from the conversion of "natural" ecosystems to agroecosystems by CSs [9]. Clearing land for agriculture, humans target wild species for harvest or elimination. Humans have so tamed nature that few locations in the world remain without human influence [22] (Fig. 1). The whole notion of a "virgin rainforest" may be erroneous, with extensive prehistoric human activity evident in what were once thought to be untouched forests in the Amazon and Congo [25]. Global maps of human impact indicate that, as of 1995, only 17% of the world's land area had escaped direct influence by humans [22]. Between 1700 and 1980, the total area of cultivated land worldwide increased 466%, and yields increased dramatically, particularly because of selectively bred, high-yielding varieties, fertilizers, pesticides, irrigation, and machinery. For example, irrigation increased corn yields in eastern Colorado by 400–500% from 1940 to 1997 [9]. Roughly 50% of the world's surface area has been converted to grazed land or cultivated crops [26]. Cropping systems (areas where at least 30% of the landscape is in croplands, confined livestock production, or freshwater aquaculture) now cover a quarter of the Earth's surface, partly by conversion of temperate grasslands, Mediterranean climate forests, and many tropical ecosystem types. More than half of the world's forests have been lost in this land conversion [26]. Forests have essentially disappeared from 25 countries, with 9.4 million hectares lost annually from the Earth's surface [27]. Species and populations of species are being lost at unprecedented rates, while at the same time, the global biota is becoming homogenized, owing to the introductions of alien species to new regions. These examples represent major losses of pieces of the biosphere machinery, which have a serious impact on the delivery of ecosystem-regulating services – impacts such as greater prevalence of infectious diseases in disrupted ecosystems, adverse effects on local climates by ecosystem modification, and the loss of flood protection [27].

Environmental damages have come from agricultural nutrients that pollute aquatic and terrestrial habitats and groundwater, and from pesticides, especially bioaccumulating or persistent organic agricultural pollutants. Agricultural nutrients enter other ecosystems through leaching, volatilization, and the waste streams of livestock and humans. Pesticides can also harm human health, as can pathogens, including antibiotic-resistant pathogens associated with certain animal production practices [9]. Many CSs have degraded soil quality and necessitated the expense of increased fertilization, irrigation, and energy to maintain productivity [28]. Today, only 30–50% of applied nitrogen

fertilizer [29] and ~45% of phosphorus fertilizer [30] is taken up by crops. A significant amount of the applied nitrogen and a smaller portion of the applied phosphorus are lost from agricultural fields. Nitrogen fertilization can increase the emission of gases that have critical roles in tropospheric and stratospheric chemistry and air pollution. Nitrogen oxides (NO_x) emitted from agricultural soils and through combustion increase tropospheric ozone, a component of smog that impacts human health, agricultural crops, and natural ecosystems. NO_x from agroecosystems can be transported atmospherically over long distances and deposited in terrestrial and aquatic ecosystems. Finally, nitrogen inputs to agricultural systems contribute to emissions of the greenhouse-gas nitrous oxide. Rice paddy agriculture and livestock production are the most important anthropogenic sources of the greenhouse-gas methane [31]. Much of agriculture in the developed world had for a time embraced potentially non-sustainable systems for economic reasons, over-utilizing monocropping, specialization and mechanization, which was damaging to soils and the environment [32]. Modern agroecosystems are also depleted in biodiversity and habitat heterogeneity, often with a reduction in resilience as a result of their biological monotony [22].

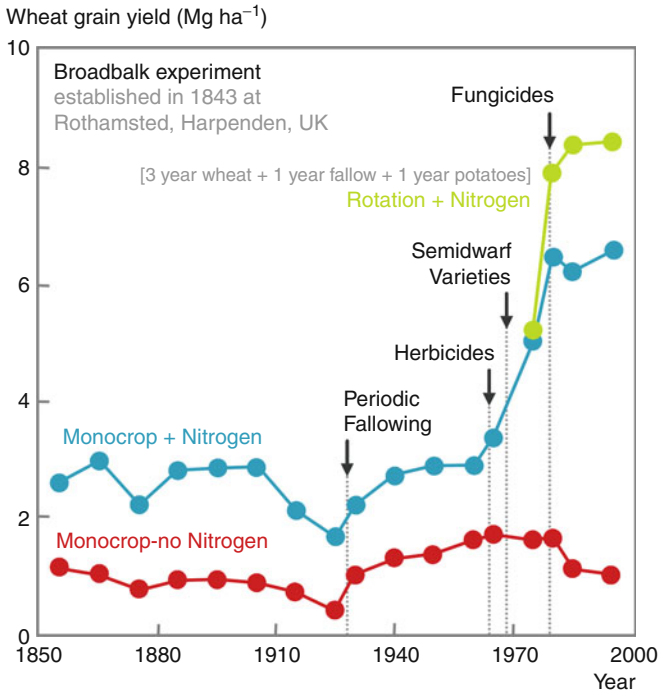
Cities are a good place to start when considering the broader implications of agroecosystems. The cumulative resource demands of cities are often expressed as the total land area required to supply those resources, called the "ecological footprint" [33]. Every city imports resources and exports waste into a region that is spatially much larger than the city's area. However, there is substantial variation in per capita ecological footprints between rich and poor regions, with the average resident of the United States using 6 times the area of the average sub-Saharan African [34].

Praise of Agricultural Science

To a conservationist interested mainly in biodiversity, we have degraded nature, but to an agronomist, we have shaped the nature to make it better serve humans [22]. It is paradoxical that current urban dwellers look down upon and lack an appreciation of agriculture when it is the key to our existence and to obtaining the energy needed for their daily activities. There is no question that humans have been successful in their efforts to produce food, thereby enhancing their well-being [22]. Contrary to Malthus's predictions, food production has kept up with, and even outpaced, human population growth [35]. Malthus did not take into account that "there is no gene for the human spirit" [36]. The massive increase in food supply has been achieved by focusing efforts on planting and consuming a small variety of plants [22]. As of 1999, barley, maize, rice, and wheat occupied almost 40% of global cropland [37].

Nowadays, few scientists think of agriculture as the chief, or model science. Many, indeed, do not consider it a science at all. Yet it was the first science – the mother of all sciences; it remains the science that makes human life possible; and it may well be that, before the century is over, the success or failure of Science as a whole will be judged by the success or failure of agriculture [38]. For too many years, the agricultural sciences have been disparaged in the science and education communities, perhaps because agronomy, soil science, plant pathology, and animal science use a problem-solving approach rather than simply seeking knowledge [39]. But as we move into a new era of shared accountability and responsibility, let's keep in mind that agricultural sciences affect us all, and when agricultural science is thriving, our communities likely are thriving too [39]. Agriculture is not seen as a source of solutions to many of the world's most pressing challenges. When science research funds are handed out, agriculture often gets left off the list. It is possible to suspect this because policy-makers and some scientists see "agriculture" as synonymous with "agribusiness" rather than as a purely scientific discipline, and they assume private funding will take care of agriculture-related research needs [39].

The benefits of agriculture have been immense. Before the dawn of agriculture, the hunter–gatherer lifestyle supported about four million people globally [40]. Modern agriculture now feeds 6,000 million people. Global cereal production has doubled in the past 40 years, mainly from the increased yields resulting from greater inputs of fertilizer, water and pesticides, new crop strains, and other technologies of the "Green Revolution" [41] (Fig. 2). This has increased the global per capita food supply, reducing hunger, improving nutrition (and thus the ability of people to



Cropping Systems: Shaping Nature. Figure 2
Changes with time in winter wheat yields from 1850 to 2000 with explanations for the trends due to new agricultural practice technologies introduced. Treatments show how important is nitrogen fertilization and crop rotation (Adapted from [42])

better reach their mental and physical potential), and sparing natural ecosystems from conversion to agriculture [43].

Agriculture is the science that has had and continues to have the greatest impact on humanity. The advancements are the result of countless researchers unknown to the general public and who deserve proper tribute and acknowledgement. We should be eternally grateful to people, many of whom are consigned to oblivion, such as Justus von Liebig, (1803–1873), who is known as the "father of the fertilizer industry," for his discovery of nitrogen as an essential plant nutrient and his formulation of the Law of the Minimum which described the effect of individual nutrients on crops; Sir John Bennet Lawes, (1814–1900) who experimented with crops and manures at his farm at Harpenden, nowadays known as Rothamsted, and established the first long-term field experiment in the world, the Broadbalk (1843); Daniel Albone, who invented, in 1902, the world's first successful light farm tractor; Fritz Haber and Carl Bosch, whose work led to the synthesis of the first N fertilizer in 1913, seeing that without the use of synthetic fertilizers, world food production could not have increased at the rate it did and more natural ecosystems would have been converted to agriculture [9]; Sir Ronald Aylmer Fisher, who, working in the long-term experiments at Rothamsted in 1925, established the basis of the modern statistics that so greatly benefited agricultural research and all other sciences; Erling Johnson, who in 1927 developed an industrial method for producing nitrophosphate; Paul Hermann Müller, who discovered that DDT was a very effective insecticide in 1939, in spite of the fact that it was later discovered to be hazardous for many living beings, the good it accomplished for humanity is immeasurable as it controlled mosquitoes spreading malaria and lice transmitting typhus; Judah Hirsch Quastel, who working at Rothamsted, developed the first herbicide (2,4-D) in 1946; Norman Ernest Borlaug, father of the "green

revolution," led the introduction of high-yielding varieties, doubling wheat grain yield in many countries during the 1960s (Fig. 2); John E. Franz, who discovered Glyphosate in 1970, a broad-spectrum systemic herbicide; Marc Van Montagu and Jeff Schell discovered the gene transfer mechanism, developing the first transgenic plant in the 1980s; in addition to a long list of anonymous researchers who contributed their part to providing humanity with the food needed through techniques which have become increasingly more respectful of the environment and safer for consumption.

Eradication of Fallow, Tillage, and Monoculture

There are three agricultural practices that must tend to disappear from present and future CSs because it has been proven, in general, that they are more harmful than beneficial. Two of these, tillage and fallow, have been used since the beginning of agriculture, while the third, monoculture, emerged during the last century as a consequence of the incorporation of other techniques that made it apparently viable in the context of a great demand for food. These practices must be considered errors of the past that the present has allowed us to discover.

Soil is not naturally intended to be in a state of bare fallow. CSs must be inspired by or consider nature in their design. Soil is meant to be covered by vegetation as can be observed in most terrestrial ecosystems; therefore, when man included bare fallow in its group of agricultural practices, he committed a big mistake. Even in the conditions employed to allow low-fertility soils to rest, the most ideal option would be a change in crop, or if necessary, another option would be to not remove the crop. Although it was already known at the end of the last century that bare fallow was not useful, there have been policies, as is the case of the EU, which encouraged this practice as a result of market forces. Efforts to stabilize production of cereal crops led to the adoption of wheat–fallow CSs. This system, while popular with producers because it required limited equipment and managerial skills, has proven to be agronomically inefficient and environmentally unsustainable as shown through poor precipitation-use efficiency and decreased soil quality [44, 45]. In fact, at least 60% of the precipitation received during fallow is lost to evaporation [46]. Recognition of the drawbacks of wheat–fallow as well as advances in weed and residue management technology led to a reduction in the frequency of fallow [13]. Fallow time must be limited to those periods in which no water is available. Leaving a soil without residue shifts away from natural processes since in naturally dry ecosystems, the dry pasture leaves its residue protecting the soil against aggressive climatic events. All of the suggestions for intensifying cropping under dry-land conditions are contingent on maximum water capture and minimum losses to weeds, runoff, and evaporation. Ideally, crops should be synchronized so that there is always one crop in the field available to intercept possible rainfall [44]. Increased emphasis on crop diversity within annual CSs has allowed producers to take advantage of positive agronomic benefits derived from crop rotations [14]. Realizing these benefits requires knowledge of soil-water depletion and recharge characteristics for individual crops. Such knowledge is especially critical for areas where soil-water status can vary greatly between growing seasons [47].

Tillage, as is, is also not observed in nature. It was man, with the purpose of domesticating nature, who introduced it. No-tillage was used since ancient times by indigenous cultures. This was because tillage to any depth required more energy and power than was generally possible with hand labor. The ancient Egyptians and the Incas in the Andes of South America used a stick to make a hole in the ground and put seeds by hand into unprepared soil [48]. For thousands of years, agriculture and tillage were considered synonymous. It was simply not thought possible to grow crops without first tilling the soil before planting and for weed control [49]. Intensive tillage and use of heavy machinery have accelerated soil erosion, soil-C loss and nutrient depletion, soil compaction, acidification, pollution, and salinization [50]. The advent of modern herbicides permitted no-tillage to be developed and practiced on actual working family farms. No-tillage is generally defined as planting crops in unprepared soil with at least 30% mulch cover [49]. Adoption of no-tillage after its successful demonstration in the 1950s was slow. Today, approximately 23% of the total cropland in the United States is planted using no-tillage [49]. No-tillage has revolutionized agricultural systems because it allows individual producers to manage greater amounts of land with reduced energy, labor,

and machinery inputs. Maintenance of surface residue cover is essential to optimize CS performance as residue coverage minimizes erosion, enhances retention of limited precipitation, and improves soil quality [51]. Moreover, no-tillage substantially improves soil carbon, an area that is currently of great importance [45]. Crop residues, including the presence of crop stubble, can alter the soil environment in a number of ways by acting as a physical barrier, exudate of chemical suppressants (allelochemicals) from the residue, or enhancer of biological activity, and by providing a habitat for weed seed predators. These factors, as well as the buffering effect of crop residues on soil moisture and soil temperature and impacts on light availability, have a significant impact on weed-seed germination and emergence [52]. Lastly, the adoption of no-tillage has an economic benefit for farmers, reducing crop costs [53].

Monoculture, the lack of biodiversity, was a contributing factor to several agricultural disasters in history. Monoculture causes a loss of biodiversity in the "ecosystem" soil as a result of the monotony, leading to a yield loss with respect to a crop in rotation [54]. Practices that change species' composition or reduce biodiversity in agricultural systems may also diminish goods and services because the ability of ecosystems to provide some services depends both on the number and type of species in an ecosystem [9]. The supply of agricultural products and ecosystem services are both essential to human existence and quality of life. However, recent agricultural practices that have greatly increased global food supply have had inadvertent, detrimental impacts on the environment and on ecosystem services, highlighting the need for more sustainable agricultural methods [9]. Wheat, rice, and maize crops have become the three most abundant plants on Earth. A central conclusion of epidemiology is that both the number of diseases and disease incidence should increase proportionally to host abundance, and this disconcerting possibility illustrates the potential instability of a global strategy of food production in which just three crops account for so high a proportion of production [9]. The relative scarcity of outbreaks of diseases on these crops is a testament to plant breeding and cultivation practices. For all three cereals, breeders have been successful at improving resistances to abiotic stresses, pathogens, and diseases, and at deploying these defenses in space and time so as to maintain yield stability despite low crop diversity in continuous cereal systems [9]. However, it is unclear if such conventional breeding approaches can work indefinitely. Both integrated pest management and biotechnology that identifies durable resistance through multiple gene sources should play increasingly important roles [55]. Nonetheless, the evolutionary interactions among crops and their pathogens mean that any improvement in crop resistance to a pathogen is likely to be transitory [9]. Although the "Green Revolution" significantly increased rice yields in Asia, yield increases have not occurred in the past 15–20 years. The genetic "yield potential" has increased for wheat, but the yield potential for rice has not increased since 1966, and the yield potential for maize has "barely increased in 35 years." It takes a decade or two for herbicide-resistant weeds to emerge, and insects become resistant to insecticides within about a decade. Within about one or two decades of the introduction of each of seven major herbicides, herbicide-resistant weeds were observed. Crop rotation helps to prevent resistances [9].

Biological Synergism and Technical Synchronization

Agriculture should fulfill the economic, environmental, and social objectives of sustainable development. It is thus necessary to tailor current CSs to meet these needs as they are often too intensive and dependent on external inputs [56]. It is clear that the more we imitate nature or encourage natural processes, the greater the benefits for the CSs and fewer inputs that will be required. Two basic concepts should illustrate the design and management of CSs of the future: biological synergism and technical synchronization.

Biological Synergism

Biological synergic CSs should synchronize edaphoclimatic resources with the natural rhythms of crops, this being a question of common sense that has not always been applied, and clearly speaks of the diversity which has been lost with intensive systems. Therefore, the increase in biodiversity in CSs is a clear consequence of the search for biological synergism in addition to the promotion of natural processes. Biodiversity is essential to ecosystem processes in ways that

are not yet fully understood [57], and it is considered worth protecting in its own right. Crop diversification by itself, however, is of limited use without knowledge of how individual crops affect each other in a sequence. Consequently, a thorough understanding of crop sequencing effects – both positive and negative – on agronomic parameters is necessary to optimize CS performance [13, 46]. One problem associated with synergic CSs is how to choose and sequence crops to develop the inherent internal resources of the system while taking advantage of external resources such as weather, markets, government programs, and new technology [14]. Present CSs rely on extensive use of fertilizer and pesticides and the low cost of fossil fuel energy [46]. Future challenges for CSs will exploit synergism through crop sequencing to improve crop yields without additional inputs and to reduce deterioration of the environment [58].

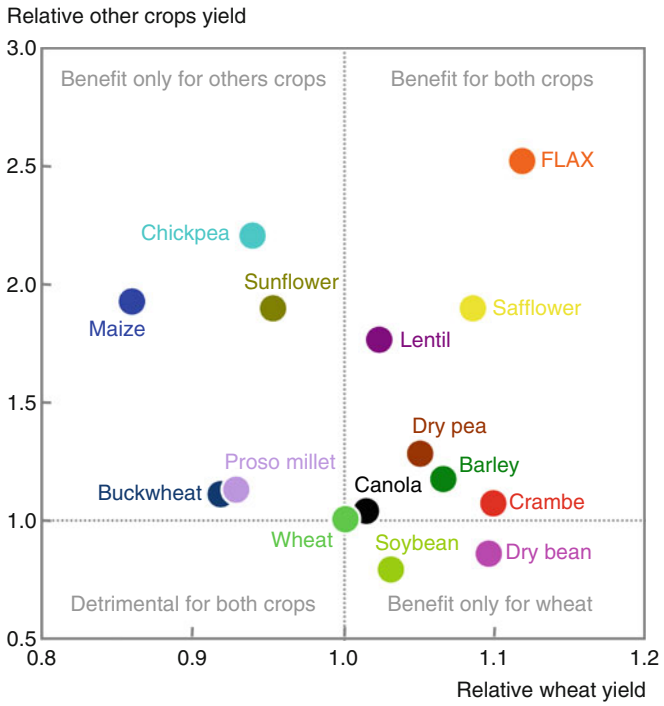
Diversification of farming systems cannot be managed on a farm or field scale alone but also requires management on a regional scale. To improve biodiversity, the interaction with other stakeholders in the region is necessary as part of regional planning processes. This has consequences for agronomic research which thus far has had only limited attention for the regional scale. Spatial planning aimed at multifunctional agriculture can be seen as a negotiation process on environmental, social, and economic aspects of land use. Complexity arises due to the high number of stakeholders and due to limited knowledge, which is often organized along disciplinary divides [59].

Crop rotation is defined as the growing of different crops (spatial and temporal), in recurring succession, on the same land in contrast to monoculture cropping. Rotation usually is done to replenish soil fertility, create a diverse soil organism, and reduce pest populations in order to increase the potential for high levels of production in future years [2, 46]. Each crop or a closely related crop species should not be grown more than every 4 years because of increased pest problems [60, 61]. One of the most effective and inexpensive methods to control plant diseases in CSs is through crop rotation. Through the use of this practice, the need to breed for new disease resistance and to discover new pesticides can be reduced. Recently, an important and costly pathogen of rice was controlled in a large region of China by planting alternating rows of two rice varieties [62]. This tactic increased profitability and reduced the use of a potent pesticide. Specific crop-rotation effects on plant diseases, however, are poorly understood and contradictory results have been reported [13].

Changes in the weed flora of agroecosystems can occur as long-term changes or temporary fluctuations in species composition [52]. Agricultural weeds are a unique group of plant species because of their ability to infest and thrive in intensively disturbed habitats despite extensive efforts to eliminate them. Weed floras differ between fields, farms, regions, climatic zones, and CSs. Weeds are successful because they are generally plastic plants that adapt to and survive changes in the environment. In continuous CSs, changes in the environment may be as a result of differences in crop species, tillage, fertilizers, herbicides, and other weed-control tactics. Long-term population shifts are usually observed after repeated use of a control measure, which exerts a high selection pressure on a population, for example, the increased incidence of some annual grass weed species due to the intensification of cereal production and selective herbicides [52]. Studies have shown that the more diverse the CS, the more diverse the weed community, with less dominant and troublesome species as would occur in a simple rotation.

With diversified continuous cropping, there is greater opportunity to utilize crops that vary in N requirements [63]. Rotations with legumes are of great interest for strengthening the synergism of a CS. Legume crops are often planted to enhance nutrient cycling and availability to other crops in the rotation. Crops with high N demands are usually grown after legume crops. Grain legumes conserve soil N by fixing atmospheric N, thereby leaving residual N in place for the next crop. Wheat yields are often higher following legumes than following other cereals [54] and the N use efficiency of a wheat crop is greater following legumes than others crops [64]. The inclusion of a legume crop decreases its reliance on external fertilizer, supplying a significant proportion of the N for the next crop and may moderate nitrate levels in the soil to avoid the potential for nitrate leaching [65] (Fig. 3).

Alternatives such as plurispecific CSs are of considerable interest for biological synergism because plant associations can provide environmental benefits in terms of better use of resources and provision of



Cropping Systems: Shaping Nature. Figure 3
Effect of 14 previous crops on relative wheat yield and the effect of wheat, as a previous crop, on those crops in the Great Plains (Adapted from [46, 66])

ecological services [67]. Yet they should also be able to maintain a level of productivity and production stability that fits with farmers' objectives. Intercrops (grass cover in the inter-rows) are now being introduced to an increasing extent due to the potential positive impacts on perennial crop and their environment: increased infiltration rate and decreased runoff due to modified soil surface characteristics [68], mitigation of soil erosion [69], limitation of herbicide use and weed control, better water-resource utilization by roots [70], and limitation of risks of diseases by reducing vegetative development [71]. However, intercropping also induces competition for soil resources, and vegetative development and yield can consequently be limited [56]. These different functions should be promoted through simultaneous adapted management of the two crops of the system.

Finally, an important link of biological synergism is the return provided by livestock to the CSs. There was always a crop–livestock pairing in ancient agriculture, but specialization caused this very important aspect of biodiversity and synergism to disappear. Research is needed to better understand interactions between crops and livestock with the intention of identifying management practices that maximize agroecosystem productivity and operational efficiency. This would save having to remove residues, part of which would be used directly by the livestock, partially allowing the recycling of nutrients. Inclusion of livestock in a CS complements both crops and livestock by adding value to grain, improving nutrient-use efficiency, and providing alternative uses for forages and crop residues [72]. It may create synergisms between the two enterprises, resulting in far greater productivity than either enterprise could attain alone [13, 73]. The inclusion of livestock in CSs would also help alleviate the problems generated by livestock: high-density animal production operations can increase livestock disease incidence, the emergence of new, often antibiotic-resistant diseases, and air, groundwater, and surface water pollution associated with animal wastes. Current livestock operations are vulnerable to catastrophic loss

of animals to disease. The handling and disposal of animal wastes are significant problems of high-density animal confinement facilities. Manure lagoons can release high levels of hydrogen sulfide and other toxic gases, volatilize ammonium that greatly increases regional nitrogen deposition, and contaminate surface and ground waters with nutrients, toxins, and pathogens. These animal wastes pose health and environmental risks similar to those of human wastes and should be treated accordingly. For example, animal wastes could be treated by composting to create a crop fertilizer that no longer harbors pathogens, and that is applied at appropriate rates and times and with methods that minimize nutrient leaching. This closing of the nutrient cycle decreases dependence on synthetic fertilizer production, and is more efficient when animal and crop production are combined locally [9].

Technical Synchronization

Technical synchronization must be defined as the concept on which precision agriculture is based, i.e., the temporal and spatial synchronization of agricultural techniques. All agricultural practices must be synchronized in time with the rhythms of the agroecosystem (time-specific management). This synchronization must take into account the growth stage of the crop, different soil characteristics (nutrients and water), weather forecasts, etc. On the other hand, there is the spatial synchronization that divides the land into homogeneous units for management (site-specific management) [63]. A basic principal of technical synchronization is the energetic efficiency when making decisions in relation to the agricultural practices to be carried out.

The key to the application of technical synchronization in a CS is the acquisition of data in order to make management decisions in time and space. There are an increasing number of data acquisition devices available to assist with decision making: global positioning (GPS), satellites or aerial images, information management tools (GIS), weather stations, soil sensors (water content, temperature, etc.), optical sensors (e.g., chlorophyll meters), and remote sensors (visible and near infrared spectral), etc. These tools allow the farmer to have a better understanding of the interaction of climate and management that causes tremendous year-to-year variation in on-farm yields and crop requirements.

Nonetheless, technical synchronization poses a problem when applied by the farmer. There is a large body of published research on technologies for increasing the technical synchronization of agricultural practices, but relatively few have been adopted by farmers because they are not cost-effective or practical. Adoption of improved technologies typically requires additional skills and labor or investments in new equipment. Information on expected costs and economic returns from such investments is required to convince farmers of the benefits from adoption [12]. A possible solution is cooperativism for the acquisition and sharing of these new technologies.

Dynamic Cropping Systems

It could be said that, until now, CSs have been static, i.e., year after year the same group of agricultural practices have been repeated, causing soil monotony in the agroecosystem. A dynamic CS must be flexible and go against repeated management practices, and the decisions must be made based on measured parameters and their determining factors. A dynamic CS is only the consequence of the application of the two previously stated principles: biological synergism and technical synchronization. Cropping systems need to be inherently flexible to take advantage of economic opportunities and/or adapt to environmental realities [13, 18]. The implementation of a management strategy requires knowing, understanding, planning, measuring, monitoring, and record keeping at each step of the production process. Dynamic CSs require the development and implementation of agricultural production systems that are highly productive, energy efficient, and environmentally non-degrading [12].

Dynamic CSs are a form of agricultural production that relies on an annual or pluri-annual strategy to optimize the outcome of (1) production, (2) economic, and (3) resource-conservation goals using ecologically based management principles [74]. Implicit to this strategy is the need for producers to possess information necessary to respond to continual change. The key factors associated with dynamic CSs are diversity, adaptability, reduced input cost, multiple enterprise

systems, and awareness of environment and information [14]. Greater crop diversity and sequencing flexibility within dynamic CSs may result in reduced weed, pest and disease infestations, greater nutrient- and precipitation-use efficiency, decreased requirements of exogenous inputs, and lower production risk [74]. To remain competitive in agricultural markets, increased diversity of systems will be required to minimize inputs and improve economic margins.

To increase responsiveness to externalities, opportunity/flex CS [75] or dynamic CS [14] concepts have been developed. These systems allow producers to adjust CS intensity and/or diversity based on externalities as well as management goals, such as soil-water status at planting, soil residual nitrogen, market demands, etc. This approach to crop sequencing possesses an inherent flexibility to adapt to high-risk conditions, and therefore may be more economically and environmentally sustainable than other approaches to crop sequencing [13]. However, the authors who established the principles of dynamic CSs have focused solely on rotation selection, which is erroneous, since a CS is more than its rotation selection, although the crop selection is without a doubt the most important decision made in a CS. Perhaps the concepts should be called dynamic or flexible crop election system. However, a dynamic CS makes more sense if each year all of the crop techniques used are reconsidered, i.e., crop selection, tillage, residue management, nitrogenous fertilization, etc. in such a way that there are dynamics in the selection of each and every one of the agricultural practices that depend on externalities.

Future Challenges

Agricultural science is ripe for a renaissance [39] as a consequence of the future challenges it must confront. The widespread adoption of CSs that are sustainable and environmentally benign is essential for the long-term survival of civilization [32]. Furthermore, through the use of strategic alliances, cooperation among producers on a regional basis may eventually lead to greater integration and diversification than could be achieved for the individual farm operation [15]. The primary challenges facing CSs in this century are: covering the demands of an increasing world population; water shortage; the inclusion and management of GM crops; harmonization of the production of biofuels and food production; and climate change, and the focus it brings to agricultural research. Obviously, there are many other challenges which have not been presented here, but which are intimately related to those mentioned such as: the biodiversity of CSs – as already previously mentioned – which should redound to a reduction of inputs and, therefore, a reduction of the negative effects on the environment, strategies to manage anthropogenic carbon emissions from terrestrial systems as well as fossil fuel and industrial sources, etc.

Nevertheless, together with these future challenges are a series of forces that may contribute or hinder their fulfillment: research policies, transference, and financing. In view of these challenges, governmental policies play an important role that may help or hurt the achieving of these objectives. For this reason, policymakers have a great responsibility, and their decisions should be guided by agricultural science professionals. Political intervention has an economic facet that arises as the result of the following question: How can society accomplish the dual objectives of improving food production and stability and of preserving the quality and quantity of ecosystem services provided by the Earth's land and water resources? Clearly, appropriate incentives are needed [9]. What incentives and policies could lead to the adoption of sustainable agricultural practices? Several policy initiatives have tried to level the playing field between agricultural production and the production of ecosystem services.

Another problem in the attempt to fulfill these objectives is the transference and application of new technologies in CSs. The earlier paradigm of science being developed at the international or perhaps national level and then disseminated to farmers should be replaced by an active exchange of information among scientists and farmers. Scientists in developing countries who understand the ecosystems, human culture, and demands on local agricultural systems must be actively trained, promoted, and brought into the international scientific community [9].

The economic investment in agricultural research is the third problem. Organized public and private investment in agricultural R&D was a primary driver of the comparatively rapid growth in agricultural

productivity experienced in the latter half of the twentieth century [76]. Substantially greater public and private investments in technology and human resources are needed internationally, especially in low-income nations, to make agricultural systems more sustainable. Global research expenditures are less than 2% of agricultural gross domestic product worldwide [77], being roughly 5.5% of agricultural GDP in developed countries, but less than 1% in developing countries (where most of the increased food demand will occur during the next 50 years). At present, there are few incentives for the private sector to increase investments in lower-income developing countries [78]. Moreover, funds have been redirected away from farm productivity toward other concerns, such as the environmental effects of agriculture; food safety and other aspects of food quality; and the medical, energy, and industrial uses of agricultural commodities. Without adequate investments, yield gains and environmental protection may be insufficient for a transition to sustainable agriculture [9].

Feeding the World: Priority Aim

In a recent update of earlier estimates, the Food and Agriculture Organization [79] of the United Nations reported that more than one billion people now suffer malnutrition [80]. By 2050, global population is projected to be 50% larger than at present and global grain demand is projected to double [28, 82], especially the economic growth of the fast-growing economies of Asia [81]. This doubling will result from a projected 2.4-fold increase in per capita real income and from dietary shifts toward a higher proportion of meat (much of it grain fed) associated with higher income. Further increases in agricultural output are essential for global political and social stability and equity. Doubling food production again and sustaining food production at this level are major challenges [82]. Doing so in ways that do not compromise environmental integrity and public health is a greater challenge still [9]. Agricultural productivity growth will be a pivotal determinant of long-term growth in the supply, availability, and price of food over the coming decades [81]. However, a slowdown in growth of agricultural productivity and grain yields has been documented. If this slowdown in productivity persists, it could have profound implications for food-price trends in the future [81].

A key trade-off in cultivated systems is between increasing the amount of cropland needed to meet growing food needs versus increasing the productivity of each hectare of cropland. The "land-sparing" impact of modern farming practices has mainly been achieved by yield increases from the use of crop monocultures with improved crop varieties, fertilizer inputs, and irrigation. For example, if yields of the six major crop groups that are cultivated on 80% of the total cultivated land area had remained at 1961 yield levels, it would require an additional 1.4 billion hectares of land in 2004 – more than double the amount currently used. This represents 34% of the total land area suitable for crop cultivation and would have required conversion of large areas of uncultivated land that support rain forests, grassland savannahs, and wetlands. In Asia alone, it would require an additional 600 million hectares, which represent 25% more land area than is suitable for cultivation on this continent. Asia would now be heavily dependent on food imports if crop yields had remained at 1961 yield levels. Although this increase in productivity has saved some land from conversion, it has resulted in greater impact on other services through water withdrawals, excessive nutrient loads and pesticide use. The key ecological question is, therefore, whether environmental services – other than food production at regional and global scales – would be enhanced by focusing food production on less land under intensive management with high yields, versus expanding cultivated area in lower-yielding systems using farming practices that preserve environmental services at the field and local levels. Few studies have addressed this issue using sound, ecological, analytical methods [27].

Water: Colorless Gold

Water is the principal factor limiting crop yield and is considered a luxury in many agroecosystems. In many developing countries, water is a major factor constraining agricultural output, and income of the world's rural poor [83]. Forty percent of crop production comes from the 16% of agricultural land that is irrigated [84]. Irrigated lands account for a substantial portion of increased yields obtained during the Green

Revolution [9]. Unless water-use efficiency is increased, greater agricultural production will require increased irrigation. However, the global rate of increase in irrigated area is declining, per capita irrigated area has declined by 5% since 1978, and new dam construction may allow only a 10% increase in water for irrigation over the next 30 years [84]. Irrigation return-flows typically carry more salt, nutrients, minerals, and pesticides into surface and ground waters than in source water, impacting downstream agricultural, natural systems, and drinking water. Technologies such as drip and pivot irrigation can improve water-use efficiency and decrease salinization while maintaining or increasing yields. They have been used in industrialized nations on high-value horticultural crops, but their expanded use currently is not economically viable for staple food crops. In developing countries, 15 million hectares have experienced reduced yields owing to salt accumulation and waterlogging [85]. The water-holding capacity of soil can be increased by adding manure or reducing tillage and by other approaches that maintain or increase soil organic matter. Cultivation of crops with high water-use efficiency and the development – through the use of biotechnology or conventional breeding – of crops with greater drought tolerance can also contribute to yield increases in water-limited production environments. Investment in such water-efficient technologies, however, is best facilitated when water is valued and priced appropriately [9].

However, most of the world's agricultural area corresponds to dry-land areas and therefore the challenge is to increase the efficiency of water use in these areas. Efficient use of limited water supplies in dry-land CSs is critical to system success. In the future, increases to semiarid dry-land system water-use efficiency and precipitation-use efficiency may come from continued improvement in managing residues (especially no-tillage), cropping sequences that minimize fallow periods, herbicides, and crop choice. However, continuous cropping under dry-land conditions remains risky due to the limited precipitation (erratic in distribution and frequency) and high-potential evaporation. In the future, the following are potentially fruitful areas of research that may improve system water-use efficiency [86]: (1) Increasing the amount and persistence of crop residues. (2) Implementation of flexible rotations (i.e., opportunity cropping). The occurrence of precipitation and, hence, the availability of adequate stored soil water for a crop is highly variable, especially in semiarid regions. Sometimes stored soil water at normal planting times for a crop in a given CS is limited; at other times, adequate water for a crop is available when the planting of a crop had not been planned. By practicing opportunity cropping, some crop generally could be planted when water becomes available. The goal should be to grow a crop whenever conditions are or become favorable and not according to some predetermined schedule [87]. (3) Matching of crop cultivar selection to prevailing weather conditions. Genetic yield potential is linked positively with maturity, so cultivar evaluation trials conducted under conditions of adequate soil water often favor longer-maturity cultivars and influence farmer choice. (4) Improvement of the timeliness of cultural operations, including early seeding of crops and optimum timing of weed control, and time operations to coincide with favorable conditions as predicted by short-term (48–72 h) weather forecasts.

Genetic Modified Crops: Essential

Genetically modified crops are a very important part of the "second Green Revolution," which may help to break the tendency toward the stabilization of work agricultural production. Genetically modified crops may offer the best hope for producing crops that can withstand drought, impoverished soils, and disease. Future GM products are likely to carry traits that will improve nutrition and health, help guard against drought, heat and cold, and allow plants to access and more efficiently utilize plant nutrients. All of these technologies have more benefits to offer society, and especially, poor farmers and consumers even more than rich ones [88].

Therefore, GM crops are an essential tool in feeding the growing world population and reducing the negative effects on the environment from the use of pesticides. Nevertheless, this technology must be used properly by growers. Growers who adopt herbicide or pest tolerant crops in all cycles of the crop rotation will be at greater risk than those who choose to rotate crops

and/or use other management strategies [89]. However, farmers must be proactive in protecting the longevity of the technology by reducing the likelihood of resistance occurring and providing alternative selection pressures on weeds and pests [52]. We must not forget that life always finds a way through barriers [90].

Biofuel Crops: A Controversial Birth

Recent analyses of the energy and greenhouse-gas performance of alternative biofuels have ignited a controversy that may be best resolved by applying two simple principles. In a world seeking solutions to its energy, environmental, and food challenges, society cannot afford to miss out on the global greenhouse-gas emission reductions and the local environmental and societal benefits when biofuels are done right. However, society also cannot accept the undesirable impacts of biofuels done wrong [91]. Biofuels done right can be produced in substantial quantities with little or no competition with food production.

The biofuel industry could have many positive social and environmental attributes, but it could also suffer from many of the sustainability issues if not implemented the right way. Putting biofuel crops on marginal lands, rather than on our most productive croplands, could mean preventing competition with food production and concomitant effects on commodity prices, as well as minimizing or even avoiding the carbon debt associated with land clearing. However, marginal lands can also be rich in biodiversity, may require sizable inputs of nutrients and water to make production economically viable, and may carry the opportunity cost of forgone future carbon sequestration [92].

Globally, the production of an important amount of energy with biofuels will require a large amount of land – perhaps as much as is in row crop agriculture today. This will change the landscape of Earth in a significant way. The identification of unintended consequences early in the development of alternative fuel strategies will help to avoid costly mistakes and regrets about the effects on the environment. Policies that support long-term sustainability of both our landscapes and our atmosphere are essential if we are to chart a low-carbon economy that is substantially better than business as usual. Sustainable biofuel production systems could play a highly positive role in mitigating climate change, enhancing environmental quality, and strengthening the global economy. But it will take sound, science-based policy and additional research effort to make this so [93].

Until now, the most convincing option for the production of biofuel has been the use of perennial plants as they require fewer inputs and can be grown on degraded lands abandoned from agricultural use [91]. Use of such lands minimizes competition with food crops. This also minimizes the potential for direct and indirect land-clearing associated with biofuel expansion, as well as the resultant creation of long-term carbon debt and biodiversity loss. Some initial analyses on the global potential of degraded lands suggest that they could meet meaningful amounts of current global demand for liquid transportation fuels [92, 94, 95]. The option of using crop residues from food crops, such as corn stover and straw from rice and wheat is a more controversial matter. Recent research suggests that it is to the benefit of farmers to leave substantial quantities of crop residues on the land [96], but that, nonetheless, even conservative removal rates can provide a sustainable biomass resource about as large as that from dedicated perennial crops grown on degraded lands.

Good public policy will ensure that biofuel production optimizes a bundle of benefits, including real energy gains, greenhouse-gas reductions, preservation of biodiversity, and maintenance of food security. Performance-based policies that provide incentives proportional to the benefits delivered are needed. This is a complex question that cannot be addressed with simplistic solutions and sound bites. It needs a new collaboration between environmentalists, economists, technologists, the agricultural community, engaged citizens, and governments around the world [91].

Climate Change: Technical Plasticity

The scenario produced, if indeed climate change becomes a greater reality, is unclear. There is a “climate of suspicion” as published in the journal Nature at the beginning of January 2010. Climate

researchers must emphasize that – although many holes remain to be filled – there is little uncertainty about the overall conclusions: greenhouse-gas emissions are rising sharply, they are very likely to be the cause of recent global warming and precipitation changes, and the world is on a trajectory that will shoot far past 2°C of warming unless emissions are cut substantially [97]. It is evident that CSs must have the ability to adapt to a more unpredictable amount of rainfall and high temperatures. According to the IPCC definition, the extent to which CSs are vulnerable to climate change depends on the actual exposure to climate change, their sensitivity, and adaptive capacity [98]. The adaptive capacity refers to the ability to cope with climate change, including climate variability and extremes, in order to (1) moderate potential damages, (2) take advantage of emerging opportunities, and/or (3) cope with its consequences [99].

Nonetheless, in spite of the uncertainty regarding climate change, primarily as a consequence of the increase in atmospheric CO_2 levels, there are some positive effects. When you step into a commercial greenhouse, the chances are you are stepping into the future. To plants, CO_2 is food, and greenhouse operators, knowing this, use it to fatten them up. While today's atmosphere contains about 380 parts per million of CO_2, commercial greenhouses often contain CO_2 concentrations of twice that or more – the sort of concentration that we might expect in the open air at the end of the century [100]. Many crop scientists believe that this carbon dioxide fertilization effect will go at least some way toward offsetting the losses in yield to be expected as a result of the higher temperatures, flooding, drought, and rising sea levels that the CO_2 greenhouse effect will bring. But some are not so sure. These researchers point to the known negative effects that increased CO_2 concentrations have on the protein content of crops. When Bruce Kimball started out in the 1970s, available technology for high-CO_2 research was limited. Based on current knowledge, he says, the net effect of increasing CO_2 is a good one: "As far as crops go, I think higher CO_2 is a definite benefit. Yes, a little less nutrition than before, but we get more food" [100]. But while Kimball thinks that, in general, the gains in yield are the most important thing, he is not blind to the drop in protein levels.

Agricultural Research: Return to the Field

In recent years, an important movement in agricultural research has emerged on an economic as well as human resource level, toward molecular aspects of plant growth and development. Progress in understanding plant molecular biology has been impressive, and useful applications are evident [101]. However, agronomy has been marginalized, with its field laboratories which require much more time to produce results. This migration could have something to do with the scientific productivity stimulated by the present scientific system since many agricultural research areas that do not conduct field work require less than a year from the start of experimental design until the results are published. On the contrary, field research requires at least 2 years before the results can be accepted by the scientific community. Most records used to assess ecosystem changes are based on short-term data or satellite imagery spanning only a few decades. In many instances, it is impossible to disentangle natural variability from other, potentially significant trends in these records partly because of their short time scale [102]. It is evident that the improvement of CSs requires research in CSs, and specially long-term experiments. Unless a return is made to the roots of research under field conditions, many of the challenges faced will not be overcome. The return to field research will contribute to the global public good by restoring and sustaining productivity growth over the long run, which in turn will mitigate hunger and poverty and, at the same time, reduce pressure on the natural resource base [81].

Nevertheless, in recent years, some agronomists have begun to once again stress the importance of this marginalized dimension of agricultural research, which is time, in relation to the consequences that CSs have on productivity and the environment. Within this framework, long-term experiments acquire a noteworthy relevance. The aim is to compare the biological and economic productivity of different CSs. The high annual fluctuations in rainfall, and resulting crop yields, may make several crop cycles necessary in order to detect significant differences. Likewise, the differences between soils, water–soil relationship, and variability of pathogens and plagues could provide information that explains the differences in yield

among CSs and suggest strategies in which crop sequences and management practices can be selected to increase efficiency in the use of water and nutrients, and control the populations of weed, plagues, and diseases. In this respect, long-term studies act as "laboratories" in which specific problems or mechanisms can be studied in continuous field conditions, where the crop and "input/output" history is well known, and also where tendencies of the quality of resources over time and crop yields can be examined [103]. The most convincing evidence regarding the sustainability of an agroecosystem comes from long-term experiments with positive results.

Bibliography

1. ASA (2010) Glossary of terms. American Society of Agronomy, https://www.agronomy.org
2. CRS (2005) Agriculture: a glossary of terms, programs, and laws. Congressional Research Service, Washington, DC
3. Sadras V, Roget D, Krause M (2003) Dynamic cropping strategies for risk management in dry-land farming systems. Agric Syst 76:920–948
4. Shrestha A (2003) Cropping systems: trends and advances. Food Products Press, Binghamton
5. FAO (2008) Analysis of farming systems. UN Food and Agriculture Organization, Rome
6. Acquaah G (2004) Agricultural production systems principles of crop production, theories, techniques and technology. Prentice Hall, Upper Saddle River, pp 283–317
7. Gold MV (1999) Sustainable agriculture: definitions and terms. USDA National Agriculture Library, Beltsville
8. Earles R, Williams P (2005) Sustainable agriculture: an introduction. ATTRA National Sustainable Agriculture Information Service, Fayetville
9. Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671–677
10. Whitfield J (2006) How green was my subsidy? Nature 439:908–909
11. Marris E, Dodds P, Glover J, Hibberd J, Zhang JH, Sayre R (2008) Agronomy: five crop researchers who could change the world. Nature 456:563–568
12. Cassman KG, Dobermann A, Walters DT (2002) Agroecosystems, nitrogen-use efficiency and nitrogen management. Ambio 31:132–140
13. Liebig MA, Tanaka DL, Krupinsky JM, Merrill SD, Hanson JD (2007) Dynamic cropping systems: contributions to improve agroecosystem sustainability. Agron J 99:899–903
14. Tanaka DL, Krupinsky JM, Liebig MA, Merrill SD, Ries RE, Hendrickson JR, Johnson HA, Hanson JD (2002) Dynamic cropping systems: an adaptable approach to crop production in the Great Plains. Agron J 94:957–961
15. Halloran JM, Archer DW (2008) External economic drivers and US agricultural production systems. Renew Agr Food Syst 23:296–303
16. Bergez JE, Colbach N, Crespo O, Garcia F, Jeuffroy MH, Justes E, Loyce C, Munier-Jolain N, Sadok W (2010) Designing crop management systems by simulation. Euro J Agron 32:3–9
17. Loyce C, Wéry J (2006) Les outils des agronomes pour l'évaluation et la conception des systèmes de culture. In: Doré T, Le Bail M, Martin P, Ney B, Roger-Estrade J (eds) L'agronomie aujourd'hui. QUAE, Paris, pp 77–95
18. Wery J, Langeveld JWA (2010) Introduction to the EJA special issue on "Cropping systems design: new methods for new challenges". Euro J Agron 32:1–2
19. Washington-Ottombre C, Pijanowski B, Campbell D, Olson J, Maitima J, Musili A, Kibaki T, Kaburu H, Hayombe P, Owango E, Irigia B, Gichere S, Mwangi A (2010) Using a role-playing game to inform the development of land-use models for the study of a complex socio-ecological system. Agric Syst 103:117–126
20. Diamond J (2002) Evolution, consequences and future of plant and animal domestication. Nature 418:700–707
21. Diamond J (1997) Guns, germs, and steel: the fates of human societies. Norton, New York
22. Kareiva P, Watts S, McDonald R, Boucher T (2007) Domesticated nature: shaping landscapes and ecosystems for human welfare. Science 316:1866–1869
23. Foley JA, DeFries R, Asner GP, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, Helkowski JH, Holloway T, Howard EA, Kucharik CJ, Monfreda C, Patz JA, Prentice IC, Ramankutty N, Snyder PK (2005) Global consequences of land use. Science 309:570–574
24. Sanderson EW, Jaiteh M, Levy MA, Redford KH, Wannebo AV, Woolmer G (2002) The human footprint and the last of the wild. Bioscience 52:891–904
25. Willis K, Gillson L, Brncic T (2004) How "virgin" is virgin rainforest? Science 304:402–403
26. Millennium Ecosystem Assessment (2005) Ecosystems and human well-being: current state and trends. Island Press, Washington, DC
27. Mooney H, Cropper A, Reid W (2005) Confronting the human dilemma. Nature 434:561–562
28. Cassman KG (1999) Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. Proc Natl Acad Sci USA 96:5952–5959
29. Smil V (1999) Nitrogen in crop production: an account of global flows. Glob Biogeochem Cycl 13:647–662
30. Smil V (2000) Phosphorus in the environment: natural flows and human interferences. Annu Rev Energy Environ 25:53–88
31. Prather MD et al (2001) Atmospheric chemistry and greenhouse gases. In: Houghton JT et al (eds) Climate change 2001: the scientific basis. Cambridge University Press, Cambridge, pp 239–287
32. Boquet DJ, Breitenbeck GA (2004) Book reviews. Cropping systems: trends and advances. Crop Sci 44:2285
33. Wackernagel M, Onisto L, Bello P, Callejas Linares A, López Falfán IS, Méndez García J, Suárez Guerrero AI, Suárez Guerrero

MG (1999) National natural capital accounting with the ecological footprint concept. Ecol Econ 29:375–390
34. van Vuuren DP, Bouwman LF (2005) Exploring past and future changes in the ecological footprint for world regions. Ecol Econ 52:43–62
35. Oki T, Kanae S (2006) Global hydrological cycles and world water resources. Science 313:1068–1072
36. Niccol A (1997) Gattaca trailer. Jersey Films, Columbia Pictures
37. Tilman D (1999) Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. Proc Natl Acad Sci USA 96:5995–6000
38. Mayer A, Mayer J (1974) Agriculture: island empire. Daedelus 103:83–95
39. Levine AS (2009) "Agriculture" is not a dirty word. Science 324:1140
40. Cohen JE (1995) How many people can the earth support? Norton, New York
41. Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science 292:281–284
42. Rasmussen PE, Goulding KWT, Brown JR, Grace PR, Janzen HH, Körschens M (1998) Long-term agroecosystem experiments: assessing agricultural sustainability and global change. Science 282:893–896
43. Waggoner PE (1995) How much land can ten billion people spare for nature? Does technology make a difference? Technol Soc 17:17–34
44. López-Bellido RJ, López-Bellido L, Benítez-Vega J, López-Bellido FJ (2007) Tillage system, preceding crop, and nitrogen fertilizer in wheat crop: II water utilization. Agron J 99:66–72
45. López-Bellido RJ, Fontán JM, López-Bellido FJ, López-Bellido L (2010) Carbon sequestration by tillage, rotation, and nitrogen fertilization in a Mediterranean Vertisol. Agron J 102:310–318
46. Tanaka DL, Anderson RL, Rao SC (2005) Crop sequencing to improve use of precipitation and synergize crop growth. Agron J 97:385–390
47. Merrill SD, Tanaka DL, Krupinsky JM, Ries RE (2004) Water use and depletion by diverse crop species on Haplustoll soil in the northern Great Plains. J Soil Water Conserv 59:176–183
48. Derpsch R (1998) Historical review of no-tillage cultivation of crops. JIRCAS Working Rep. 13:1–18. Japan International Research Centre for Agricultural Sciences, Ibaraki, Japan
49. Triplett GB Jr, Dick WA (2008) No-tillage crop production: a revolution in agriculture! Agron J 100:153–165
50. Lal R, Reicosky DC, Hanson JD (2007) Evolution of the plow over 10, 000 years and the rationale for no-till farming. Soil Tillage Res 93:1–12
51. Wienhold BJ, Pikul JL, Liebig MA, Mikha MM, Varvel GE, Doran JW, Andrews SS (2006) Cropping system effects on soil quality in the Great Plains: synthesis from a regional project. Renew Agr Food Syst 21:49–59
52. Murphy CE, Lemerle D (2006) Continuous cropping systems and weed selection. Euphytica 148:61–73
53. Nail EL, Young DL, Schillinger WF (2007) Diesel and glyphosate price changes benefit the economics of conservation tillage versus traditional tillage. Soil Tillage Res 94:321–327
54. López-Bellido L, López-Bellido RJ, Castillo JE, López-Bellido FJ (2000) Effects of tillage, crop rotation, and nitrogen fertilization on wheat under rainfed Mediterranean conditions. Agron J 92:1054–1063
55. DeVries J, Toenniessen G (2001) Securing the harvest: biotechnology, breeding, and seed systems for African crops. CAB International, Wallingford
56. Ripoche A, Celette F, Cinna JP, Gary C (2010) Design of intercrop management plans to fulfil production and environmental objectives in vineyards. Eur J Agron 32:30–39
57. Hooper CIIIFS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setälä H, Symstad AJ, Vandermeer J, Wardle DA (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecol Monogr 75:3–35
58. Kirschenmann F (2002) Why American agriculture is not sustainable. Renewable Resour J 20:6–11
59. Groot JCJ, Jellema A, Rossing WAH (2010) Designing a hedgerow network in a multifunctional agricultural landscape: balancing trade-offs among ecological quality, landscape character and implementation costs. Eur J Agron 32:112–119
60. Bailey KL, Gossen BD, Lafond GR, Watson PR, Derksen DA (2001) Effect of tillage and crop rotation on root and foliar diseases of wheat and pea in Saskatchewan from 1991 to 1998: univariate and multivariate analyses. Can J Plant Sci 81:789–803
61. Krupinsky JM, Bailey KL, McMullen MP, Gossen BD, Turkington TK (2002) Managing plant disease risk in diversified cropping systems. Agron J 94:198–209
62. Zhu Y, Chen H, Fan J, Wang Y, Li Y, Chen J, Fan J, Yang S, Hu L, Leung H, Mew TW, Teng PS, Wang Z, Mundt CC (2000) Genetic diversity and disease control in rice. Nature 406:718–722
63. Schlegel AJ, Grant CA, Havlin JL (2005) Challenging approaches to nitrogen fertilizer recommendations in continuous cropping systems in the Great Plains. Agron J 97:391–398
64. López-Bellido RJ, López-Bellido L (2001) Efficiency of nitrogen in wheat under Mediterranean conditions: effect of tillage, crop rotation and N fertilization. Field Crop Res 71:31–46
65. Grant CA, Peterson GA, Campbell CA (2002) Nutrient considerations for diversified cropping systems in the northern Great Plains. Agron J 94:186–198
66. Tanaka DL, Krupinsky JM, Merrill SD, Liebig MA, Hanson JD (2007) Dynamic cropping systems for sustainable crop production in the Northern Great Plains. Agron J 99:904–911
67. De Costa WAJM, Surentham P (2005) Tree-crop interactions in hedgerow intercropping with different tree species and tea in Sri Lanka: 1. Production and resource competition. Agroforest Syst 63:199–209
68. Léonard J, Andrieux P (1998) Infiltration characteristics of soils in Mediterranean vineyards in southern France. Catena 32:209–223
69. Battany MC, Grismer ME (2000) Rainfall runoff and erosion in Napa Valley vineyards: effects of slope, cover and surface roughness. Hydrol Process 14:1289–1304

70. Monteiro A, Lopes CM (2007) Influence of cover crop on water use and performance of vineyard in Mediterranean Portugal. Agric Ecosyst Environ 121:336–442
71. Valdés Gomez H, Fermaud M, Roudet J, Calonnec A, Gary C (2008) Grey mould incidence is reduced on grapevines with lower vegetative and reproductive growth. Crop Prot 27:1174–1186
72. Tanaka DL, Karn JF, Liebig MA, Kronberg SL, Hanson JD (2006) An integrated approach to crop/livestock systems: forage and grain production for swath grazing. Renew Agr Food Syst 20:223–231
73. Villano R, Fleming E, Fleming P (2010) Evidence of farm-level synergies in mixed-farming systems in the Australian wheat-sheep zone. Agric Syst 103:146–152
74. Hanson JD, Liebig MA, Merrill SD, Tanaka DL, Krupinsky JM, Stott DE (2007) Dynamic cropping systems: increasing adaptability amid an uncertain future. Agron J 99:939–943
75. Peterson GA, Westfall DG, Peairs FB, Sherrod L, Poss D, Gangloff W, Larson K, Thompson DL, Ahuja LR, Koch MD, Walker CB (2000) Sustainable dryland agroecosystem management. Agric Exp Stn Tech Bull TB00–3. Colorado State University, Fort Collins
76. Evenson RE (2002) Economic impacts of agricultural research and extension. In: Gardner BL, Rausser GC (eds) Handbook of agricultural economics, vol 1A, chapter 11. Elsevier, New York, pp 573–628
77. Pardey PG, Alston JM, Piggott RR (2006) Agricultural R&D in the developing world: too little, too late? International Food Policy Research Institute, Washington, DC
78. Falcon W, Fowler C (2002) Carving up the commons – emergence of a new international regime for germplasm development and transfer. Food Policy 27:197–222
79. FAO (2008) Hunger on the rise: soaring prices add 75 million people to global hunger rolls. Briefing paper, FAO, Rome
80. FAO (2009) More people than ever are victims of hunger. Press release, FAO, Rome
81. Alston JM, Beddow JM, Pardey PG (2009) Agricultural research, productivity, and food prices in the long run. Science 325:1209–1210
82. Alexandratos N (1999) World food and agriculture: outlook for the medium and longer term. Proc Natl Acad Sci USA 96:5908–5914
83. Namara RE, Hanjra MA, Castillo GE, Ravnborg HM, Smith L, Van Koppen B (2010) Agricultural water management and poverty linkages. Agric Water Manage 97:520–527
84. Postel SL, Daily GC, Ehrlich PR (1996) Human appropriation of renewable fresh water. Science 271:785–788
85. Naylor R (1996) Energy and resource constraints on intensive agricultural production. Annu Rev Energy Environ 21:99–123
86. Nielsen DC, Unger PW, Miller PR (2005) Efficient water use in dryland cropping systems in the Great Plains. Agron J 97:364–372
87. Miller PR, McConkey BG, Zentner RP, Campbell CA, Cochran VL (2003) Flexible cropping systems in the semiarid northern Great Plains. In: Hanson JD, Krupinsky JM (eds) Proceedings of dynamic cropping systems: principles, processes, and challenges, Bismark, ND, 4–7 Aug 2003. USDA-ARS, Mandan, pp 87–104
88. Borlaug NE (2007) Sixty-two years of fighting hunger: personal recollections. Euphytica 157:287–297
89. Diggle AJ, Neve PB, Smith FP (2003) Herbicides used in combination can reduce the probability of herbicide resistance in finite weed populations. Weed Res 43:371–382
90. Crichton M (1991) Jurassic park. Ballantine Books, The Random House Publishing Group, New York
91. Tilman D, Socolow R, Foley JA, Hill J, Larson E, Lynd L, Pacala S, Reilly J, Searchinger T, Somerville C, Williams R (2009) Beneficial biofuels – the food, energy, and environment trilemma. Science 325:270–271
92. Field CB, Campbell JE, Lobell DB (2008) Biomass energy: the scale of the potential resource. Trends Ecol Evol 23:65–72
93. Robertson GP, Dale VH, Doering OC, Hamburg SP, Melillo JM, Wander MM, Parton WJ, Adler PR, Barney JN, Cruse RM, Duke CS, Fearnside PM, Follett RF, Gibbs HK, Goldemberg J, Mladenoff DJ, Ojima D, Palmer MW, Sharpley A, Wallace L, Weathers KC, Wiens JA, Wilhelm WW (2008) Sustainable biofuels redux. Science 322:49–50
94. Tilman D, Hill J, Lehman C (2006) Carbon-negative biofuels from low-Input high-diversity grassland biomass. Science 314:1598–1600
95. Campbell JMF, Lobell DB, Genova RC, Field CB (2008) The global potential of bioenergy on abandoned agriculture lands. Environ Sci Technol 42:5791–5794
96. Wilhelm WW, Johnson JMF, Karlen DL, Lightle DT (2007) Corn stover to sustain soil organic carbon further constrains biomass supply. Agron J 99:1665–1667
97. Editorial (2010) Climate of suspicion. Nature 463:269
98. IPCC (2001) Climate change 2001. Impacts, adaptation and vulnerability. Cambridge University Press, Cambridge
99. Reidsma P, Ewert F, Oude Lansink A, Leemans R (2010) Adaptation to climate change and climate variability in European agriculture: the importance of farm level responses. Eur J Agron 32:91–102
100. Stafford N (2007) The other greenhouse effect. Nature 448:526–528
101. Borras L, Slafer GA (2008) Agronomy and plant breeding are key to combating food crisis. Nature 453:1177
102. Willis KJ, Birks HJB (2006) What is natural? The need for a long-term perspective in biodiversity conservation. Science 314:1261–1265
103. ICARDA (1995) Farm resource management program. Annual Report for 1994. International Center for Agricultural Research in the Dry Areas, Aleppo, Syria

Dairy Cattle Breeding

Filippo Miglior[1,2], Sarah Loker[3], Roger D. Shanks[4]
[1]Guelph Food Research Centre, Agriculture and Agri–Food Canada, ON, Canada
[2]Canadian Dairy Network, Guelph, ON, Canada
[3]Department of Animal and Poultry Science, University of Guelph, Guelph, ON, Canada
[4]Department of Animal Sciences, University of Illinois, Urbana, IL, USA

Article Outline

Glossary

Allele A variant form of a gene. Differences between alleles of a gene are a result of alternate DNA sequences.

Breeding value The sum of the independent allele effects on the trait of interest (i.e., the additive genetic worth).

Generation interval The average age of the parent when he/she is replaced by their offspring.

Genome-wide selection Selection of animals based on the value of their genomic profile. Animals are genotyped for several (thousands) of markers spanning the entire genome. These markers are so close together that they are thought to be linked with all genes in the genome.

Heritability A population measure depicting the strength of the relationship between performance and breeding value.

Indicator trait A trait genetically correlated with the trait of interest, but is easier, cheaper, or more convenient to measure and select in hopes of indirectly affecting the trait of interest in the population.

Mendelian sampling Describes the genetic variation of progeny of the same parents. More specifically, full-sibs are not expected to be genetically identical because of random segregation and recombination of genes from the sire and dam.

Reliability Regarding estimated breeding values, reliability, or accuracy of the estimated breeding value reflects the strength of the relationship between the estimated breeding value and the true breeding value.

Definition of the Subject

Dairy cattle breeding is the process of selecting and mating individuals in accordance with breeding goals, with the aim of changing genetic merit of future generations and bringing about an improvement in economic efficiency. For instance, a breeding goal may be designed to improve milk production, health, and fertility. Selection would then be for individuals who will produce offspring that genetically will earn greater profit through improved production at a lower cost (due to improved health and fertility).

Many factors have contributed to the vast improvement in dairy cattle production over the last century. One of the most important factors is the regular recording of phenotypic records. It is from these phenotypic records that the industry has estimated genetic worth. Improvement in methods for genetically evaluating dairy cattle is a large contributor to the substantial genetic improvement seen in this species. Such methods include the use of BLUP (Best Linear Unbiased Prediction), a method first proposed by C.R. Henderson in 1949 [1]. Another important milestone in the improvement of dairy cattle breeding was the development of techniques to freeze and store bovine semen in the early 1950s [2]. Because of this, semen can be stored for longer, shipped further, and therefore shared internationally. A large contributor to the success of dairy cattle breeding has been the implementation of progeny testing programs in the 1950s, which allowed for reliable genetic evaluations for bulls, especially for traits only expressed in daughters (such as

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

milk production) [2]. To aid in the trade and use of dairy cattle genetics on an international basis, Schaeffer in 1994 developed MACE (multiple-trait across-country evaluation) [3]. This methodology allowed genetic evaluations to be converted to different countries' scales. In 2006, Shook [4] described the remarkable increase in yield traits from 1980 to 2000, revealing an increase of 3,500 kg of milk, 130 kg of fat, and 100 kg of protein per cow per lactation. While this increase is due to improvements of many factors, including genetics, nutrition, and management, Shook [4] determined that 55% of the gains in yield traits were due to genetics and that genetic change (versus altering environmental conditions) is permanent and cumulative.

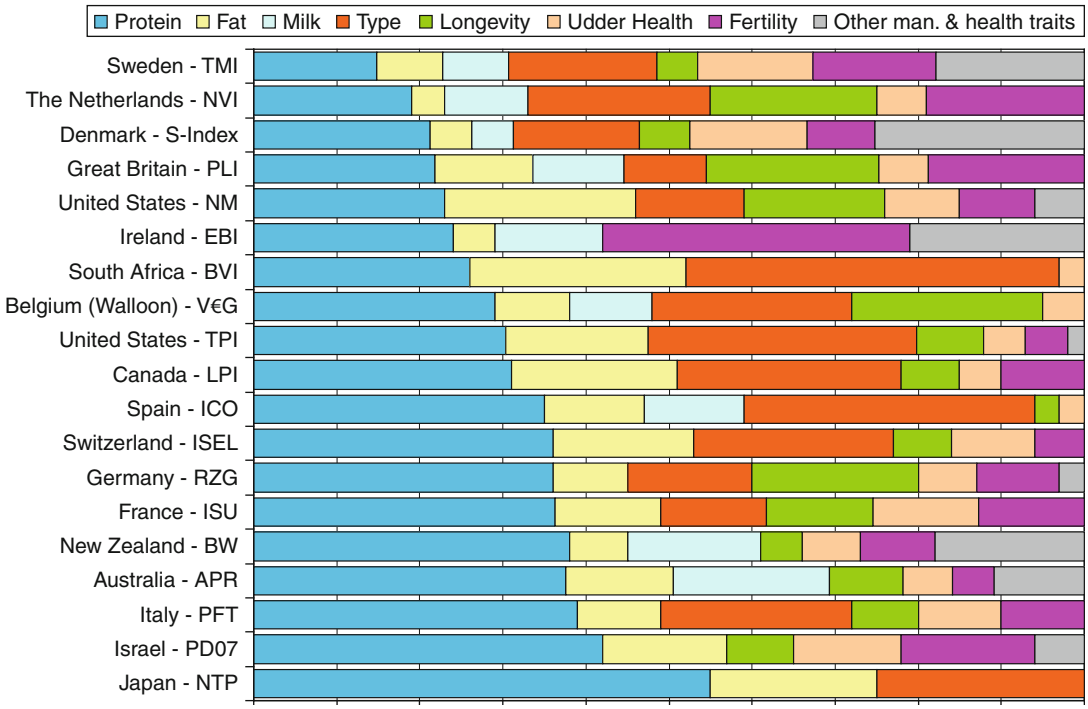
Introduction

As seen in previous entries, genetic improvement in any livestock species requires: (a) identification of breeding goals; (b) accurate data collection, animal identification, and pedigree registration; (c) breeding scheme; and (d) genetic evaluation of measured traits. In dairy cattle breeding, artificial insemination is highly used and traits of interest are usually only expressed in females. Both points determine that males are very important in breeding scheme and genetic progress, but generation interval will be longer than in other species, given that males need to be proven based on progeny performance instead of their own. Another important aspect of dairy cattle breeding is an open international market for dairy genetics, where the male side is controlled through semen sales by a large number of AI organizations, some national and some multinational based. The female side, by contrast, is controlled by the dairy producers. Being an international market with high exchange of semen, and somewhat lower but still common exchange of embryos and live animals, a constant need is to obtain genetic values of foreign animals on local scales, a service provided via international genetic evaluations by the Interbull Centre in Sweden. Finally, in the last 2 years, the full sequence of the bovine genome has opened the way for genome-wide selection. The advent of genomic selection has provided new opportunities and challenges in the global dairy semen market. The market has already seen a partial shift from progeny tested sires to young genotyped bulls. After this transition time, provided one can confirm over the next few months that the genetic level and accuracy of evaluation of these young bulls are as high as expected, genomic selection will revolutionize dairy cattle breeding, and will decrease the importance of progeny testing for some bulls. This chapter will present all the characteristics of traditional dairy cattle breeding, the international aspects of this species breeding as well as the current application of genomic selection and its consequences.

Breeding Goals

Generally, the breeding goal of a dairy producer is to maximize the profitability of his/her dairy farm. The main return in a dairy farm derives from sales of milk production. Cost of milk varies across and within countries based on supply and demand and whether a quota system is present. Additionally, premiums are paid for high-quality milk, and higher percentages of fat and protein. Furthermore, penalization will apply for milk with somatic cell count (SCC) higher than a given threshold. The second return in a dairy farm originates from the sale of breeding stock, primarily young or pregnant heifers. This type of return is less common, being present only in dairy farms with high-genetic-value cows. The same type of farm will generate return from embryo sales by multiple flushings of their top cows. A more common but low return derives from the sale of male calves and cull cows. The most important variable cost in dairy farms is represented by feed costs, followed by veterinary and breeding costs.

For many years, most selection programs worldwide focused on increasing milk production. National selection indices were based on improving milk yield and gradually shifted toward improving protein yield and, outside North America, toward increasing fat and especially protein content. This was true for most countries with the exception of the Scandinavian countries, whose selection indices also included health and reproduction; and North American countries, whose selection indices included conformation together with production. In the last 10 years, a growing interest has broadened selection indices to include functional traits such as reproduction and health. Main reasons for this shift were quota-based milk marketing systems, price constraints, or both, together with increasing producer and consumer concerns associated with the observed



Dairy Cattle Breeding. Figure 1
Relative emphasis of traits for various national selection indexes around the world

deterioration of the health and reproduction of dairy cows. Labor costs have increased relatively more than milk price in some countries. Several studies have shown that selection for production alone causes negative effects on udder health [5] and reproductive performance [6–8].

Figure 1 shows the relative emphasis on traits in national selection indices in October 2009. The main difference between selection indices in various countries was the relative emphasis on production. However, every country has now broadened their index by adding longevity, health, and reproduction to the usual production and conformation traits [9]. The search for the ideal balance between all of these important traits continues.

Data Collection, Identification, and Pedigree Registration

Proper identification, pedigree recording, and performance recording are crucial for genetic improvement of dairy cattle. Without them, accurate genetic evaluations would not be possible. In the past, animal identification was more important within the herd for management purposes [10]. However, it is now important to have proper animal identification for genetic evaluation purposes, which means that an animal's ID should be unique outside its herd. In some countries, a unique animal ID is mandatory. In the Canadian dairy cattle industry, a herd's lactation records only qualify for official publication if 80% of its first lactation animals are registered in a breed association herd book with a unique animal identification. Unique animal identification is not without error, however. Larger ID numbers associated with unique national or international identifications are at risk of recording errors, and ID tags can become worn-out or lost. Identification errors can also lead to inaccurate pedigree recording, though pedigree errors can occur for various reasons. Banos et al. [11] and Israel and Weller [12] showed that pedigree errors resulted in biased estimated breeding values and reduced genetic gain. Also, faulty equipment and human error can lead to inaccurate data recording. Fortunately, several

techniques correct or accommodate erroneous outliers in the data (e.g., robust procedures described by Jamrozik et al. [13]).

In summary, numerous errors can occur when recording performance and pedigree information. Therefore, to ensure better quality data, it is mandatory in most countries to follow the rules and standards established by the International Committee for Animal Recording before records can be used for genetic evaluation. Several traits are of economic importance in the dairy industry, but the relative importance of each depends on the country (Fig. 1).

Test-day models are used for the genetic evaluation of dairy cattle for milk production traits in many countries. These models necessitate the regular recording of milk production traits. A good recording scheme can therefore require records for 24-h milk, fat, protein, and somatic cell count (SCC) to be taken once monthly. These are called test-day records. Generally, production traits such as milk, fat, and protein yield are moderately heritable.

While milk production traits are important, many other traits are of economic importance in dairy cattle breeding, including conformation, longevity, reproduction, health, and workability traits. Conformation (or "type") traits describe the physical attributes of the cow that are generally associated with survival, health, and reproduction. Many traits (e.g., body condition score) require visual appraisal by the recorder, and are considered to be more subjective. In these cases, it is vital that assessors are highly trained to ensure repeatable and accurate recording. Many "type" traits are moderately heritable.

Because of the negative genetic correlations between milk production and fertility or health traits, long-term selection for improved milk production has led to reduced fertility and health in dairy cattle. As a result, routine genetic evaluations of reproductive and health traits are becoming more common, despite low heritabilities. A major challenge is that direct health data has not been recorded for very long and can be difficult to measure. In many cases, countries use indicator traits instead of measuring the health trait directly. An example of this is using somatic cell count as an indicator of mastitis.

Longevity (or survival) describes the length of a cow's survival in the herd, and has a low heritability as this trait can be greatly affected by herd management and other nongenetic factors. Workability includes traits such as milking speed and temperament during milking. Milking temperament has a low heritability, while milking speed has a moderate heritability.

Breeding Scheme

In general, a breeding scheme is the amalgamation of the processes involved in the selection and mating of livestock for the purpose of genetic improvement. Because of artificial insemination in the dairy cattle industry, semen from a single male can be used widely throughout the population. Therefore, genetic improvement is achieved largely through intense selection of males. However, most of the economically important traits in the industry (such as milk production traits) are expressed in the female. As a result, dairy cattle breeding relies on progeny testing schemes for genetic improvement. Data on various milk production and performance traits from daughters are collected and used to calculate estimated breeding values for bulls. The more daughters and daughter records are available for a bull, the greater the accuracy of the estimated breeding values. "Proven bulls" are bulls with very reliable estimated breeding values because they have many daughters with performance records. Estimated breeding values for bulls without progeny records are less reliable because they are calculated using the average of the estimated breeding values of the parents.

The major factors influencing rate of genetic progress for a given trait are the components of "the key equation" of animal breeding:

$$\frac{\Delta BV}{t} = \frac{r_{BV,\,\widehat{BV}}\, i \sigma_{BV}}{L} \tag{1}$$

where BV is true breeding value, t is time, $\widehat{\mathrm{BV}}$= estimated breeding value, $r_{\mathrm{BV},\,\widehat{\mathrm{BV}}}$= accuracy of the estimated breeding value relative to the true breeding value (also the correlation between the estimated and true breeding values), i = selection intensity (a function of the proportion of the population chosen to be parents of the next generation), σ_{BV} is additive genetic standard deviation, and L is generation interval. The aim is to choose animals with superior genetics to be parents of the next generation to improve the genetics of the population. Increasing the reliability (accuracy)

of prediction, selecting only the best animals as parents (increasing selection intensity), and decreasing the generation interval are important factors for increasing genetic improvement per unit time.

A major challenge in dairy cattle breeding is developing an optimum breeding program that maximizes genetic progress while minimizing cost. AI organizations are generally responsible for breeding schemes [14], and a typical AI organization can spend millions of dollars per year progeny testing bulls to find the best bull to market to the world [15]. However, dairy breeding is entering a new era in which genomic selection is possible. With genomic selection, bulls can be genotyped and selected at a young age. This improves the industry's traditional breeding scheme by reducing reliance on progeny testing (a lengthy and costly process), and will theoretically increase response to selection via a reduced generation interval [15]. Also, genomic values will increase the accuracy of genetic evaluations, especially for young sires for which traditional estimated breeding values are derived from parent averages [16].

Purebred and Crossbred Cows

The group of animals selected as parents of the next generation are expected to possess alleles that the industry considers favorable. Therefore, through selection, the frequency of favorable alleles in the population should increase with each generation while that of unfavorable alleles should decrease. The result is an increase in average breeding value, and improved performance of the dairy cattle population. The change in average breeding value over time defines the genetic trend.

As mentioned previously, artificial insemination has allowed for intense selection of sires for increased genetic improvement over time. This means that a few top bulls, with the best collection of favorable alleles, can be mated widely throughout the population. While this is a good way to progress more quickly toward fixing favorable alleles in the population, it reduces the effective population size of the breed which could raise inbreeding and reduce performance from the associated inbreeding depression. The dairy industry needs to find a compromise between selection of the best sires for use in artificial insemination, and minimization of inbreeding depression. This is, of course, less of a problem initially with crossbreeding, as sire and dam are unrelated. However, while several benefits exist with crossbreeding in general, heterosis obtained is too low to lead to more profitable animals than purebred Holsteins (the most widely used dairy breed) [17, 18]. Therefore, in the dairy industry, selection tends to occur within breeds.

Again, a large degree of dairy cattle genetic progress is achieved through the selection and use of semen of a few top bulls. However, it is important to understand that selection and consequent genetic progress within a breed is achieved via four selection pathways, all of which center around the progeny testing scheme.

Progeny testing is required so that the genetic merit of bulls can be calculated reliably via analysis of many performance records on many daughters. Every year, genetically superior bulls and cows are mated using artificial insemination to produce young bulls with high predicted genetic merit. On average, young bulls resulting from these matings will have high true genetic merit, but because of Mendelian sampling, it is not certain that these young bulls will be genetically superior. The young bulls therefore need to be proven via progeny testing. If a young bull is in fact of high genetic merit, he will yield daughters that perform well for traits of interest. The more daughters he produces with superior performance, the more certain it is that he is a genetically superior bull.

So far, two selection pathways have been discussed: selection of sires of young bulls, and of dams of young bulls. The sires of young bulls are proven and their semen can be used widely, so they can be selected very intensely, and their estimated breeding values are very reliable. Referring to Eq. 1, increased selection pressure and reliability will lead to increased genetic progress per unit time. Dams of young bulls can be selected intensely because not many young bulls need to be produced for progeny testing (only about 400 a year in Canada and about 6,000 Holsteins worldwide). However, the reliability of estimated breeding values for dams of young bulls is not as high as that of the sires because the dams have fewer close relatives with records.

In a dairy herd, replacement heifers are required. Therefore, further potential for increasing genetic progress of the population is through two more selection pathways: selection of sires of cows and dams of

cows. It takes several years for young bulls to be fully proven. However, when these bulls have a genomic evaluation or some daughter records (but not yet enough to achieve the reliability of a proven bull), they can be selected to produce replacement heifers with a reasonably high selection intensity. Dams of these future cows, however, cannot be selected so intensely for several reasons. Many replacement heifers are required, and because a female cannot breed as many times as a male, most cows will be selected for breeding. Also, female fertility in the dairy cattle population is typically low, so the industry cannot afford to be very selective with this pathway.

Genetic Evaluation

For progress in the dairy industry, it is important to accurately select genetically superior animals as parents of the next generation. Traditionally, genetic worth could only be estimated by evaluation of phenotypic records, which are a result of a combination of genetic and environmental factors (and sometimes an interaction of the two). Again, genomics is revolutionizing the way the industry evaluates dairy cattle, making it possible to genotype animals instead of waiting for phenotypic records. However, genomics is just one part of the process, and the collection of phenotypic records will still be important for some time.

The additive genetic value of an animal for a particular trait is the sum of the independent effects of that individual's alleles on that trait. On average, half of an animal's additive genetic worth is passed on to its offspring. The greater the animal's genetic worth, the more genetically superior its offspring are expected to be. The additive genetic value is therefore appropriately termed "breeding value." Breeding values of animals can be estimated from many different sources of information, including observations on the animal itself and observations from a variety of relatives. This reiterates the importance of quality phenotypic and pedigree data. True breeding value can never be known, only estimated from a very large (effectively infinite) number of genes with alleles each of which has a small effect on the trait of interest. Breeding values are estimated from limited phenotypic data using models that are not perfect. The accuracy of estimated breeding values (EBV) depends on a variety of factors, including the degree of relationship between the animals providing the phenotypic information and the animal being evaluated, the number of records available, and the heritability of the trait of interest. Of course, through genomics, perhaps one day the effect that each allele in the genome has on each trait could be quantified, bringing the industry closer to an animal's true breeding value for each trait.

Several traits of economic importance to the dairy industry were already discussed. So, a dairy bull or cow has several estimated breeding values, one for each trait. This makes selection complicated. For example, perhaps a potential sire has excellent estimated breeding values for milk production traits, but terrible values for health and fertility traits. Therefore, countries devise a national economic selection index, which incorporates estimated breeding values for traits of interest and their respective monetary worth into an equation that gives a single score for profitability ("aggregate breeding value") of each animal [19]. This makes selection much easier, as animals with the most favorable combination of estimated breeding values (e.g., high milk production and good health and fertility) are the most profitable.

Traditionally, selection index methods were used to combine weighting factors with adjusted phenotypic records from various sources (i.e., own records and records from various relatives) to derive estimated breeding values for traits. The phenotypic records were first adjusted for a variety of environmental influences including, for example, age of animal and effect of contemporary group. These methods of adjustment were not always effective for disentangling genetic effects from environmental influences. To improve the application of selection index methods, the dairy breeding industry began to use the statistical method known as best linear unbiased prediction, or BLUP, in the 1970s. This method was first proposed by C.R. Henderson in 1949 [1]. Without going into too much detail, BLUP is able to simultaneously estimate environmental effects and predict breeding values while taking into account pedigree relationships.

Both the traits and the methodologies involved in national genetic evaluations vary substantially among countries [20]. Therefore, EBVs for one trait in one country may not be representative of EBVs for the same trait in another country. This makes comparing

animals from different countries difficult. Dairy cattle genetics are shared internationally, especially sire genetics via artificial insemination. Therefore, the Interbull Centre was created to provide international evaluations. Specifically, the procedure carried out is called the multiple-trait across-country evaluation, or MACE [3]. This procedure allows Interbull to provide a separate list of International Genetic Evaluations to each participating country, expressed on that country's scale.

Future Directions

As previously mentioned, genome-wide selection is revolutionizing dairy cattle breeding. Young bulls benefit the most with a large increase in reliability of estimated breeding values at an earlier time in their lives, reducing the generation interval of these animals and hence increasing the speed of genetic improvement. It is fairly certain that future dairy cattle genetics research will focus on the improvement of genomic techniques.

Over the years, as quantitative geneticists improve upon techniques surrounding genome-wide selection for animal breeding, it is important to keep in mind the application of such techniques to human health. In 2008, Mardis [21] predicted that sequencing the entire human genome for $1,000 will be feasible in the near future. While animal breeders are currently using genomics to predict the genetic value of animals for complex traits, it may one day be possible to utilize genomics to predict human individuals' genetic risk for complex, multifactorial diseases, such as Crohn's disease [22]. Research in genomics in animal breeding will certainly pave the way for research and development of genomics applied to human health.

Bibliography

1. Henderson CR (1949) Estimation of changes in herd environment. J Dairy Sci 32:706 (Abstr)
2. Funk DA (2006) Major advances in globalization and consolidation of the artificial insemination industry. J Dairy Sci 89:1362–1368
3. Schaeffer LR (1994) Multiple-country comparison of dairy sires. J Dairy Sci 77:2671–2678
4. Shook GE (2006) Major advances in determining appropriate selection goals. J Dairy Sci 89:1349–1361
5. Heringstad B, Chang YM, Gianola D, Klemetsdal G (2003) Genetic analysis of longitudinal trajectory of clinical mastitis in first-lactation Norwegian cattle. J Dairy Sci 86: 2676–2683
6. Haile-Mariam M, Morton JM, Goddard ME (2003) Estimates of genetic parameters for fertility traits of Australian Holstein-Friesian cattle. Anim Sci 76:35–42
7. Kadarmideen HN, Thompson R, Coffey MP, Kossaibati MA (2003) Genetic parameters and evaluations from single- and multiple- trait analysis of dairy cow fertility and milk production. Livest Prod Sci 81:183–195
8. Veerkamp RF, Koenen EPC, De Jong G (2001) Genetic correlations among body condition score, yield, and fertility in first-parity cows estimated by random regression models. J Dairy Sci 84:2327–2335
9. Miglior F, Muir BL, Van Doormaal BJ (2005) Selection indices in Holstein cattle of various countries. J Dairy Sci 88:1255–1263
10. Jr Hooven NW (1978) Cow identification and recording systems. J Dairy Sci 61:1167–1180
11. Banos G, Wiggans GR, Powell RL (2001) Impact of paternity errors in cow identification on genetic evaluations and international comparisons. J Dairy Sci 84:2523–2529
12. Israel C, Weller JI (2000) Effect of misidentification on genetic gain and estimation of breeding value in dairy cattle populations. J Dairy Sci 83:181–187
13. Jamrozik J, Strandén I, Schaeffer LR (2004) Random regression test-day models with residuals following a Student's-*t* distribution. J Dairy Sci 87:699–705
14. Miglior F (2000) Impact of inbreeding – Managing a declining Holstein gene pool. In: Proceedings from 10th world Holstein Friesian federation conference – 30 Apr – 3 May 2000, Sydney, pp 108–113
15. Schaeffer LR (2006) Strategy for applying genome-wide selection in dairy cattle. J Anim Breed Genet 123:218–223
16. VanRaden PM, Van Tassell CP, Wiggans GR, Sonstegard TS, Schnabel RD, Taylor JF, Schenkel FS (2009) Invited review: reliability of genomic predictions for North American Holstein bulls. J Dairy Sci 92:16–24
17. Gunjal K, Menard L, Shanmugam R (1997) Economic analysis of crossbreeding dairy cattle. Agric Syst 54:327–339
18. VanRaden PM, Sanders AH (2003) Economic merit of crossbred and purebred US dairy cattle. J Dairy Sci 86:1036–1044
19. Hazel LN (1943) The genetic basis for constructing selection indexes. Genetics 28:476–490
20. Mark T (2004) Applied genetic evaluations for production and functional traits in dairy cattle. J Dairy Sci 87:2641–2652
21. Mardis ER (2008) Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet 9:387–402
22. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH et al (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 40:955–962

Disease-Resistant Transgenic Animals

Caroline Lassnig, Mathias Müller
Institute of Animal Breeding and Genetics & Biomodels Austria, University of Veterinary Medicine Vienna, Vienna, Austria

Article Outline

Glossary

Disease resistance/susceptibility The interplay of the genetically determined ability of an individual to prevent the reproduction of a pathogen or to reduce pathogen growth, host–pathogen interactions, and changing environmental conditions/factors decides on resistance/susceptibility.

Gene targeting Integration of exogenous DNA into the genome of an organism at specific sites as a result of homologous recombination. It can be used to disrupt or delete a gene, to remove or add sequences as well as to introduce point mutations at a given locus. Gene targeting can be permanent, i.e., ubiquitous with respect to tissue and developmental stage, or conditional, i.e., restricted to a specific time during development/life or limited to a specific tissue.

Genetic engineering Technological process resulting in a directed alteration of the genotype of a cell or organism. It combines recombinant nucleic acid technologies, in vitro culture technologies for gametes, embryos, tissues, or organisms, methods for the delivery of nucleic acids to the host genomes (gene transfer), and if needed, reproductive technologies to produce transgenic embryos and transfer them to foster organisms. With respect to inheritance ('transmission') to offspring, germline and somatic gene transfer methods are distinguished.

Knockdown Downregulation of expression of a specific gene by RNAi-based technologies.

Knockout/knockin Incorporation of a sequence into a specific site by homologous recombination (gene targeting) that results in disruption of gene function/altered gene function.

Quantitative trait loci (QTL) Genetic loci or chromosomal regions that contribute to variability in complex traits, as identified by statistical analysis. The genetic basis of these traits generally involves the effects of multiple genes and gene–environment interactions.

RNA interference (RNAi) The silencing of gene expression by the introduction of dsRNA that triggers the specific degradation of a homologous target mRNA, often accompanied by an attendant decrease in the production of the encoded protein.

Single nucleotide polymorphism (SNP) A variation in DNA sequence in which one nucleotide position is substituted for another by either nucleotide exchange, or deletion, or insertion. SNPs are the most frequent type of polymorphism in the genome.

Somatic cell nuclear transfer (SCNT) The nonsexual generation of nuclear genome-identical offspring ("cloned animals") by reconstitution of an enucleated oocyte with the diploid nucleus of a somatic cell to a zygote, which under appropriate culture conditions leads to reprogramming of the genome, enabling embryonic and fetal development.

Zoonotic infection The ability of a given pathogen to cross the host species barrier, from its current or long-term evolutionary host to animals and humans and thereby causing disease.

Definition of the Subject and Its Importance

Infectious diseases of livestock are a major risk to global animal health and welfare. In addition, human health is influenced due to the zoonotic potential of some of these infections.

Moreover, livestock diseases significantly impair food production and safety and cause enormous economic losses worldwide.

Transgenic technology was first developed as a research tool for studying gene function in mice in

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

the early 1980s. The technique was extended and applied to other mammals in 1985. An interesting and challenging focus of agricultural transgenesis was the potential to increase disease resistance and/or reduce disease susceptibility by introducing new genes and/or deleting deleterious genes. The laborious improvements to original and recently developed transgene technologies lead to the generation of transgenic farm animals with improved resistance to infectious diseases, demonstrating the proof of principle that genetic engineering may potentially improve animal health and aid infectious disease control in livestock.

Introduction

Phenotype-driven traditional animal breeding and marker-assisted selection based on quantitative trait loci (QTLs) have been successfully used for the genetic improvement of many agricultural production traits such as body weight, carcass composition, or milk yield. However, these genetic selection strategies have not yet resulted in a significant increase in the resistance of farm animals to disease.

Currently, genomic sequences are available for several livestock species [1] and as a by-product of the sequencing, a huge number of single nucleotide polymorphisms (SNPs) were discovered. The large panels of available SNPs were used in genome-wide association (GWA) studies for mapping and identifying genes [2]. GWA studies have already been successful in identifying causal genes and mutations for monogenic traits [3], but not for complex or quantitative traits such as resistance or susceptibility to disease.

Furthermore, traditional strategies in combating devastating infectious diseases of livestock, such as vaccination, antibiotic treatment, or even culling, have, to date, been unsuccessful (Fig. 1). Parasites evolved to resist chemical or vaccine control measures and bacteria developed resistance to many antibiotics. So far, a single infectious viral disease in livestock, rinderpest (cattle plague) could be eliminated through large-scale vaccination.

As an alternative to the traditional approaches, genetic engineering of livestock species may assist in the fight against infectious diseases.

The oldest and probably the most robust technique to produce transgenic farm animals is the injection of DNA sequences into the pronucleus of recently fertilized zygotes [4–6]. Pronuclear microinjection was successfully used to generate the most important livestock species, mainly for production of highly valuable human therapeutics. A more recent method for generating transgenic animals is the nuclear transfer technology, that is, 'cloning' [7, 8], which, together with a gene-targeting strategy allows the generation of specific gene-targeted animals [9, 10]. Recently, lentiviral vector-based strategies have been established which results in highly efficient production of transgenic livestock [11, 12]. This method in combination with the RNAi-technology may lead to the generation of disease-resistant transgenic livestock in the near future [13].

In the following section, the authors present an overview of the various transgenic methods used for the genetic enhancement of animal resistance to infectious diseases. Many studies were initially done using transgenic mouse models as this model often provides useful preliminary results prior to initiation of livestock studies.

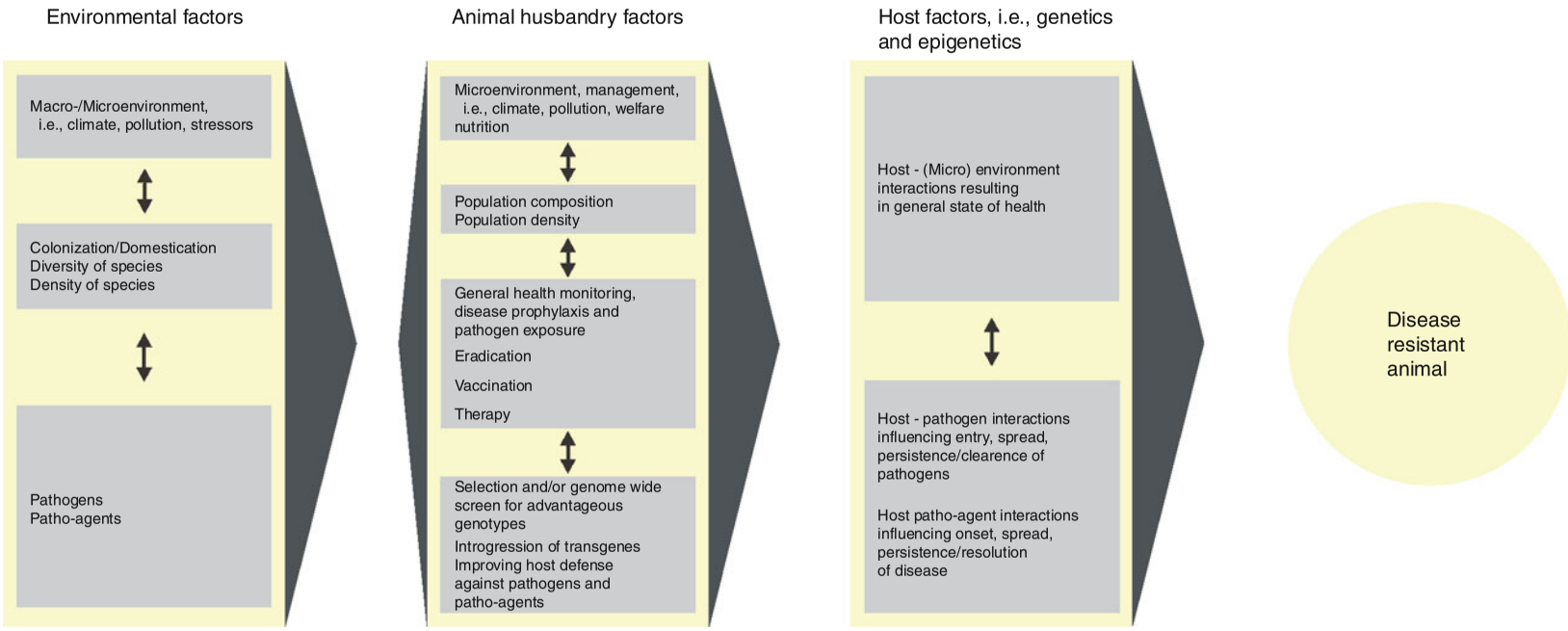
Disease-Resistant Transgenic Animals

Reducing farm animal susceptibilty to infectious diseases via genetic engineering has been an ambitious goal since the first transgenic livestock was generated more than 20 years ago. Various transgenic strategies for improving animal health are described elsewhere [14–17].

In general, disease-resistant transgenic farm animals can be generated by two approaches: (1) introduction of resistance genes into the genome of the host (gain-of-function strategy) and (2) specific targeting of endogenous or exogenous susceptibility genes (loss or exchange-of-function strategy).

Improving Animal Health through Gain-of-Function Gene Transfer

In most cases, susceptibility to pathogens originates from the interplay of numerous genes, meaning susceptibility to pathogens is polygenic in nature. The murine *Mx* gene is one of the few examples of a single genetic (monogenic) locus encoding a disease-resistance trait. Mice and mouse fibroblast cell lines carrying the autosomal dominant *Mx1* allele are



Disease-Resistant Transgenic Animals. Figure 1

Horizontal and vertical interrelations influencing the animal's health status, diseased versus nondiseased. The most specific measures to disrupt the pathogen/patho-agent flow toward the animal and/or improve the animal's disease defense mechanisms are highlighted

resistant to influenza virus infection [18, 19]. The transfer of the *Mx1* gene was able to restore virus resistance in mice lacking the *Mx1* allele [20] and inhibited influenza virus replication in avian cells [21]. However, the introduction of the murine *Mx1* gene into swine via pronuclear microinjection failed to produce influenza-resistant pigs [22]. The constitutive *Mx1* expression seemed to be detrimental to the pigs, whereas the expression from an inducible promoter was too low to produce detectable levels of *Mx1* protein. In the meantime, *Mx* genes of different farm animals have been identified, but their importance for disease susceptibility is not yet clear [23–25]. However, the ongoing detailed deciphering of the genomes of different farm animals, the improved techniques in generating transgenic animals [26–28], and the new tools for controlling transgene expression levels [29, 30] might allow the idea of generating influenza-resistant livestock by transferring a disease resistance gene to be addressed once more.

Antimicrobial peptides (AMPs) are an important component of the innate defense of most living organisms and there is a growing body of evidence to show that their role in defense against microbes is as important to the host as antibodies and innate and adaptive immune cells [31, 32]. AMPs are usually composed of 12–50 amino acids and synthesized by microorganisms as well as multicellular organisms, including plants and animals. They can have broad-spectrum antibacterial, antifungal, antiviral, antiprotozoan, and antisepsis properties. In addition to the wide range of these naturally occurring AMPs, many new ones have also been synthesized [33, 34]. Based on three-dimensional structural studies, the peptides are broadly classified into five major groups namely: (1) peptides that form alpha-helical structures; (2) peptides that form beta-sheets; (3) peptides rich in cysteine residues; (4) peptides rich in regular amino acids namely histatin, arginine, and proline; and (5) peptides composed of rare and modified amino acids [35, 36]. They can induce complete lysis of the organism by disrupting the membrane or by perturbing the membrane lipid bilayer, which allows for leakage of specific cellular components as well as dissipating the electrical potential of the membrane.

In initial engineering studies, the endogenous production of antimicrobial compounds in transgenic animals was shown to enhance disease resistance. Recombinant bovine tracheal antimicrobial peptide (bTAP) isolated from milk from transgenic mice, showed antimicrobial activity against *Escherichia coli*, without any deleterious side effects in suckling pups [37]. The antimicrobial activity of the synthetic alpha-helical peptide *Shiva 1a* was confirmed in transgenic mice, challenged with *Brucella abortus* [38]. The expression of the recombinant peptide significantly reduced both the bacterial colonization and the associated pathological changes in the genetically engineered mice.

Mastitis which is caused by bacterial infection of the mammary gland is reported to be the most costly disease in animal agriculture. It seriously affects animal well-being and is the most common reason for antibiotic use in diary cattle and the most frequent cause of antibiotic residues in milk [39]. The major contagious mastitis pathogen, *Staphylococcus aureus* is sensitive to lysostaphin, an antibacterial peptide naturally produced by a related bacterium, *Staphylococcus simulans* [40]. Kerr and colleagues showed that mammary gland expression of a bioactive variant of lysostaphin conferred protection against *S.aureus* infection in mice [41]. The staphylolytic activity in the milk of transgenic mice appeared to be 5–10 fold less active than bacterially derived lysostaphin, but was sufficient to confer substantial resistance to staphylococcal mastitis. Transgene production appeared to have no apparent effect on the physiology of the animal, the integrity of the mammary gland, or the milk it produces. Using nuclear transfer techniques, this approach was successfully extended to cattle, recently [42]. Transgenic dairy cows secreting lysostaphin constitutively in their milk were more resistant to *S. aureus* infections than nontransgenic animals. Lysostaphin concentrations in the milk of transgenic animals remained fairly constant during lactation. The recombinant lysostaphin was approximately 15% as active as bacterially derived protein. Challenge studies with *S. aureus* clearly demonstrated a direct correlation between the extent of protection against *S. aureus* infection with lysostaphin levels in the milk. Transgenic cows have been previously generated, primarily as bioreactors for large-scale production of pharmaceuticals and nutraceuticals. Thus, lysostaphin-transgenic cattle are the first example for enhancing disease resistance and animal welfare in

livestock, and may allow substantial reductions in antibiotic use. This in turn will help to control the spread of antibiotic-resistant bacteria and to reduce bacterial and antibiotic contamination of milk and milk products.

The antibacterial effect of lysostaphin is restricted to *S. aureus* only and transgenic cows are not protected against other mastitis-causing pathogens. The additional expression of secondary antibacterial compounds in the milk might be necessary for further enhancing mastitis resistance.

Human lysozyme (hLZ), a bacteriostatic milk protein that is known to attack the peptidoglycan component of bacterial cell walls, was expressed in the mammary gland of transgenic mice [43] and transgenic dairy goats [44]. Milk from the transgenic animals showed significant bacteriostatic activity and slowed the growth of several bacteria responsible for causing mastitis and the cold-spoilage of milk. The somatic cell count (SCC) is applied as a measure for udder health and milk quality and a high SCC in milk is directly correlated with mastitis and an impairment of milk quality [45]. Analyzing the SCC in milk samples of transgenic diary goats revealed a significant lower SCC compared to milk samples from control animals suggesting an improved udder health in the transgenic animals [46]. Lysozyme plays a role in the defense against gastrointestinal pathogens and reduces gastrointestinal illness in breastfed infants [47]. Feeding trials were conducted in pigs to evaluate putative health-promoting functions of hLZ-transgenic milk. Pigs are monogastric animals with a digestive system similar to humans and therefore are commonly used to study human health. Brundige and colleagues demonstrated that the consumption of pasteurized milk from hLZ-transgenic goats improved the gastrointestinal health of young piglets and was beneficial against a gastrointestinal infection with enteropathogenic *E. coli* [48].

A Chinese group enabled synthesis and secretion of bioactive bovine lactoferrin and bovine tracheal antibacterial peptides in goat mammary cells by use of plasmid-mediated gene transfer techniques [49], and the milk samples collected from these animals exhibited bacteriostatic effects against different mastitis-causing pathogens.

The authors summarize that genetic engineering for secretion of a broad range of AMPs in the mammary gland of dairy goats and cows reduces susceptibility to various microbial pathogens and is therefore a realistic approach to combat mastitis. Enhanced mastitis resistance will not only improve animal health and well-being, but also reduces bacterial contamination of milk and milk products in addition to reducing the costs incurred during disease prevention and cure.

Transgenic mice, expressing and processing a human enteric alpha-defensin peptide exclusively in specialized epithelia of the small intestinal crypt were generated, and were immune to an oral challenge with virulent *Salmonella typhimurium* [50].

Protegin-1 (PG-1) that is normally expressed in porcine myeloid cells and resides in secretory granules of neutrophils is another potent antimicrobial peptide targeting both gram-negative and gram-positive bacteria [51]. The ectopic expression of PG-1 in transgenic mice conferred enhanced respiratory resistance to an intranasal challenge with *Actinobacillus suis* [52], an opportunistic pathogen that may cause pneumonia, abortion, and fatal septicemia in pigs of all ages [53, 54]. Extending this concept to pigs and other somatic tissues beyond neutrophils will be another step toward the development of disease-resistant livestock.

The overexpression of dominant-negative mutants of viral proteins or pathogen receptors is another potent strategy to enhance animal disease resistance. The major focus has been to block viral attachment and penetration into a host cell by (1) producing viral proteins that block cellular receptors (antireceptor) or (2) altering known host molecular components, such as replacing host receptor genes with a modified version which is able to perform the receptor's physiological function but prevents attachment of the virus [55]. The first successful introduction of pathogen-mediated disease resistance in animals was reported 20 years ago. Transgenic chickens expressing the viral envelope of a recombinant avian leukosis retroviral genome were resistant to the corresponding subgroup of avian leukosis virus due to blockage of the virus receptors by the viral envelope proteins [56]. Using the same strategy, Clements et al. generated transgenic sheep expressing the maedi-visna virus envelope (E) gene, which is responsible for virus attachment to the host cells [57]. Maedi-visna virus is a prototype of ovine lentiviruses that cause encephalitis, pneumonia, and arthritis in sheep. Transgenic lambs expressing the

viral E glycoprotein in monocytes/macrophages, the target cells for virus replication, were healthy and neither deleterious effects nor clinical abnormalities from the transgene was observed. However, up to date, challenge studies to determine the susceptibility of these animals to ovine lentiviruses have not been reported.

Transgenic mice expressing a soluble form of porcine nectin-1, the cellular receptor for α-herpesviruses were generated. These mice displayed high resistance to pseudorabies virus (PRV) infections [58]. In pigs, PRV causes lethal encephalitis, acute respiratory syndrome, abortion and infertility, and latent infections [59]. Analysis of transgenic mouse lines, ubiquitously expressing different soluble forms of the cellular receptor for the viral glycoprotein D revealed that the transgene encoding the soluble form of the entire ectodomain of porcine nectin-1 fused to the human IgG1 conferred highest resistance to intranasal and intraperitonal PRV infections without any side effects [60]. Surprisingly, the expression of a fusion protein consisting of the first Ig-like domain of nectin-1 and the Fc portion of porcine IgG1 not only resulted in reduced virus resistance but also caused microphthalmia and the lack of vitreous bodies [61, 62]. Before implementing this promising approach to the generation of α-herpesviruses-resistant swine, further investigations examining the interactions of different soluble forms of nectin-1, endogenous nectins, and viral glycoprotein D and analysis of the influence of Fc domains of different species are required.

An alternative transgenic approach to protect livestock against infectious diseases is the expression of genes directing the synthesis of defined antibodies which target specific pathogens and thus induce immediate immunity without prior exposure to that pathogen.

Initial studies to express gene constructs encoding monoclonal antibodies in transgenic livestock were conducted nearly 20 years ago [63, 64]. However, the recombinant antibodies expressed in transgenic rabbits, sheep, and pigs showed aberrant sizes and only low antigen binding affinity. Nevertheless, following this idea, transgenic mice expressing coronavirus-neutralizing antibodies in the mammary gland were generated [65, 66]. High antibody expression titres throughout the lactation period provided complete protection against the enteric infection of newborns with transmissible gastroenteritis virus (TGEV), a pathogen which produces high mortality in suckling piglets, and also against a murine hepatitis virus (MHV)-induced encephalitis. Following this strategy, manipulating the lactogenic immunity in farm animals could improve the protection of suckling newborns through colostrium-delivered antibodies [67].

Enhancing Disease Resistance by Targeting Endogenous Susceptibility Genes

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative disorders of the central nervous system which are termed scrapie in goat and sheep and bovine spongiform encephalopathy (BSE) in cattle. According to current knowledge, the causative agent of the brain pathology in diseased animals is the prion. Prion diseases are characterized by the accumulation of the abnormally folded and protease-resistant isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) of the host [68, 69]. The generation of prion-free livestock resistant to TSE has been an ambitious goal since the BSE epidemic in cattle in the UK and the appearance of a new and highly lethal variant of Creutzfeldt-Jakob disease (vCJD) in humans. Early studies in mice revealed that reduction or loss of PrP^{C} expression did not affect normal development of the mice, but conferred protection against scrapie disease after inoculation with PrP^{Sc} prions [70–73]. With the development of nuclear transfer cloning techniques using genetically modified embryonic or somatic cell donors [7–10], the possible 'knock out' of the prion gene in transgenic sheep, goats, and cattle has opened new perspectives for the generation of disease-resistant livestock. A decade ago, Denning and colleagues generated the first PrP^{C}-targeted lambs. However, none of the cloned sheep survived more than 12 days [74]. Analyses of the targeted fetuses and lambs revealed defects that have been described in other nuclear transfer experiments with nontransfected cells and therefore, the authors expected that the early death of the lambs was not a consequence of the PrP^{C} disruption per se, but was probably due to the nuclear transfer procedures and/or the prolonged culture and drug selection of the primary fibroblasts used for nuclear transfer.

The functional disruption of the caprine PrP^{C} gene in cloned goats was first described by Yu et al. [75] and resulted in two goats lacking the prion protein [76].

The scientists confirmed the complete PrP^C ablation at mRNA and protein levels, and at 2 months age, the PrP^C null goats were healthy and showed no developmental or behavioral defects. The scientific community is awaiting the final proof of the concept – scrapie resistance of PrP^C-deficient goats after infection with PrP^{Sc} prions.

Richt and colleagues described the generation of the first PrP^C-deficient cattle [77]. They used a sequential gene targeting strategy which was demonstrated for the first time by the group of Kuroiwa et al. [78]. Male Holstein primary fibroblasts were transfected with two knockout vectors to sequentially disrupt the two alleles of the PrP^C gene. PrP^C-deficient fetal cell lines were established at 40–75 days of gestation and recloned for the generation of calves. The impact of PrP^C deficiency on calf development, on the immune system, on growth, and general health of the cattle for at least 20 months was analyzed in detail, and no negative influence of PrP^C ablation on animal health and well-being was detected. Importantly, brain homogenates from 10-month-old PrP^C-deficient cattle prevented PrP^{Sc} propagation in vitro, whereas in brain homogenates from wild-type cattle PrP^{Sc} proliferated. The researchers concluded that the presence of the endogenous bovine PrP^C is essential for PrP^{Sc} propagation and that there are no other host-derived cellular factors that can support the in vitro PrP^{Sc} propagation in the absence of the endogenous bovine PrP^C. In vivo tests of resistance to prion propagation in PrP^C-deficient cattle are under way, but still will require some years to complete. Analyses of several PrP^C-targeted mouse lines indicated that the loss of the normal cellular function of PrP^C may adversely affect the animals. For example, PrP^C-deficient mice developed ataxia and cerebellar neurodegeneration [79, 80], slight alterations in sleep–wake circadian rhythm [81], and altered synaptic functions [82]. To date, none of the above-described alterations in PrP^C null mice could be observed in PrP^C-deficient cattle and goats, respectively, but further investigations on aged transgenic animals will be necessary to exclude these altered phenotypes.

Small interfering RNAs (siRNAs) can silence/shut down specific targeted genes by interfering with the RNA transcripts they produce [83, 84]. For a transient gene 'knock down,' synthetic siRNAs can be directly transfected into cells or early embryos. However, for stable gene expression and germline transmission, the siRNA sequences are incorporated into gene constructs which express short hairpin (sh)RNAs that are processed to siRNAs within the cell. Through stably integrated shRNA expression vectors, additional genetic information is introduced into an organism (gain-of-functions strategy), which then produces a 'knock down' phenotype that is functionally similar to a 'knock out' (loss of function). Thus, RNAi-transgenics is an interesting alternative to the homologous gene targeting strategies which are traditionally used for the generation of 'knock out' livestock.

One of the most interesting susceptibility genes in livestock is the PrP^C gene and in a preliminary in vitro experiment, it was demonstrated that siRNA suppression of the PrP^C gene abrogates the PrP^C synthesis and inhibits the formation of PrP^{SC} protein in chronically scrapie-infected murine neuroblastoma cells [85]. Shortly after, Golding and colleagues combined this RNAi-based technique with lentiviral transgenesis for targeting the PrP^C gene in an adult goat fibroblast cell line, which was then used for somatic cell nuclear transfer to produce a cloned goat fetus [13]. Protein analyses of brain tissues demonstrated that PrP^C expression was reduced >90% in the cloned transgenic fetus when compared with a control. In a further experiment, they injected the recombinant lentivirus directly into the perivitelline space of bovine ova. Development of more than 30% of injected ova to blastocysts and expression of the shRNA targeting the PrP^C gene in more than 70% provides strong evidence that this RNAi approach may be useful in creating genetically engineered farm animals with natural resistance to prion diseases.

In two further approaches, lentiviral-mediated delivery of shRNA expression vectors into the brain of scrapie-infected mice resulted in a clear reduction of the PrP^C protein level and a prolonged survival of infected mice [86, 87], inferring that RNAi-technology may also be used for therapeutic applications.

Enhancing Disease Resistance by Targeting Exogenous Susceptibility/Viral Genes

Another application of the RNAi-technology is the silencing of exogenous viral genes through the

introduction of specific dsRNA molecules into cells, where they are targeted to essential genes or directly to the viral genome, thus inhibiting viral replication [88, 89]. Currently, the use of RNAi-based strategies for generation of viral disease-resistant livestock focuses on three pathogens: food and mouth disease virus (FMDV), bovine viral diarrhea virus (BVDV), and influenza A viruses.

FMDV is an extremely contagious pathogen that affects cattle, swine, and other livestock worldwide [90]. FMD is difficult to control by vaccination and impossible to eliminate by conservative natural breeding. Initial studies tested specific FMDV-siRNAs for their ability to inhibit virus replication in BHK-21 cells [91]. Transfection of BHK-21 cells with a mixture of siRNAs targeting highly conserved sequences of the 3B region and the 3D polymerase gene in all FMDV serotypes resulted in nearly 100% suppression of virus growth.

In another approach, siRNAs were designed to specifically target the viral VP1 gene, which plays a key role in virus attachment. This resulted in a nearly 90% reduction in FMDV VP1 expression and conferred resistance to FMDV challenge in cultured cells which are susceptible to this virus [92]. Encouragingly, pretreatment with siRNAs before infection made suckling mice significantly less susceptible to FMDV, and expression of siRNAs directed against the viral nonstructural protein 2B clearly inhibited virus replication in infected porcine cells [93].

Another RNAi target of agricultural interest is the bovine viral diarrhea virus (BVDV), an ubiquitously occurring pathogen that affects cattle herds worldwide resulting in respiratory disorders and increased susceptibility to other pathogens [94]. Lambeth and his group demonstrated that BVDV replication in bovine cells can be efficiently suppressed by RNAi [95]. They transfected shRNA expression vectors and siRNAs targeting the 5′ nontranslated region (NTR) and the region encoding the C protein of the viral genome into MDBK cells. After challenging with BVDV, they detected reduced virus titres by both siRNA and shRNA-mediated RNAi.

Farm animals, in particular swine and poultry, serve as key links between the natural reservoir of influenza A viruses and epidemics and pandemics in human populations. Due to repeated reassortment or mixing of RNA segments between influenza viruses from different species, virulent strains emerge periodically and often lead to devastating human catastrophes [96]. However, the emergence of the RNAi technology has opened many new options for preventing influenza virus infections in animals.

In initial studies, a set of siRNAs specific for conserved regions of the influenza virus genome could potently inhibit virus production in MDCK cells and embryonated chicken eggs [97]. In subsequent approaches, this strategy was extended to an established animal model of influenza infections by two independent groups. Tompkins and colleagues used siRNAs for targeting highly conserved regions of the viral nucleoprotein (NP) and acidic polymerase (PA). After administration of influenza virus-specific siRNAs via hydrodynamic i.v. injection [98], BALB/c mice were infected intranasally with influenza A/H1N1. Virus titre in lung homogenates were significantly reduced in siRNAs-treated mice when compared to control mice 48 h p.i [99]. In addition, they demonstrated that influenza-specific siRNA treatment can protect mice from otherwise lethal virus challenges.

Ge and coworkers administered influenza virus-specific siRNAs intravenously along with lentiviral shRNA expression vectors into C57BL/6 mice. They demonstrated that siRNAs as well as shRNAs can reduce influenza virus production in the lung when given either before or after virus infection and that the simultaneous use of two or more siRNAs specific for different virus genes resulted in a more severe reduction of virus titres [100].

A promising approach for the generation of influenza-resistant livestock was published by Wise and colleagues [101]. They used shRNA expression vectors, targeting the viral NP and PA gene for lentiviral-mediated generation of transgenic mice. Expression of the siRNAs was confirmed by an RNAse protection assay, and thus far, stable transmission of the transgene was observed up to the third generation. Currently, transgenic mice are mated to generate homozygous lines for delivering the final proof for influenza virus resistance in vivo.

Recently the generation of transgenic chicken expressing a shRNA molecule able to inhibit influenza virus polymerase activity [115] was reported. Although

the transgenic chicken did not exhibit a higher resistance to high challenge doses of H5N1, a highly pathogenic avian influenza virus, they showed strongly reduced transmission of the infection to transgenic and even non-transgenic birds housed in direct contact with them, demonstrating that this strategy may be used to prevent transmission and propagation of an infection at the flock level.

Future Directions

The past decade was dominated by large-scale and high throughput nucleic acid analyses allowing comparative genome sequencing and expression profiling projects. The comprehensive and ongoing analysis of the huge data sets led to the need for an updated definition of the term 'gene' and the introduction of the term 'epigenetics.' Taking into account that Mendel's and Morgan's elements of heredity include multifunctional protein coding, structural, regulatory, and RNAs of unknown functions and gene regulation is more complex than previously assumed, the 'gene' is suggested to be 'a union of genomic sequences encoding a coherent set of potentially overlapping functional products' [102] and 'epigenetics' is defined to describe 'stably inheritable phenotypes resulting from changes in a chromosome without alterations of the DNA-sequence' [103]. The future challenge of the postgenomic era is subsumed as integrative, quantitative, and/or systems biology. 'Systems biology is the comprehensive and quantitative analysis of the interactions between all of the components of biological systems over time' [104]. 'Systems biology involves an iterative cycle, in which emerging biological problems drive the development of new technologies and computational tools' [104]. The further understanding of disease mechanisms also depends on these emerging disciplines.

The ongoing genome sequencing programs for various animal species and the increasing densities of SNP arrays will lead to the discovery of new QTLs underlying economically important traits such as disease resistance and susceptibility. In addition, complete genome sequences of many disease-causing pathogens are becoming available. Hence, genome data on host intrinsic factors and host–pathogen interactions causing disease can be used to increase the health of individuals or populations. Conventional breeding and genomic selection will increasingly benefit from the natural variations identified among the populations. This can be supplemented with gene transfer technologies allowing a more targeted approach toward desired animal breeding without the limitation of species barriers.

The future of transgene technologies is dependent on the simplification of the gene delivery systems along with targeted manipulation of animal genomes. The former aim is achieved by using lentiviral vectors which are highly efficient for domesticated animals including poultry [105, 106] and pets [107]. Gene targeting in species other than mice is limited as embryonic stem (ES) cells of farm or pet animals are unavailable and gene targeting via homologous recombination of embryonic and somatic cells and subsequent nuclear transfer is highly inefficient. However, the advent of the RNAi-technology offers new possibilities for specific gene targeting in animal species and will have a huge impact on transgenesis in the near future. Furthermore, the zinc finger nuclease (ZNF) technology has shown to be an attractive alternative to ES cell targeting and nuclear transfer technology [108] and was already applied successfully for targeted gene disruption in rats [109].

For further reading concerning the use of ZFN-technology in farm animals we refer to Kues and Niemann [116]. In addition, site-directed mutagenesis of genomes can be achieved by TALENs (transcription activator-like effector nucleases) which were originally identified in plant pathogens and recently were successfully used to generate knockout rats [117]. These site-specific nucleases may complement/enlarge the well established ZFN-technology for efficient gene targeting in livestock [118].

Last but not least, the cross-species generation of pluripotent/embryonic cell lines has gained new impetus through the induced pluripotent stem cell (iPS) technology, i.e., the reprogramming of somatic cells making them capable of embryogenesis (reviewed in [110]) and the recent isolation of authentic embryonic stem cells from rat blastocysts by novel culture conditions [111, 112]. In the future, animal transgenetics and animal disease resistance will be important in basic research and in the understanding of disease mechanisms. Bridging the gap between model and man by

generating transgenic animals is fundamental to the development of novel therapeutics and disease prevention strategies.

Increased availability of genomic information of livestock species along with more sophisticated transgenic tools offers the potential to generate animal models to combat livestock diseases to a larger extent than ever before. However, animal geneticists/scientists must consider several important aspects. (1) The dissemination of the trait of interest such as disease resistance, introduced by a transgene will neither be simple nor fast, therefore cost–benefit calculations will probably decide on implementation of transgenic animals. For example, transgenic BSE-resistant cattle [77] will probably never gain importance in agriculture where culling is considered to meet demands with respect to cost efficiency and biosafety. However, BSE-resistant cattle may be engineered for the production of pharmaceuticals and therefore will have an enormous impact on providing safer drugs. (2) There is general public opposition to the use of transgenic livestock. However, if animals were resistant to zoonotic diseases, therefore resulting in reduced frequency of pandemics and epidemics such as those caused by influenza virus, attitudes of human societies might change [113]. In this context, recently, a trypansome lytic factor (TLF) from baboons that protected mice both from animal and human-infective *Trypanosoma* subspecies was identified and suggested to be transferred to livestock [114]. Animal trypanosomiasis is one of the major parasitic diseases of livestock flocks and livestock are the major reservoir for human-pathogenic trypanosomes. (3) Scientists and society should clearly keep in mind that pathogens readily change their antigenic determinants and create novel subtypes to escape the 'resistant' host's immune system. Attempts to introduce resistance traits into animal populations either by conventional breeding or transgenesis should be subjected to thorough cost/detriment–benefit analyses.

Bibliography

1. de Koning DJ, Archibald A, Haley CS (2007) Livestock genomics: bridging the gap between mice and men. Trends Biotechnol 25:483–489
2. Goddard ME, Hayes BJ (2009) Mapping genes for complex traits in domestic animals and their use in breeding programmes. Nat Rev Genet 10:381–391
3. Charlier C, Coppieters W, Rollin F, Desmecht D, Agerholm JS, Cambisano N, Carta E, Dardano S, Dive M, Fasquelle C, Frennet JC, Hanset R, Hubin X, Jorgensen C, Karim L, Kent M, Harvey K, Pearce BR, Simon P, Tama N, Nie H, Vandeputte S, Lien S, Longeri M, Fredholm M, Harvey RJ, Georges M (2008) Highly effective SNP-based association mapping and management of recessive defects in livestock. Nat Genet 40:449–454
4. Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
5. Brem G, Brenig B, Goodmann HM, Selden RC, Graf F, Kruff B, Springman K, Hondele J, Meyer J, Winnacker E-L, Kräußlich H (1985) Production of transgenic mice, rabbits and pigs by microinjection into pronuclei. Reprod Domest Anim 20:251–252
6. Pursel VG, Hammer RE, Bolt DJ, Palmiter RD, Brinster RL (1990) Integration, expression and germ-line transmission of growth-related genes in pigs. J Reprod Fertil Suppl 41:77–87
7. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM (1998) Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280:1256–1258
8. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813
9. Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66
10. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130–2133
11. Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4:1054–1060
12. Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA (2004) Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett 571:233–236
13. Golding MC, Long CR, Carmell MA, Hannon GJ, Westhusin ME (2006) Suppression of prion protein in livestock by RNA interference. Proc Natl Acad Sci USA 103:5285–5290
14. Muller M, Brem G (1994) Transgenic strategies to increase disease resistance in livestock. Reprod Fertil Dev 6:605–613
15. Muller M, Brem G (1996) Intracellular, genetic or congenital immunisation–transgenic approaches to increase disease resistance of farm animals. J Biotechnol 44:233–242
16. Muller M, Brem G (1998) Transgenic approaches to the increase of disease resistance in farm animals. Rev Sci Tech 17:365–378
17. Whitelaw CB, Sang HM (2005) Disease-resistant genetically modified animals. Rev Sci Tech 24:275–283
18. Haller O, Arnheiter H, Gresser I, Lindenmann J (1981) Virus-specific interferon action. Protection of newborn Mx

carriers against lethal infection with influenza virus. J Exp Med 154:199–203
19. Staeheli P, Haller O, Boll W, Lindenmann J, Weissmann C (1986) Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. Cell 44:147–158
20. Arnheiter H, Skuntz S, Noteborn M, Chang S, Meier E (1990) Transgenic mice with intracellular immunity to influenza virus. Cell 62:51–61
21. Garber EA, Chute HT, Condra JH, Gotlib L, Colonno RJ, Smith RG (1991) Avian cells expressing the murine Mx1 protein are resistant to influenza virus infection. Virology 180:754–762
22. Muller M, Brenig B, Winnacker EL, Brem G (1992) Transgenic pigs carrying cDNA copies encoding the murine Mx1 protein which confers resistance to influenza virus infection. Gene 121:263–270
23. Ko JH, Jin HK, Asano A, Takada A, Ninomiya A, Kida H, Hokiyama H, Ohara M, Tsuzuki M, Nishibori M, Mizutani M, Watanabe T (2002) Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res 12:595–601
24. Thomas AV, Palm M, Broers AD, Zezafoun H, Desmecht DJ (2006) Genomic structure, promoter analysis, and expression of the porcine (Sus scrofa) Mx1 gene. Immunogenetics 58:383–389
25. Ellinwood NM, McCue JM, Gordy PW, Bowen RA (1998) Cloning and characterization of cDNAs for a bovine (Bos taurus) Mx protein. J Interferon Cytokine Res 18:745–755
26. Whitelaw CB, Lillico SG, King T (2008) Production of transgenic farm animals by viral vector-mediated gene transfer. Reprod Domest Anim 43(Suppl 2):355–358
27. Pfeifer A (2004) Lentiviral transgenesis. Transgenic Res 13:513–522
28. Park F (2007) Lentiviral vectors: are they the future of animal transgenesis? Physiol Genomics 31:159–173
29. Aigner B, Klymiuk N, Wolf E (2010) Transgenic pigs for xenotransplantation: selection of promoter sequences for reliable transgene expression. Curr Opin Organ Transplant 15(2):201–206
30. Kues WA, Schwinzer R, Wirth D, Verhoeyen E, Lemme E, Herrmann D, Barg-Kues B, Hauser H, Wonigeit K, Niemann H (2006) Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. FASEB J 20:1200–1202
31. Hancock RE, Diamond G (2000) The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 8:402–410
32. Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. Nat Immunol 6:551–557
33. Lee DG, Hahm KS, Shin SY (2004) Structure and fungicidal activity of a synthetic antimicrobial peptide, P18, and its truncated peptides. Biotechnol Lett 26:337–341
34. Powers JP, Hancock RE (2003) The relationship between peptide structure and antibacterial activity. Peptides 24:1681–1691
35. Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1462:11–28
36. Reddy KV, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 24:536–547
37. Yarus S, Rosen JM, Cole AM, Diamond G (1996) Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice. Proc Natl Acad Sci USA 93:14118–14121
38. Reed WA, Elzer PH, Enright FM, Jaynes JM, Morrey JD, White KL (1997) Interleukin 2 promoter/enhancer controlled expression of a synthetic cecropin-class lytic peptide in transgenic mice and subsequent resistance to Brucella abortus. Transgenic Res 6:337–347
39. Erskine RJ, Walker RD, Bolin CA, Bartlett PC, White DG (2002) Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J Dairy Sci 85:1111–1118
40. Kumar JK (2008) Lysostaphin: an antistaphylococcal agent. Appl Microbiol Biotechnol 80:555–561
41. Kerr DE, Plaut K, Bramley AJ, Williamson CM, Lax AJ, Moore K, Wells KD, Wall RJ (2001) Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. Nat Biotechnol 19:66–70
42. Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N, Hawk HW (2005) Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. Nat Biotechnol 23:445–451
43. Maga EA, Anderson GB, Huang MC, Murray JD (1994) Expression of human lysozyme mRNA in the mammary gland of transgenic mice. Transgenic Res 3:36–42
44. Maga EA, Shoemaker CF, Rowe JD, Bondurant RH, Anderson GB, Murray JD (2006) Production and processing of milk from transgenic goats expressing human lysozyme in the mammary gland. J Dairy Sci 89:518–524
45. Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky L, Gonzalez RN (2003) Monitoring udder health and milk quality using somatic cell counts. Vet Res 34:579–596
46. Maga EA, Cullor JS, Smith W, Anderson GB, Murray JD (2006) Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. Foodborne Pathog Dis 3:384–392
47. Lonnerdal B (2003) Nutritional and physiologic significance of human milk proteins. Am J Clin Nutr 77:1537S–1543S
48. Brundige DR, Maga EA, Klasing KC, Murray JD (2008) Lysozyme transgenic goats' milk influences gastrointestinal morphology in young pigs. J Nutr 138:921–926
49. Zhang J, Li L, Cai Y, Xu X, Chen J, Wu Y, Yu H, Yu G, Liu S, Zhang A, Cheng G (2008) Expression of active recombinant human lactoferrin in the milk of transgenic goats. Protein Expr Purif 57:127–135
50. Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL (2003) Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature 422:522–526
51. Panyutich A, Shi J, Boutz PL, Zhao C, Ganz T (1997) Porcine polymorphonuclear leukocytes generate extracellular microbicidal activity by elastase-mediated activation of secreted proprotegrins. Infect Immun 65:978–985

52. Cheung QC, Turner PV, Song C, Wu D, Cai HY, MacInnes JI, Li J (2008) Enhanced resistance to bacterial infection in protegrin-1 transgenic mice. Antimicrob Agents Chemother 52:1812–1819
53. Mauch C, Bilkei G (2004) Actinobacillus suis, a potential cause of abortion in gilts and low parity sows. Vet J 168:186–187
54. McManus MT, Sharp PA (2002) Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3:737–747
55. Gavora J (1996) Resistance to livestock to viruses: mechanisms and strategies for genetic engineering. Genet Sel Evol 28:385–414
56. Salter DW, Crittenden LB (1989) Artificial insertion of a dominant gene for resistance to avian leukosis virus into the germ line of the chicken. Theor Appl Genet 77:457–461
57. Clements JE, Wall RJ, Narayan O, Hauer D, Schoborg R, Sheffer D, Powell A, Carruth LM, Zink MC, Rexroad CE (1994) Development of transgenic sheep that express the visna virus envelope gene. Virology 200:370–380
58. Ono E, Amagai K, Taharaguchi S, Tomioka Y, Yoshino S, Watanabe Y, Cherel P, Houdebine LM, Adam M, Eloit M, Inobe M, Uede T (2004) Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock. Proc Natl Acad Sci USA 101:16150–16155
59. Mulder WA, Pol JM, Gruys E, Jacobs L, De Jong MC, Peeters BP, Kimman TG (1997) Pseudorabies virus infections in pigs. Role of viral proteins in virulence, pathogenesis and transmission. Vet Res 28:1–17
60. Ono E, Tomioka Y, Watanabe Y, Amagai K, Taharaguchi S, Glenisson J, Cherel P (2006) The first immunoglobulin-like domain of porcine nectin-1 is sufficient to confer resistance to pseudorabies virus infection in transgenic mice. Arch Virol 151:1827–1839
61. Tomioka Y, Morimatsu M, Amagai K, Kuramochi M, Watanabe Y, Kouda S, Wada T, Kuboki N, Ono E (2009) Fusion protein consisting of the first immunoglobulin-like domain of porcine nectin-1 and Fc portion of human IgG1 provides a marked resistance against pseudorabies virus infection to transgenic mice. Microbiol Immunol 53:8–15
62. Yoshida K, Tomioka Y, Kase S, Morimatsu M, Shinya K, Ohno S, Ono E (2008) Microphthalmia and lack of vitreous body in transgenic mice expressing the first immunoglobulin-like domain of nectin-1. Graefes Arch Clin Exp Ophthalmol 246:543–549
63. Lo D, Pursel V, Linton PJ, Sandgren E, Behringer R, Rexroad C, Palmiter RD, Brinster RL (1991) Expression of mouse IgA by transgenic mice, pigs and sheep. Eur J Immunol 21:1001–1006
64. Weidle UH, Lenz H, Brem G (1991) Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs. Gene 98:185–191
65. Castilla J, Pintado B, Sola I, Sanchez-Morgado JM, Enjuanes L (1998) Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. Nat Biotechnol 16:349–354
66. Kolb AF, Pewe L, Webster J, Perlman S, Whitelaw CB, Siddell SG (2001) Virus-neutralizing monoclonal antibody expressed in milk of transgenic mice provides full protection against virus-induced encephalitis. J Virol 75:2803–2809
67. Saif LJ, Wheeler MB (1998) WAPping gastroenteritis with transgenic antibodies. Nat Biotechnol 16:334–335
68. Aguzzi A, Calella AM (2009) Prions: protein aggregation and infectious diseases. Physiol Rev 89:1105–1152
69. Aguzzi A, Sigurdson C, Heikenwaelder M (2008) Molecular mechanisms of prion pathogenesis. Annu Rev Pathol 3:11–40
70. Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C (1993) Mice devoid of PrP are resistant to scrapie. Cell 73:1339–1347
71. Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature 356:577–582
72. Prusiner SB, Groth D, Serban A, Koehler R, Foster D, Torchia M, Burton D, Yang SL, DeArmond SJ (1993) Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. Proc Natl Acad Sci USA 90:10608–10612
73. Weissmann C, Bueler H, Fischer M, Sailer A, Aguzzi A, Aguet M (1994) PrP-deficient mice are resistant to scrapie. Ann NY Acad Sci 724:235–240
74. Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, de Sousa P, Travers A, Wilmut I, Clark AJ (2001) Deletion of the alpha (1, 3) galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat Biotechnol 19:559–562
75. Yu G, Chen J, Yu H, Liu S, Xu X, Sha H, Zhang X, Wu G, Xu S, Cheng G (2006) Functional disruption of the prion protein gene in cloned goats. J Gen Virol 87:1019–1027
76. Yu G, Chen J, Xu Y, Zhu C, Yu H, Liu S, Sha H, Xu X, Wu Y, Zhang A, Ma J, Cheng G (2009) Generation of goats lacking prion protein. Mol Reprod Dev 76:3
77. Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, Sathiyaseelan J, Wu H, Matsushita H, Koster J, Kato S, Ishida I, Soto C, Robl JM, Kuroiwa Y (2007) Production of cattle lacking prion protein. Nat Biotechnol 25:132–138
78. Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, Tomizuka K, Ishida I, Robl JM (2004) Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet 36:775–780
79. Moore RC, Lee IY, Silverman GL, Harrison PM, Strome R, Heinrich C, Karunaratne A, Pasternak SH, Chishti MA, Liang Y, Mastrangelo P, Wang K, Smit AF, Katamine S, Carlson GA, Cohen FE, Prusiner SB, Melton DW, Tremblay P, Hood LE, Westaway D (1999) Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. J Mol Biol 292:797–817
80. Rossi D, Cozzio A, Flechsig E, Klein MA, Rulicke T, Aguzzi A, Weissmann C (2001) Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. EMBO J 20:694–702

81. Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA, Manson JC (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. Nature 380:639–642
82. Collinge J, Whittington MA, Sidle KC, Smith CJ, Palmer MS, Clarke AR, Jefferys JG (1994) Prion protein is necessary for normal synaptic function. Nature 370:295–297
83. Melo EO, Canavessi AM, Franco MM, Rumpf R (2007) Animal transgenesis: state of the art and applications. J Appl Genet 48:47–61
84. Hannon GJ (2002) RNA interference. Nature 418:244–251
85. Daude N, Marella M, Chabry J (2003) Specific inhibition of pathological prion protein accumulation by small interfering RNAs. J Cell Sci 116:2775–2779
86. Pfeifer A, Eigenbrod S, Al-Khadra S, Hofmann A, Mitteregger G, Moser M, Bertsch U, Kretzschmar H (2006) Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. J Clin Invest 116:3204–3210
87. White MD, Farmer M, Mirabile I, Brandner S, Collinge J, Mallucci GR (2008) Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease. Proc Natl Acad Sci USA 105:10238–10243
88. Berkhout B, Haasnoot J (2006) The interplay between virus infection and the cellular RNA interference machinery. FEBS Lett 580:2896–2902
89. Colbere-Garapin F, Blondel B, Saulnier A, Pelletier I, Labadie K (2005) Silencing viruses by RNA interference. Microbes Infect 7:767–775
90. Grubman MJ, Baxt B (2004) Foot-and-mouth disease. Clin Microbiol Rev 17:465–493
91. Kahana R, Kuznetzova L, Rogel A, Shemesh M, Hai D, Yadin H, Stram Y (2004) Inhibition of foot-and-mouth disease virus replication by small interfering RNA. J Gen Virol 85:3213–3217
92. Chen W, Yan W, Du Q, Fei L, Liu M, Ni Z, Sheng Z, Zheng Z (2004) RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice. J Virol 78:6900–6907
93. de Los Santos T, Wu Q, de Avila Botton S, Grubman MJ (2005) Short hairpin RNA targeted to the highly conserved 2B nonstructural protein coding region inhibits replication of multiple serotypes of foot-and-mouth disease virus. Virology 335:222–231
94. Lindberg AL (2003) Bovine viral diarrhoea virus infections and its control. A review. Vet Q 25:1–16
95. Lambeth LS, Moore RJ, Muralitharan MS, Doran TJ (2007) Suppression of bovine viral diarrhea virus replication by small interfering RNA and short hairpin RNA-mediated RNA interference. Vet Microbiol 119:132–143
96. Cox NJ, Subbarao K (2000) Global epidemiology of influenza: past and present. Annu Rev Med 51:407–421
97. Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, Chen J (2003) RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc Natl Acad Sci USA 100:2718–2723
98. Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet 32:107–108
99. Tompkins SM, Lo CY, Tumpey TM, Epstein SL (2004) Protection against lethal influenza virus challenge by RNA interference in vivo. Proc Natl Acad Sci USA 101:8682–8686
100. Ge Q, Filip L, Bai A, Nguyen T, Eisen HN, Chen J (2004) Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc Natl Acad Sci USA 101: 8676–8681
101. Wise TG, Schafer DS, Lowenthal JW, Doran TJ (2008) The use of RNAi and transgenics to develop viral disease resistant livestock. Dev Biol (Basel) 132:377–382
102. Gerstein MB, Bruce C, Rozowsky JS, Zheng D, Du J, Korbel JO, Emanuelsson O, Zhang ZD, Weissman S, Snyder M (2007) What is a gene, post-ENCODE? History and updated definition. Genome Res 17:669–681
103. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A (2009) An operational definition of epigenetics. Genes Dev 23:781–783
104. Zak DE, Aderem A (2009) Systems biology of innate immunity. Immunol Rev 227:264–282
105. McGrew MJ, Sherman A, Ellard FM, Lillico SG, Gilhooley HJ, Kingsman AJ, Mitrophanous KA, Sang H (2004) Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep 5:728–733
106. Scott BB, Lois C (2005) Generation of tissue-specific transgenic birds with lentiviral vectors. Proc Natl Acad Sci USA 102:16443–16447
107. Hong SG, Kim MK, Jang G, Oh HJ, Park JE, Kang JT, Koo OJ, Kim T, Kwon MS, Koo BC, Ra JC, Kim DY, Ko C, Lee BC (2009) Generation of red fluorescent protein transgenic dogs. Genesis 47:314–322
108. Le Provost F, Lillico S, Passet B, Young R, Whitelaw B, Vilotte JL (2010) Zinc finger nuclease technology heralds a new era in mammalian transgenesis. Trends Biotechnol 28(3):134–141
109. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Menoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325:433
110. Belmonte JC, Ellis J, Hochedlinger K, Yamanaka S (2009) Induced pluripotent stem cells and reprogramming: seeing the science through the hype. Nat Rev Genet 10: 878–883
111. Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A (2008) Capture of authentic embryonic stem cells from rat blastocysts. Cell 135:1287–1298
112. Li P, Tong C, Mehrian-Shai R, Jia L, Wu N, Yan Y, Maxson RE, Schulze EN, Song H, Hsieh CL, Pera MF, Ying QL (2008) Germline competent embryonic stem cells derived from rat blastocysts. Cell 135:1299–1310
113. Chen J, Chen SC, Stern P, Scott BB, Lois C (2008) Genetic strategy to prevent influenza virus infections in animals. J Infect Dis 197(Suppl 1):S25–S28

114. Thomson R, Molina-Portela P, Mott H, Carrington M, Raper J (2009) Hydrodynamic gene delivery of baboon trypanosome lytic factor eliminates both animal and human-infective African trypanosomes. Proc Natl Acad Sci USA 106:19509–19514
115. Lyall J, Irvine RM, Sherman A, McKinley TJ, Núnez A, Purdie A, Outtrim L, Brown IH, Rolleston-Smith G, Sang H, Tiley L (2011) Suppression of avian influenza transmission in genetically modified chickens. Science 331:223–226
116. Kues WA, Niemann H (2011) Advances in farm transgenesis. PREVET, doi:10.1016
117. Tesson L, Usal C, Menoret S, Leung E, Niles BJ, Remy S, Santiago Y, Vincent AI, Meng X, Zhang L, Gregory PD, Anegon I, Cost GJ (2011) Knockout rats generated by embryo microinjection of TALENs. Nat Biotechnol 29:695–696
118. DeFrancesco L (2011) Move over ZFNs. Nat Biotechnol 29:681–684

Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii

Neil Anthony Sims
Kona Blue Water Farms, Inc, Kailua-Kona, HI, USA

Article Outline

Glossary

Sustainability The use of resources in such a manner as not to impinge upon the ability of future generations to enjoy similar use.

Open-ocean mariculture The culture of marine fish, invertebrates, or algae in exposed ocean locations.

Definition of the Subject

Open-ocean mariculture is the culture of marine fish, invertebrates, or algae in exposed ocean locations. The criteria for demarcation of open-ocean sites from inshore or nearshore aquaculture sites is often the subject of debate, with various schemes using some integration of distance from shore, depth of water, or exposure to open water and high sea conditions. A more meaningful definition is perhaps, simply, that an open-ocean mariculture site is a site where any of these conditions or criteria combine to disconnect the culture system from the surrounding substrates that might otherwise be subject to some significant environmental impact, or might become reservoirs for some ecological feedback with the culture system. Alternatively, rather than using a geographical or operational distinction, open-ocean mariculture might simply be described as culture of marine animals or plants at sites that are further offshore, or in deeper water. This definition reflects the evolving, aspirational, and incremental improvement in culture systems, rather than thinking of the open ocean as a final destination.

Whatever the definition, open-ocean mariculture offers the potential for a scalable production system for valuable, healthful seafood, with negligible environmental impacts.

Introduction

The Seafood Crisis

The oceans are in deep trouble. Even though the demand for seafood has increased, capture fisheries around the world are collapsing from overfishing, or are static. Wild stock fisheries cannot sustain any greater pressures, and clearly cannot scale to meet the growing needs of increasing population size, increasing affluence, and wider recognition of the health benefits of seafood.

The USA is both symptomatic of the seafood crisis and significantly contributive to the problem. In the USA, closures or buyback schemes to reduce effort have effectively shut down once-productive fisheries for Atlantic tunas and swordfish, the groundfish of Georges Bank and other Northeast fisheries, Pacific Coast sardines, albacore, and more recently, rockfish. Other environmental concerns for endangered species or marine mammals have seen closures or limitations placed on fisheries for shrimp in the Gulf of Mexico, purse seining for tuna in the Pacific, and long-lining for tuna and swordfish in Hawaii and the US Pacific. Currently, over 80% of the seafood consumed in the USA is imported, and more than half of those imports are from farmed sources.

Hawaii Fisheries Status The Hawaiian Islands represent a microcosm of the global fishing crisis. Increased fishing power – in terms of both the number of boats and available technologies – has seen the valuable deep bottom fish catches decline precipitously over the last few decades. GPS units allow deep bottom fishermen to return repeatedly and precisely to a "hot spot" until the fish are gone. Electric reels reduce the labor of hauling up from 60 fathoms to simply flicking

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

a switch. Powerful echo sounders allow tuna fishermen to identify the location of individual fish tens of fathoms beneath their boat. At the same time, catches of sashimi-grade tuna have plummeted due to competing gear-types (seiners, long-liners, pole-and-line) and conflicting national interests throughout the range of these highly migratory species. Some 20 years ago, over 25 commercial fishing boats worked out of the local Honokohau Harbor in Kona, Hawaii, targeting deep-water snappers and big-eye tuna in the "ika-shibi" fishery. Today, there is only one commercial fishing vessel based there, and even that is only part-time.

The decreasing catch volume and decreasing average size of fish caught in the bottom fish fishery in Hawaii is causing increasing concern. By 1996, only 20% of the onaga (*Etelis coruscans*) catch in the main Hawaiian Islands (MHI) had previously spawned; similar declines were evident among other species stocks. These species' biological characteristics make them vulnerable to recruitment overfishing; NOAA Fisheries staff estimate that an onaga attains maturity at about 4.1 years of age, at a size of 66 cm. With Federal and State data indicating significant overfishing of these stocks, increased regulation became imperative. In June 1998, new legislation went into effect, establishing limits on fishing gear, bag limits, registration of bottom fishing craft, and restricted fishing areas (up to 20% of the bottom fish ground was placed off limits) for the commercial and recreational fishery industry.

Further restrictions are still needed. Over the last two summers, the State declared all bottom fishing closed for the summer period from May 1st to September 30th, throughout the island range. State authorities have indicated that this seasonal closure may need to be repeated for coming years, as well, before there is any measurable improvement in stocks. The majority of high-value species consumed in Hawaii already are imported from other areas, such as the South Pacific and South East Asia.

Aquaculture as Part of the Solution Aquaculture offers the only viable solution to the growing demand for sustainable, healthy sources of seafood protein for human consumption. Fish farming reduces exploitative pressure on already depleted wild stocks, supports the growth of coastal and rural industries, and yields a product that is low in saturated fat and high in protein. The annual contribution of aquaculture to global aquatic production is now almost equal to that of wild catch (47% versus 53% [1]). In 1985, aquaculture represented only 5% of US fish consumption, yet today that figure stands at around 40%. Aquaculture growth is rapid, and is projected to increase in pace. The US Department of Commerce has set a goal of a fivefold increase in domestic aquaculture production value, to $5 billion, by 2025.

Domestic aquaculture production using existing methods or species cannot keep pace. Almost all US production is from freshwater species; the only marine species cultured in any quantity are salmon and striped bass, both of which are anadromous (freshwater spawning). It is almost impossible to obtain permits for nearshore farm sites; there is intense competition among different user groups for nearshore waters, and aquaculture has been besmirched in the conventional wisdom as environmentally destructive and unsustainable.

The Open-Ocean Potential Open-ocean mariculture technology has recently moved from the realm of science fiction [2, 3] to commercial reality. In the last few years, there have been dramatic advances in the legal and engineering fields, which have opened up the new fish farming frontier of offshore areas. New submersible net pen systems have been pioneered by OceanSpar, LLC, of Washington State ("Sea Station™" net pens), and Ocean Farm Technologies, Inc., of Maine ("Aquapod™" net pens). These new technologies have dramatically increased the workable extent of ocean farming, by providing seaworthy platforms for grow-out of fish in exposed offshore environments. These farms are therefore able to be located further offshore, in sites where currents, prevailing sea conditions or seasonal storm events may make surface pens inadvisable. These operations are also usually portrayed as having less environmental impact than nearshore fish farms. The rationale is that there is a cleaner and clearer disconnect between the farm and the underlying benthic substrate or the adjacent shoreline so that there is both minimal environmental impact and negligible potential for negative accumulative feedback from the environment to the culture system.

But how have these first open-ocean mariculture operations actually performed? Is there any basis in fact to these claims of environmentally sound mariculture in offshore locations? What are the opportunities for expansion of this industry, and what are the risks and precautions – both environmental, biological and economic – that should accompany such scaling?

This entry reviews some of the recent progress toward realizing this potential, as exemplified by one farm operation, in Kona, Hawaii. The environmental performance over the past 5 years of operation at Kona Blue Water Farms' site is instructive. It may demonstrate how open-ocean mariculture may represent a partial solution to the seafood crisis: a scalable, environmentally sound production system for high-value seafood. The environmental impacts of this one open-ocean mariculture site are reviewed here, in the context of both local ecosystem effects and global marine resource use efficiency: the "footprint" on the oceans.

Overview of the Kona Blue Water Farms Operation

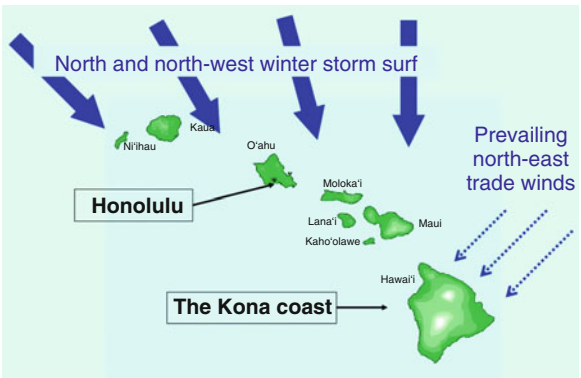
Prior to 1998, Hawaii's ocean-leasing legislation limited any potential project to a maximum of 4 acres, which had to be used for either educational or research purposes, and not for commercial gain. Through several years of work by industry aspirants, and strong leadership by the State Aquaculture Development Program, legislation was passed that allowed commercial offshore fish farms or energy projects.

The Kona Blue Water Farms principals had been involved through the legislative review, and with the passage of the bill, began research into developing hatchery culture techniques for high-value marine fish, simultaneously surveying the Kona Coastline for prospective offshore farm sites. After an extensive 3-year process of consultation and consensus-building with the community, Kona Blue was granted the requisite State and Federal permits for the original offshore farm site in March, 2004. The operation began deployment in February 2005 and first fish were harvested offshore in September, 2005. Since then, production has grown to the extent that Kona Blue has been harvesting up to 25,000 lb of sashimi-grade Kona Kampachi® per week, and up to 500 T per year. Kona Kampachi® (*Seriola rivoliana*) is also known as kahala, Long-fin Amberjack or Almaco Jack. It is related to the Japanese hamachi (*S. quinqueradiata*), but is native to Hawaii, and is distributed throughout the warm waters of the world.

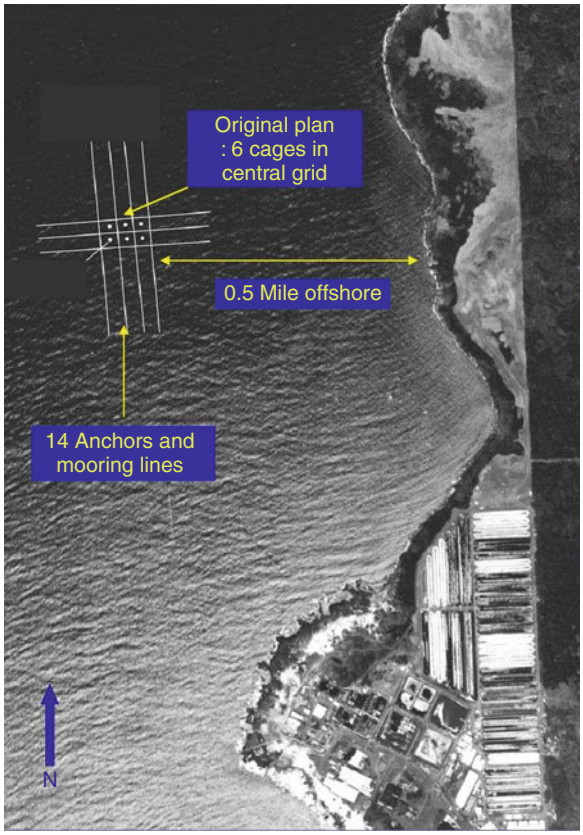
The Offshore Farm Site and Farm Operations

The Lease Area Site selection is a critical component for any mariculture operation, but is particularly so for an innovative offshore farm that is pioneering both a new permitting process and a new net pen system. The original farm lease site was selected on the basis of the following criteria:

1. The selected site was in a deep-water area, over 200 ft deep, with brisk currents.
2. There was little or no public use of this area. The farm site lay between the limits of normal recreational scuba diving (around 120 ft) and the normal depths for offshore trolling for ono (wahoo, *Acanthocybium solandri*).
3. The site afforded some protection from both Kona storms and the strong trade winds (Figs. 1 and 2). The proximity to shore also allows for future telemetry links to shore for farm control and security.
4. There was ready access from Honokohau Harbor, 5 miles to the south, which provides support facilities such as slips, fueling, and land for staging of equipment and feed.



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 1
Location of the Kona Coast on the western side of the Big Island of Hawaii affords moderate protection from trade winds and winter swells



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 2
Kona blue water farms' site located in waters over 200 ft deep over a sand bottom, a 1/2-mile offshore from a pristine coral fringing reef abutting lava cliffs. The site is a mile north of Keahole Point and west of the Kona International Airport

5. The site was directly offshore from the Kona International Airport and NELHA (Fig. 2), and, as such, its use was consistent with the adjacent land uses, and it represented no significant impact on the viewplane.

The farm site's topography and oceanography are distinguished by the depth of water, the bare sand substrate, the strong currents through the area, the exposure to high winter surf and strong trade winds, and the adjacent shoreline of a narrow coral bench reef with a steep basalt (lava) cliff. A few black sand beaches also lie along the coastline, to the north of the site, but these are little used, except by recreational fishermen. The preexisting uses of the farm lease area itself were negligible, because of its depth, the paucity of fish, and the barren benthos.

The net pens are all concentrated toward the center of the lease area (see Figs. 2 and 3), within two mooring arrays: one containing six net pens, and the other containing two net pens and the feed barge. The closest distance from the edge of the central grid array to shore is approximately 2,600 ft, or almost half a mile to the northeast, to Unualoha Point.

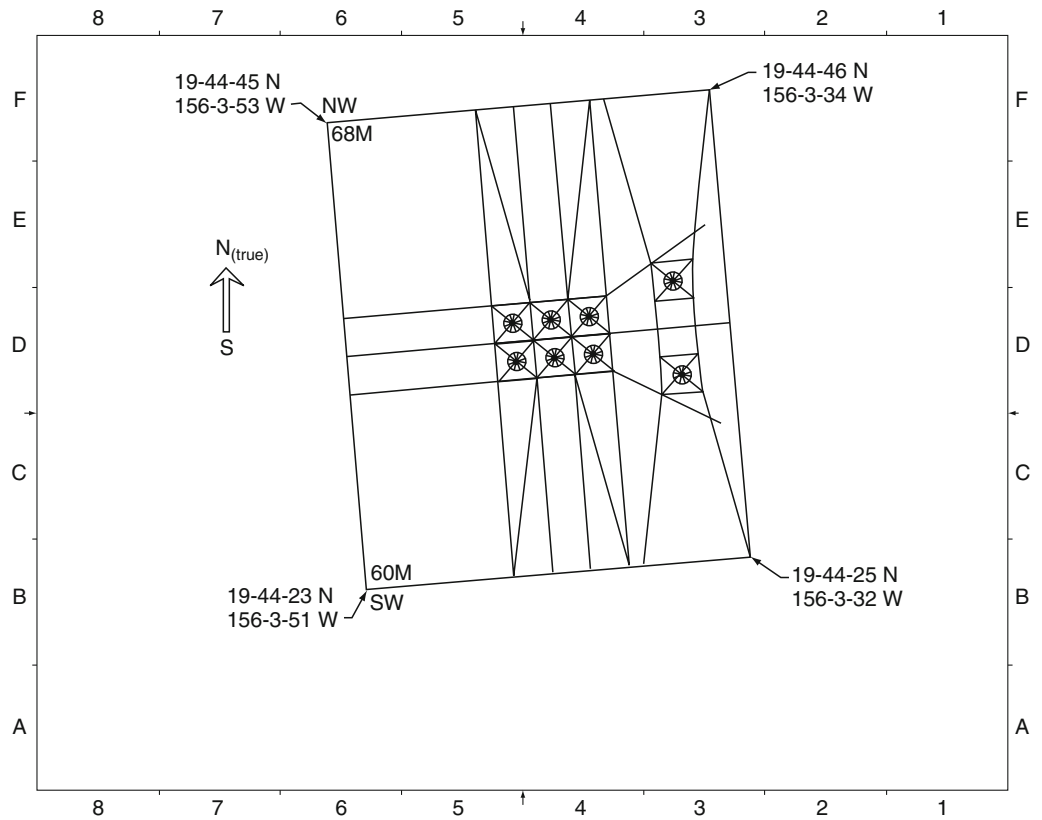
The farm site lease provides "negotiated exclusivity": Transit, trolling, hoop-net fishing and hook-and-line fishing are permitted throughout the lease area, but for liability, insurance, and safety reasons, there is no authorized anchoring, scuba diving, or swimming permitted.

The 90 acre lease area initially accommodated eight submersible Sea Station net pens, each of around 3,000 m^3 capacity (Fig. 4). The outermost area of the lease is used almost solely for mooring lines, which require a 5:1 scope. The net pens were originally tied into submerged grids that were anchored into the soft substrate using steel embedment anchors and chains. A series of buoys and weights ensure that the anchor lines are perpetually taut, to eliminate any risk of entanglement by marine mammals. Bridles from the mooring grid corners attach to the net pen rims, to hold the net pens in place in each grid square.

Farm Operations

The daily activities on the farm primarily consist of feeding the fish in the pens. Underwater video cameras inside the net pens are used to relay visual images to the operators on the feed barge. This enables the feed operators to regulate feed to ensure that no feed is wasted, and that excess feed does not fall below the net pen. Any fish carcasses are regularly removed by divers. Carcasses are disposed of as solid wastes in the county landfill.

Harvests usually occur twice each week. Fish are harvested into an ice-brine slurry, to quickly and humanely kill the animals with a minimum of damage. Fish are all transported whole, in ice-brine, to a single land-based processing facility, for packing and shipping. No fish processing occurs at sea during the harvests. Disposal of processing wastes is the responsibility



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 3
Modified mooring array and grid dimensions – plan view. The number of net pens is presently being reduced from current eight smaller pens to five larger pens, with the same overall culture capacity (24,000 m^3). The submerged central grid remains at around 30 ft (9 m) beneath the surface

of the wholesalers or other purchasers of the fish, but, at present, most trimmings from fillets go into the landfill.

Support activities for the existing operation are based out of Honokohau Harbor, where a half acre of land rented from the State accommodates containers for feed storage, gear storage areas, a closed workshop area, restroom, and office.

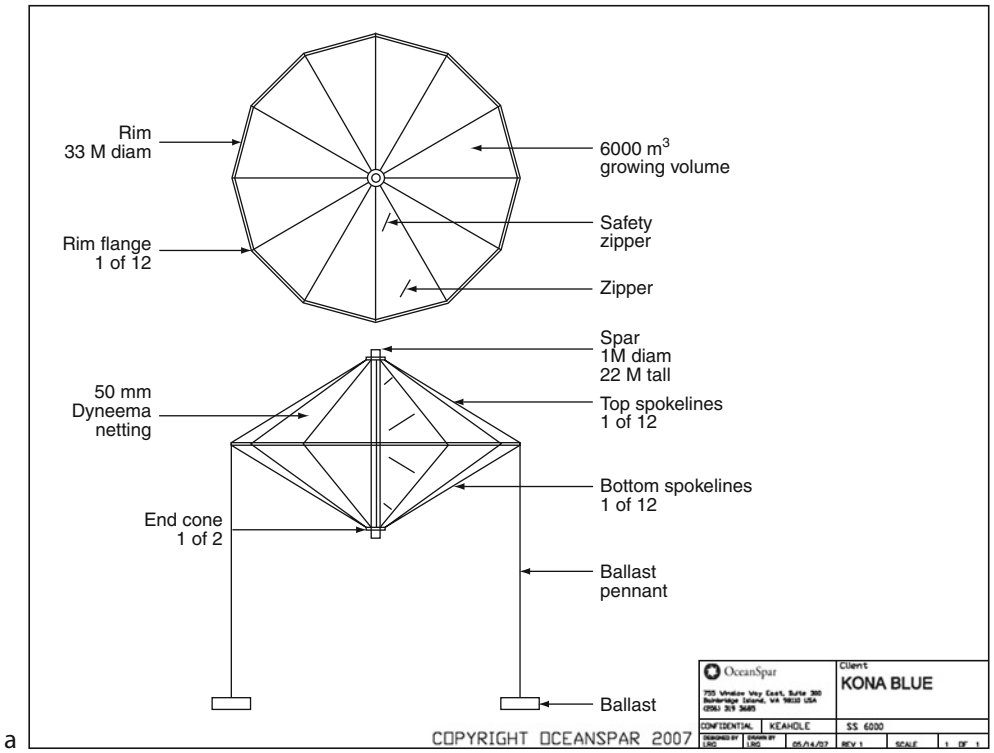
The farm is also serviced by a semipermanent feed barge/security platform vessel, which has been deployed on-site since October, 2007. A separate harvest boat – the 74 ft F.V. Kona Kampachi – transports the harvested product back from the farm site to the harbor. Several other smaller work boats are also used to support net pen and grid maintenance and cleaning, and other tasks.

Localized Environmental Concerns and Actual Impacts

The Presumed Problems

Aquaculture – or indeed, development of any food production system – brings with it attendant environmental concerns. Fish farms are widely accused of environmental degradation. The concerns that are often voiced include the following local-affect possibilities:

- Detrimental impacts on water quality
- Nutrient enrichment of the substrate beneath the farm
- Antifoulant paints from net pens to contaminate the substrate
- Therapeutant or antibiotic misuse to harm the surrounding biota



a

b

c

Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 4
Submersible Sea Station® Net Pens deployed on Kona Blue farm site. (**a**) Design of submersible SS6200 Sea Station net pens, with central steel spar and steel rim. (**b**) Sea Station SS3000 raised to rim-level on the Kona Blue farm site. (**c**) A submerged Sea Station SS3000 and diver on Kona Blue site

- Escapes to outcompete wild fish for spawning grounds or feed
- Escapes to dilute the wild fish gene pool
- Proliferation of pests, parasites, and diseases inside the net pens, which can then be transferred to wild fish
- Entanglement of whales, dolphins, and other marine mammals
- Disruption of marine mammal or other species' migratory paths
- Harmful deterrents or fatal control measures against predators

- Excessive use of fish meal and fish oil, leading to overharvesting of the smaller pelagic species targeted by industrial reduction fisheries
- Exclusion of other user groups from traditional, cultural, or recreational uses of the farm area
- Visual impact of the net pens on the viewplane

With almost 5 years' experience at the Kona Blue farm site, then, it is appropriate to evaluate the actual data and observations recorded at the Kona operation, and to compare this experience with the concerns that had been, and continue to be, voiced. Each of these issues is examined in detail, below, beginning with an evaluation of the de novo environmental status of the Kona Blue farm site, and then detailing the impacts that have occurred, their context, and their significance.

The Actual Observed Impacts: Locally

Water Quality and Effluent Impacts The water quality at the farm site is close to oceanic, with strong currents and low turbidity. Underwater visibility usually exceeds 100 ft or more.

General water movement patterns at the farm site are governed by the longshore currents past Keahole Point (the western-most point of the Big Island of Hawaii), 1 mile to the South. An S4 current meter deployed at the farm site over several periods since 2004 showed regular peak current speeds of over 50 cm/s (about 1 kt, at a depth of around 40 ft). Current headings were longshore: generally to the North (predominantly), but also to the South. The two points of first impact downstream from the farm site are therefore either Keahole Point, around 1 mile to the south of the site, or the Mahai'ula-Makalawena shelf area, around 3 miles to the north.

Because of the community concerns about potential impacts from the farm operation on water quality, the company had made commitments during the original permit process to a policy of ongoing transparency and objectivity in monitoring. These commitments included:

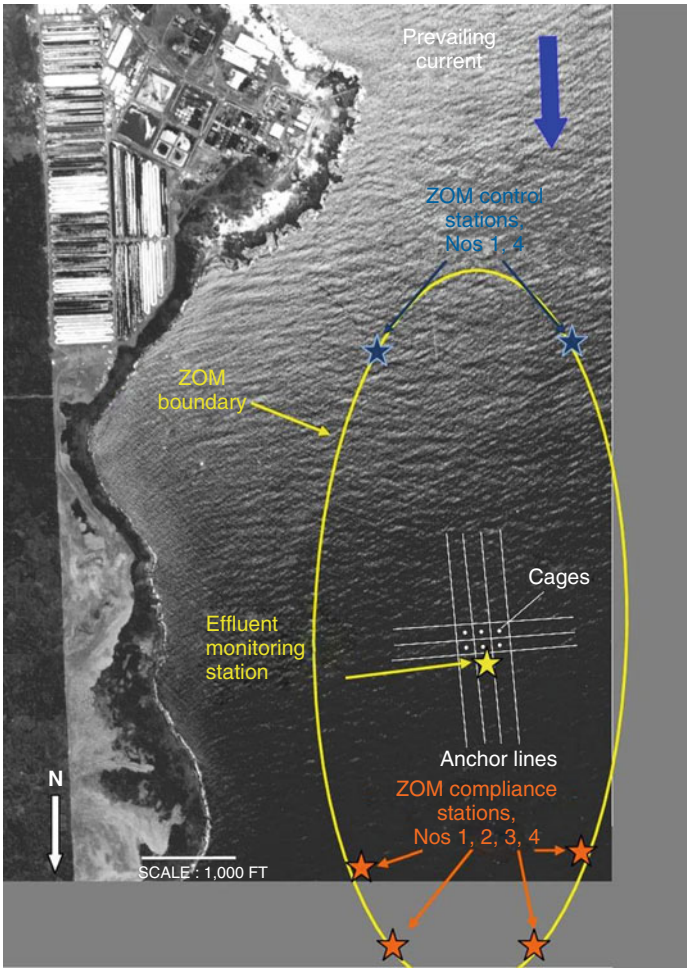
- Use of objective, third party experts to collect the water quality samples.
- Use of local water quality laboratories – such as NELHA Water Quality Lab, or local private laboratories – for conducting the sample analysis.
- Placement of copies of all monthly water quality monitoring reports at local repositories, such as the State Aquatic Resources office at Honokohau, or the NELHA library, so that local residents can review this data.
- Provision of reasonable access to Federal, State, and County officials for monitoring and oversight purposes.

Monthly measurements have been taken of ammonia and turbidity (the two most relevant water quality parameters for fish farming) at three depths (surface, mid-water – 50 ft deep, level with the submerged net pens, and at the bottom) and at a total of seven stations (two control stations upcurrent, one effluent station immediately downcurrent of the net pen with the greatest biomass, and four "zone of mixing" stations 4,000 ft downcurrent: Fig. 5). Quarterly measurements are also taken for a range of other parameters.

Detailed water quality data are available on the company's web site [4]. Figure 6 shows the mean for each sample site for turbidity for September, 2008, when the farm was at peak production of around 500 T annually. Turbidity is probably the best metric for fish feces and other particulates in the water, and so is most likely to reflect any impact from the farm's presence. These data are definitively clear – there is no discernible difference between water quality parameters at the upcurrent control sites, and the effluent site (1 m downcurrent of the net pen with the highest biomass) or the "zone of mixing" sites downcurrent. These results confirm that *there is no measureable impact* on water quality from the existing farm operations.

Benthic Impacts The substrate beneath the farm is over 200 ft deep, and almost exclusively comprises bare, coarse sand. Located along the shoreline, some 2,000 ft to the East (directly across the longshore currents) is a diverse coral reef community.

Impacts on Substrate Beneath and Around the Farm Site Prior to farm installation, a preliminary survey of the site was undertaken by repeated bounce dives, using scuba, to depths of 220 ft. Because the depth of the farm site is beyond the limits of normal safe diving, and the strength and unpredictability of the currents precluded ready use of grab samples or drop video

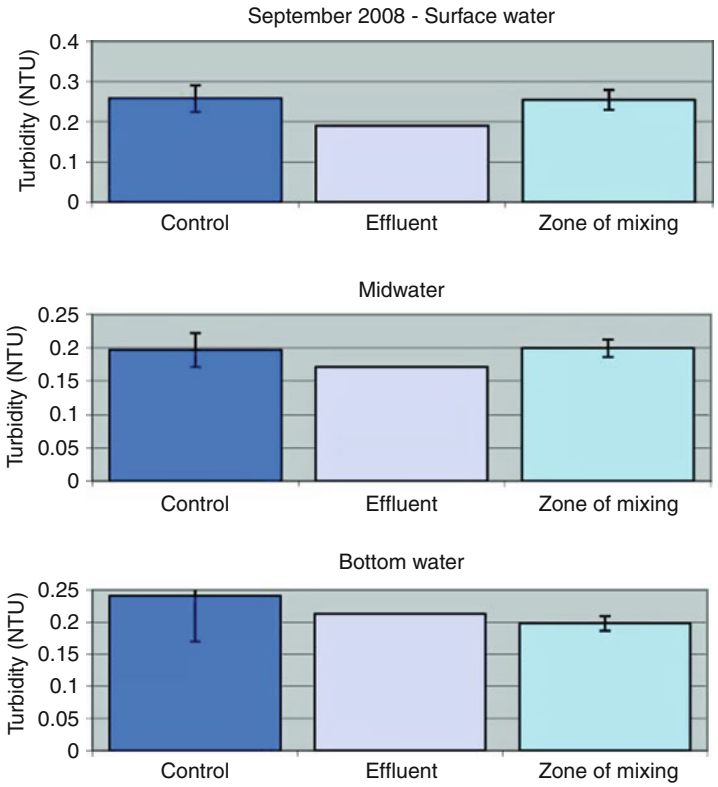


Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 5
Water quality sampling station map. Aerial photograph of the Kona Blue site showing water quality monitoring sampling station locations. Sampling stations under a prevailing N-setting current are shown. Under a S-setting current, control and compliance stations will be reversed. ZOM = Zone of Mixing

cameras, the original permit provided that no benthic monitoring would be required. Over time, however, permit requirements were tightened to include grab sample monitoring of substrate chemistry and infaunal micromollusk community structure, and video monitoring using drop cameras. These samples have been conducted quarterly, and reports and video footage have been posted on Kona Blue's website [4].

These results generally indicate that there has been no measureable impact on the benthic community around the farm site. There have been episodic perturbances of substrate chemistry immediately underneath the cage footprint, with a few instances of anoxic conditions during 2007, during periods when a new feed distribution system was being tested. This resulted in some pulverization of pellets, and reduction of feed to a "slurry," rather than discrete pellets. Once the feed system was refined, the substrate returned to its more normal condition, there was no further significant nutrient enrichment of the substrate.

Filamentous algae have also been visible in the drop-camera videos from around the farm site. These appear to have been detached from the cage mesh or the mooring lines, as the algae are not attached to the



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 6
Typical turbidity data from monthly sampling around the Kona Blue farm site. There is no discernible difference in water quality between the two Control sites (upcurrent), one Effluent site (immediately downcurrent) and four Zone of Mixing sites (4,000 ft downcurrent) of the farm site

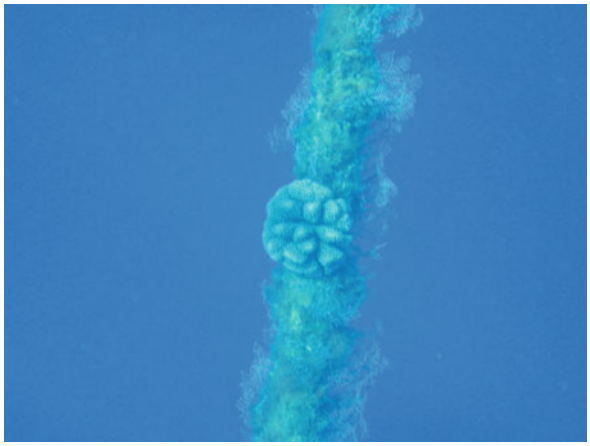
coarse sand substrate. Presumably, these algae are dispersed during periods of high current.

Monitoring of infaunal micromollusc assemblages in the substrate samples has also demonstrated that there has been no significant change in the community structure resulting from the farm presence (see reports on-line: [4]).

Impacts on the Adjacent Coral Reef Community A comprehensive survey of marine biota was conducted on the reef directly adjacent to the existing farm lease area, just south of Unualoha Point [5]. The survey of the benthic biota of the fringing reef crest used protocols identical to those employed by the State's Division of Aquatic Resources, in their West Hawaii Reef Management Task Force Survey. This provided an extensive set of "control" sites: the other benthic and fish data from the sites along the 90 miles of coastline on West Hawaii. A series of four transects of 25 m × 2 m extended parallelly to the reef crest, immediately shoreward of the seaward edge of the reef. Video footage was made of these transects, and digitized for selection of random points on the video frames.

The Makako Bay–Unualoha site has been repeatedly resurveyed since the original 2003 survey. Although no formal reports have been compiled, there have been no significant changes in benthic community composition or fish populations reported.

Biofouling on the Farm Structures There is also profuse growth of macro-invertebrate biofouling on the grid-lines and buoys of the mooring array, as well as on the bridle lines that attach the cages to the grid and the rims of the cages themselves (Fig. 7). This fouling includes diverse macroalgae, bivalves (several species of mussels and oysters: *Pteria* sp and *Pinctada*



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 7
A *Pocillopora damicornis* coral colony on a mooring grid line around the Kona Blue net pens (within about 15 m of net pen stocked with fish). The presence of coral colonies that are highly sensitive to nutrient enrichment on moorings and buoys confirms that the operation has no significant impact on marine biota in the area

spp), corals (primarily *Pocillopora* and *Porites*), sea urchins (primarily *Echinothrix calamaris*) nudibranchs (*Stylocheilus longicauda*) and sponges. These all settle out of the plankton onto the farm structures, and their presence does not represent any significant or even measureable reduction in the available recruits to the nearby coral reef area. The growth of the corals, particularly, is compelling evidence that the presence of the fish farm operation is not deleterious to benthic organisms.

Apart from the one brief instance of anoxic conditions beneath the net pens, there have been no other adverse impacts on benthic communities in, underneath, or around the net pen area.

Pests, Parasites, and Pathogens Kona Blue employs an integrated pest management strategy to optimize fish health, reduce interactions or minimize impacts on wild fish stocks, and reduce any potential environmental impacts from therapeutant use. As *Seriola rivoliana* is a new species, any therapeutant use must be conducted under an Investigational New Animal Drug (INAD) permit. INAD permits operate under the oversight of US Fish and Wildlife Service (USFWS), and the Food and Drug Administration (FDA), with State oversight through Office of Conservation and Coastal Lands (within Department of Land and Natural Resources) and Clean Water Branch (CWB, within the Department of Health). Federal Environmental Protection Agency (EPA) has oversight through the NPDES (National Pollutant Discharge Elimination System), which is administered by CWB.

As with almost all farmed animals, *S. rivoliana* is subjected to small external pests – in this case, the skin fluke, *Neobenedenia* sp, that attaches itself to the fish's skin. These flukes do not pose any risk to human health, and do not themselves detract from the quality of the harvested product, but may cause irritation to the fish. If left unchecked, the flukes can become a health problem for the animal, as the fish rub themselves on the netting to ease the irritation. Kona Blue uses occasional treatments of dilute hydrogen peroxide solution (at effective dosage rates of 200–300 ppm) to control levels of skin flukes among the fish in the net pens. Hydrogen peroxide (H_2O_2) breaks down rapidly in sunlight to form oxygen and water. Hydrogen peroxide is also considered an acceptable Organic aquaculture treatment under the draft USDA Organic aquaculture guidelines, and USDA Organic agriculture standards.

Under the permits in place at the existing site, such therapeutant use must demonstrate that there is no risk to the fish under treatment, or to the environment, or to human health. Monitoring of the effluent from any bath treatment at 100% concentration is mandated under the "Whole Effluent Toxicity" (WET Test) section of the NPDES permit. Results to date from the existing farm operation confirm that there are no significant environmental impacts from the use of the hydrogen peroxide. Ongoing effluent monitoring for WET test bioassays using larval fish (Pacific topsmelt, *Atherinops affinis*; conducted by Nautilus Laboratories in San Diego) demonstrate no significant difference in the rates of larval fish survival between control samples taken 4,000 ft upcurrent of the net pen, and samples taken of the whole effluent (100% concentration of the bath treatment water) at the conclusion of the bath treatments. There is therefore no mechanism for any measureable impact on the pelagic or benthic communities, or the surrounding water quality from the use of this therapeutant.

In addition, monitoring of wild kahala (*Seriola rivoliana*) stocks indicates that there is no significant proliferation of *Neobenedenia* sp. in the population around the farm area. Broodstock are collected periodically from around the farm area, to replenish the wild stocks in Kona Blue's hatchery. These fish are usually taken by commercial fishermen along the "drop off" of the marlin fishing grounds, about 1 mile to the South of the farm, and are sampled for ectoparasites upon capture, by immersion in a freshwater dip. Although these fish are usually infested with a number of other ectoparasites, the prevalence of *Neobenedenia* sp has never averaged much more than one individual per fish. By contrast, a parasitic copepod (sea lice, similar to *Caligus*) infests wild fish at average rates of around ten individuals per fish, and yet is not found at all on the farmed fish, and does not proliferate within the net pens.

A number of innovations, either in progress or planned, should also further reduce the proliferation of *Neobenedenia* on fish inside the net pens. The farm is being reconfigured to fewer, larger Sea Station net pens. With a planned reduction in the number of net pens, a reduction in the surface area-to-volume ratio of the remaining net pens (from double-cone net pens to a more cylindrical shape), the improved surface material characteristics and rigidity of the Kikkonet™ plastic monofilament net mesh (which make it easier to clean), and the improved access for offshore crew to regularly clean the nets from the surface (thereby breaking the skin fluke life cycle by dislodging the adhesive eggs on the mesh), the proliferative tendencies of the skin fluke should be further reduced.

Kona Blue does not use prophylactic antibiotics, but has, under the same regulatory oversights described above, and with veterinary guidance, used Florfenicol® to treat *Streptococcus iniae* infections that sometimes afflict juvenile fish after the stresses of transfer offshore. These treatments last for 10 days, and are also accompanied by WET test water quality monitoring. These WET tests have repeatedly demonstrated no impact on marine biota. A vaccine is available for *S. iniae*, which would avoid the need for these treatments, but regulatory requirements ironically prevent the use of the vaccine at this time. (The vaccine would qualify as an Organic treatment under draft USDA Organic aquaculture guidelines). *S. iniae* infections are not an issue with larger fish, once they have overcome the initial stress of transfer from the nursery to offshore.

Much of the concern over proliferative capacities for fish farm pests, parasites, or pathogens is derived from conflicts between salmon farming and wild salmon runs. Some research – though disputed – suggests that sea lice infestation rates can be exacerbated by the presence of salmon farms and can then be detrimental to survival rates of juvenile salmon as they migrate past the farms to the sea. Most marine fish, however, are broadcast spawners. Juvenile marine fish are therefore dispersed over vast areas of ocean and reef, and do not usually have vulnerable migratory patterns. Given such a distinct difference in life histories between salmonids and marine fish, there would seem to be limited applicability of the salmon and sea-lice research, or the concerns with impacts on vulnerable life stages, to open-ocean mariculture.

Interactions with Wild Fish Kona Blue cultures only Kona Kampachi® (*Seriola rivoliana*) on the offshore site, but the pertinent State permit also allows the company to possibly culture other amberjack (the other kahala species, *S. dumerili*), mahimahi (*Coryphaena hippurus*), and Pacific threadfin (*Polydactylus sexifilis*).

Aggregative Effects on Wild Fish Stocks The existing operation does have an aggregative impact on some species of fish in the area, but this is considered neither deleterious nor significant. Fish are attracted to the site for a number of possible reasons: the fouling on the net pen, the occasional release of small quantities of uneaten food from the net pen during periods of strong currents, and the aggregative nature of objects in open water (as for Fish Aggregation Devices). The makeup of the resident and transient fish communities around the net pens varies over time.

Pelagic or larger demersal fish frequently occurring around the Kona farm site include mackerel scad (opelu: *Decapterus macarellus*), ulua (giant trevally, *Caranx ignobilis*), wild kahala (*Seriola rivoliana* and *S. dumerili*), and barracuda (kaku, *Sphyraena barracuda*). Occasionally, schools of rainbow runners (kamanu, *Elegatis bipinnulatus*) and false albacore tuna (kawakawa: *Euthynnus alletteratus*) move through the net pen area. Larger pelagic fish, such as yellowfin tuna (ahi: *Thunnus alabacares*) and wahoo (ono:

Acanthocybium solandri) are also occasionally attracted to the area by the baitfish, or by the net pens themselves.

A number of other, smaller fish that are more normally associated with coral reefs settle out of the plankton and assume residence either around the subsurface buoys or around the cages themselves. Such residents include schools of Sergeant majors (*Abudefduf abdominalis*), dascyllids (*Dascyllus albisella*), chromids (primarily *Chromis hanui* and *C. ovalis*) wrasses (primarily *Coris* spp and *Thalassoma* spp), and kyphosids (*Kyphosus* spp). As these fish are settled from the plankton, their presence is not considered a significant detraction from the biomass or diversity of the fish fauna on the adjacent reef.

Escaped Fish Interaction with Wild Stocks Concerns about potential negative impacts of escaped fish are often cited as one of the reasons for objections to fish farming. However, this issue is most pressing only where non-native fish are cultured in areas where escapes might become established or compete with local species, such as Atlantic Salmon in the Pacific Coast of Canada. Kona Kampachi®, by contrast, is native to the waters of Kona. In addition, Kona Blue recognizes that the innovative net pen engineering employed means that there is some possibility of escape incidents over the initial proving period and development of refinements. In consideration of this, Kona Blue has deliberately not applied any selective breeding in the hatchery, and has not used any broodstock beyond F2 (i.e., all broodstock are either wild-caught, or first- or second-generation captive-reared). There is, therefore, no mechanism for development of any significant difference in the genetic makeup of the fish inside the net pen from the fish in the wild. This reduces any potential impact from escapes to merely direct ecological impacts.

Furthermore, the concerns with the effects of fish farm escapees on wild fish genetics are, again, largely a consequence of the conflicts between salmon-farming interests and wild salmon conservationists. Yet wild salmon stocks are unique, in that each river system or stream may have a genetically discrete stock from the adjacent watershed. Any blurring of this finer-scale differentiation, by interbreeding between escaped salmon and wild stocks, could represent a loss of genetic diversity. However, these concerns are not germane to farming of marine fish in the open ocean. As marine fish are broadcast spawners, there is only a coarse zoogeographic genetic granularity. Tagging research demonstrates that *Seriola* and other carangids migrate frequently between islands in the Main Hawaiian Archipelago. One *Seriola* migrated from French Frigate Shoals, in the Northwestern Hawaiian Islands, to the Big Island – a distance of 678 miles (over 1,000 km) over 3.6 years, at liberty [6]. The potential genetic impacts of Kona Kampachi® escapees on the wild stocks of *S. rivoliana* are therefore minimal.

Those Kona Kampachi® that have escaped from the Kona Blue net pens – either through "leakage" as divers enter or leave the pen through a submerged zipper, or from breaches in the netting – are invariably subjected to very heavy predation pressure. Individual escapees survive outside of the zipper for usually less than a minute before being eaten by either the ulua or the bottlenose dolphins that are frequently in the area. The long-term prospects for survival and reproductive success of any escapees are therefore highly dubious. In addition, any escapees that do survive in the wild are presumably entering a wide-open ecological niche, due to the severe depletion of other deep-water species – such as the deep-water snappers – by commercial fishing. There is little likelihood of escapees competing in any significant manner with the few remaining wild snapper stocks.

Other Wildlife Interactions

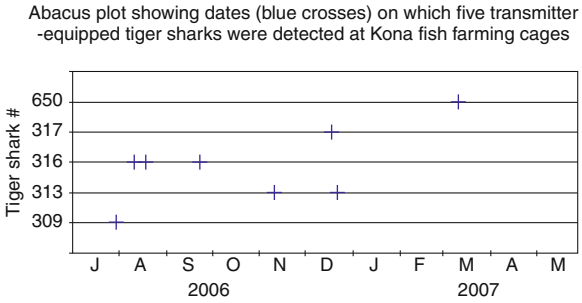
Sharks The single overarching feature of shark interaction with the offshore fish farm site has been – contrary to conventional wisdom and activist concerns prior to the farm deployment – the general absence of sharks around the net pens. For the first 8 months of operation, only one fleeting shark sighting occurred: a small tiger shark (mano: *Galeocerdo cuvier)*. Observations by farm workers suggest that there is a general pattern of brief influxes of tiger sharks to the area in the months of September and October of each year. Most of these animals appear individually, or in pairs, with a range of sizes from 8 to 15 ft in length, and they do not generally seem to take up residence on the farm site. Most tiger sharks only show interest in dead fish inside the net pens, and generally exhibit no interest in or aggression toward the farm workers.

In the first year of operation, however, a single tiger shark appeared to take up residence at the farm site. As the animal began to show aggression toward inanimate objects such as surface buoys, and then farm divers, it was humanely dispatched. Recognizing the long-term unacceptability of such predator control measures, Kona Blue sought alternative means of addressing this issue. A Shark Management Plan was then developed in consultation with State Aquatic Resources personnel in Kona and other experts, which included a range of measured responses, and nonterminal resolution if animals ever again become problematic at the site.

In subsequent years, tiger shark sightings usually increased in frequency at the farm site in the late-September early-October period. However, sharks were neither persistent, nor consistent. Farm operations had become more adept at removing dead fish, and the Shark Management Plan allowed divers to continue to work safely. One animal – or rarely two, contemporaneously – may appear at the site, and remain for an hour or so, before moving away, presenting little inconvenience to farm operations, and no real risk to diver safety.

Kona Blue has also, in collaboration with DAR and HIMB researchers, established a receiver station on the farm site, as part of the larger research program for tracking tiger shark movements along the West Hawaii coastline. The first data series obtained suggested that the observations by the farm work crews were correct – that tiger sharks only very infrequently pass by the site, and rarely do they show any interest in the operation. From July 2006 to May 2007, there were a total of eight (8) records of tagged tiger sharks in the Kona Blue farm area. None of these sharks took up residence. One animal passed by the farm site three times in 2 months, another animal was recorded twice in 2 months, and three other animals had single records. (Fig. 8).

Over 2008 and 2009, however, further tiger shark tagging trials showed that two animals appeared to regularly return to the farm site over periods of up to 5 months. Two other sharks ranged over the entire Kona coast area, but for several weeks at a time were recorded exclusively from the farm site. All animals eventually moved on; one was later detected off Maui. While these results suggested that the farm site had become a "waypoint" for the animals over a few months, the "long-term entrainment (e.g., years) of tiger sharks is unlikely" [7].



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 8
Frequency of tagged tiger shark occurrence at Kona Blue farm site: 2006–2007. Five tagged sharks were recorded over an 11-month period, with the most frequently occurring shark being present three times over a 2-month period. No animals took up residence, or showed any strong site affinity

There have also been sightings of sandbar sharks (mano: *Carcharhinus plumbeus*) around the net pens. Initially, these were rare (none in the first year of operation), but since October, 2006, the frequency of sightings and number of sandbars has increased. These animals are usually seen in small groups (one to four sharks), below the net pens at depths of over 100 ft. They rarely rise up to the level of the net pens. Sandbar sharks are more secretive, and cannot readily be distinguished by any markings. No sandbar sharks were caught during the tagging trials in 2008–2009 [7]. It is therefore unclear if these are always the same individuals, or if they represent a larger population of animals that periodically move through the area.

In the period from June to August of 2008, there were a series of breaches of varying sizes in the Dyneema® webbing of one net pen that corresponded to shark bites. The same net pen was also breached in August 2009 by a small Galapagos shark that entered the net pen. The Galapagos was captured and released alive by company divers, unharmed except for a small dorsal fin notch for later identification. In each instance, breaches were sealed immediately on discovery. These incidents underscore the vulnerability of even sturdy Dyneema® nylon mesh, and have led to a plan for wholesale installation of Kikkonet® rigid plastic webbing across the farm. This material has been used in *Seriola* culture in Japan for over 25 years,

and has been successfully used in crocodile and shark-infested waters by a sea-cage barramundi farmer in North Queensland, Australia. Kona Blue therefore anticipates that the use of Kikkonet webbing will reduce mesh breaches to negligible levels, and significantly reduce escapes and the attractant nature of the escapes to the bottlenose dolphins and sharks.

Overall, the evidence from the Kona Blue site confirms that there are no significant negative impacts from any aggregating effects of the net pens on sharks. The evolution of a nonterminal, humane plan for managing sharks on the farm site underscores the importance of commercial experience to improve open-ocean farming practices.

Turtles The threatened green sea turtle (*Chelonia mydas*) is common in the nearshore waters of the main Hawaiian Islands. The endangered hawksbill turtle (*Eretmochelys imbricata*) is infrequently found in Hawaiian waters. The principal nesting site for the green turtle is in the Northwest Hawaiian Islands, on French Frigate Shoals [8]. No turtles have been observed in the area of the farm site, but it is possible that they occasionally transit through the site. If they were to do so, the taut-line mooring system and stiff-mesh net pens will prevent animals from becoming entangled.

Seabirds The submerged net pens used by Kona Blue do not significantly impact seabird populations. The farm area itself is infrequently used as a foraging area by seabirds. Most seabird activity in the area is confined to the fishing "grounds," which extend to the northwest of Keahole Point.

Monk Seals There are four conceivable ways for open-ocean fish farming to have a significant negative impact on rare, threatened, or endangered wildlife, such as monk seals, dolphins, or whales. The project may (1) present a significant obstruction to natural migratory patterns, either (2) attract, or (3) repel the animals and thereby disrupt their normal behavior, or (4) the animals may become entangled in the ropes or mesh of the net pens or moorings.

Monk Seals have been observed at the existing farm operation on two occasions, both in association with escape incidents from the nylon mesh nets on the surface nursery pens that were previously in use at that site. (These nylon mesh surface net pens were removed in 2006, as Kikkonet was, at that time, not yet available outside of Japan.) On each of these occasions, the Monk Seal was preying on the small, escaped Kona Kampachi®, but once the school was effectively eradicated by predators, the Monk Seals moved away. A radio tag allowed movement of one monk seal to be tracked from the Unualoha site to a beach on Maui the following day, clearly affirming that the animal did not take up residence, or become conditioned to the availability of escapees.

Dolphins Makako Bay, almost half a mile to the south of the farm site, is frequented by large schools of spinner dolphins (*Stenella longisrostris*), on nearly a daily basis. These animals usually follow a diurnal pattern of movement from the Makalawena shelf area to the north, along the reef edge to the shallow areas of Makako Bay, where they rest for some time during the middle of the day. Some concerns were expressed during preliminary hearings about the potential for the farm operation to interfere with the spinner dolphin patterns of movement or resting habits [5]. There is no evidence to suggest that this has been the case. There have only been some occasions over the 5 years of operation offshore when divers or workers on the farm site have witnessed spinner dolphins coming anywhere near the net pens. The net pens clearly do not impede the usual pattern of spinner dolphin movement toward Makako Bay; they do not attract or repel the animals, nor do they affect the resting pattern of the dolphins.

Over the last 3 years, the existing farm operation has demonstrated a propensity to attract bottlenose dolphins (*Tursiops truncatus*). No bottlenose dolphins were previously present on the farm site, but the animals have begun to appear regularly at the site since about October, 2006. Patterns of dolphin movement are best characterized as one or two animals, every day or so, with occasional instances of groups of up to seven or eight animals. There is no regularity to the animals' appearance on the farm site: they may be present all day, or only in the morning, or only in the afternoon.

Kona Blue staff monitor and report on dolphin activity to HIHWNMS and NOAA's PIRO PRD. The bottlenose dolphins are probably attracted to the farm site by a combination of (1) the presence of the

mid-water structures acting as a Fish Aggregating Device and the associated fish community that is present around the net pens, (2) the occasional provisioning from "leakage" escapes when divers enter or exit a net pen, and from the rare larger escape incidents when predators have breached the Dyneema nylon webbing, and (3) interaction with divers outside of the net pen, as the divers move about the farm from boat to net pen and back.

One individual dolphin has taken up residence over 2009 and 2010. This animal was suffering from a large fishing hook and leader line that had become lodged in its jaw, and it was present on the farm site almost continuously during this period. For many months, the dolphin was lethargic and lost weight, but more recently (as of late 2009) has appeared to be more active and in better condition [9]. The aggregative effective of the net pens for this one animal might therefore be interpreted as beneficial.

No other individual bottlenose dolphin has taken up permanent residence at the farm site. There are no other animals present on the farm site on around one-quarter to one-third of days. Even when other animals are present, they are often only there for part of the day, rather than the entire day. In October–November, 2008, for example, dolphins were present for some period of time on 22 days out of 34 days [10]. There were dolphins present at the farm site for some or all of the day on 65% of the days. On 35% of days, there were no dolphins reported as observed on the site. Only on 1 day were six dolphins present. Most other days, there were one or two animals present for some portion of the day.

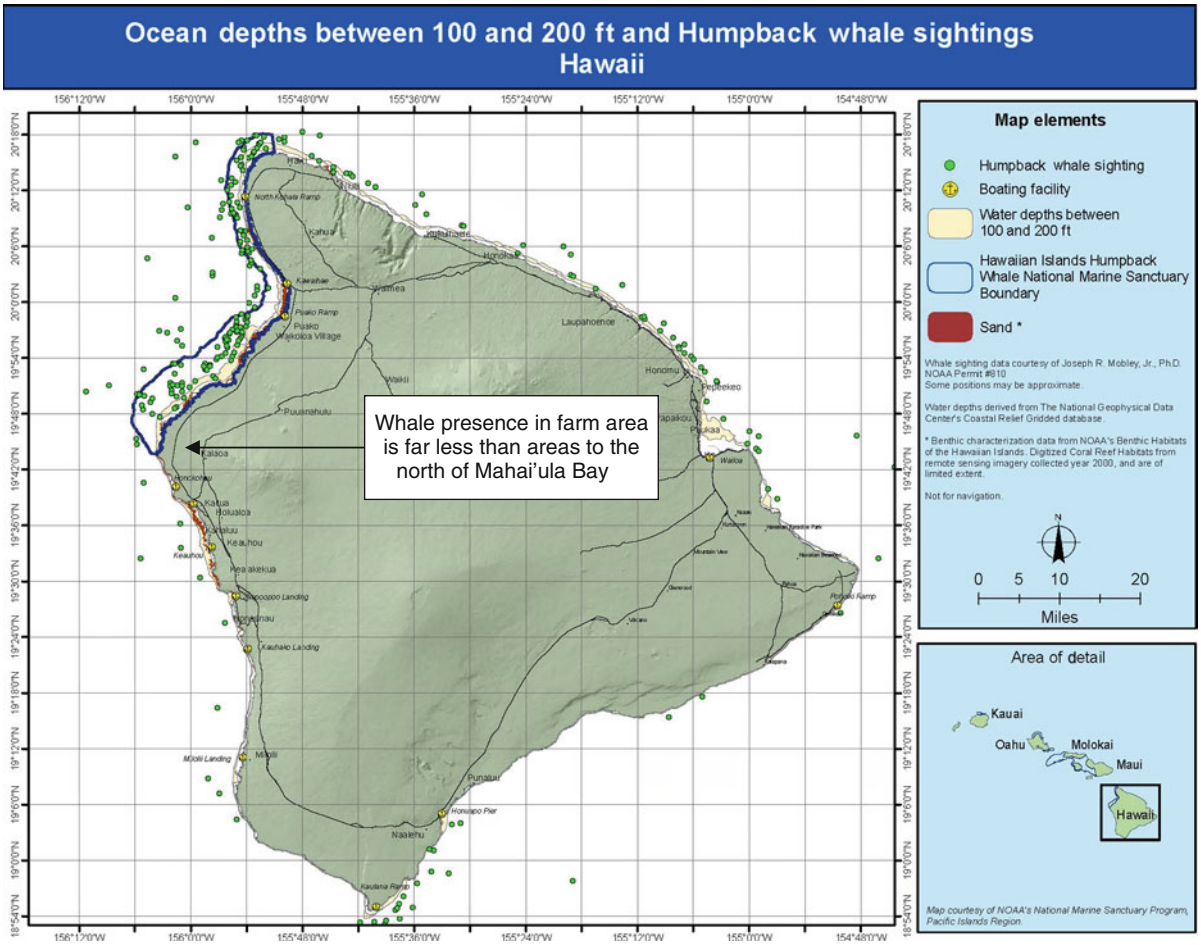
Other dolphin species may be found in and around the proposed farm lease area, but they are usually most commonly seen on the "grounds" to the south of the site. Spotted dolphins (*Stenella attenuata*), rough-toothed dolphins (*Steno bredanensis*), and false killer whales (*Pseudorca crassidens*) have all been observed on the "grounds," or in other offshore waters of the Kona Coast, but have not been reported from the farm site.

In summation, although there has been behavior modification in one compromised individual, the presence of the farm operation has not had a significant negative impact on dolphin behavior. The overall long-term impact on dolphins from the farm operation will probably be further reduced. Modifications to net pens currently under way should help to alleviate the attractive nature of the farm to the dolphins, by reducing the potential for escapes through mesh breaches, and for leakage escapes, and by reducing the amount of time that divers need to operate outside of the net pens. Kona Blue will continue with the ongoing monitoring and reporting of marine mammal activity around the farm site, and continues to collaborate in this with HIHWNMS staff, PIRO PRD staff.

Humpback Whales Populations of the endangered humpback whale (*Megaptera novaeangliae*) winter in the Hawaiian Islands, and the project site lies around 1 mile inside the southernmost boundary of the Hawaiian Islands Humpback Whale National Marine Sanctuary (HIHWNMS). Humpbacks are known to frequent the entire Kona coast area in winter. The whales move throughout the general area, usually following a longshore track (north to south, or vice versa).

Concerns about the reduction in whale habitat by the existing project were previously expressed by HIHWNMS and DLNR/DAR officials. Some concerns were also earlier expressed with the potential for entanglement of whales in the mooring lines of the net pens. A comprehensive analysis of available records of whale entanglement (NMFS Stock Assessments), a review of interactions between marine mammals and Hawaii's fisheries [11], and details of marine mammal strandings compiled by NMFS Pacific Area Office (NMFS-PAO) shows that most whale entanglement events occur in slack net mesh (such as drift nets or fish weirs), slack vertical lines (such as crab pot or lobster pot floats), or surface lines (such as long-lining gear). Among all these observations, there is no record from any US aquaculture operation of entanglement of humpback whales, or other marine mammals, in the taut moorings or net panels of fish net pens. With heavy mooring gear, and taut lines and mesh, the potential for entanglement is considered negligible [12, 13].

Furthermore, it appears that the waters in the vicinity of Keahole Point are not as heavily frequented by the whales as are other waters of the Sanctuary, further to the north (Fig. 9). Observations from workers at the farm site suggest that the farm does not interfere with the movement of the humpback whales, beyond the immediate and obvious exclusion from the waters inside the net pens. The distance of around half-mile



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 9
Typical Humpback whale sighting patterns around the Big Island of Hawaii

from the inshore side of the net pens to the shoreline offers ample room for the whales to move around the eastern end of the farm structures, without any chance for any funneling or bottleneck effects.

There is no definitive pattern of whales avoiding, or being attracted to the cages. Whales are occasionally seen within the lease area. On one instance, the farm workers witnessed a humpback on the surface inside the mooring grid array; the animal appeared to negotiate its path between the net pens and mooring lines with ease.

As part of the company's Marine Mammal Monitoring Plan (MMMP), farm workers provide data for assessing whale abundance and patterns of movement around the farm site. The MMMP describes Federal recommendations or instructions in the unlikely event of any entanglement, and also details ongoing reporting requirements for any close interaction with humpback whales, or any physical interaction between the farm array and other marine mammals.

Recreational Use Impacts The farm site lies offshore from the Natural Energy Laboratory of Hawaii Authority and the Kona International Airport, and as such, has little effect on shore-based recreation. The heavily used public recreation area of Kekaha Kai State Park (Mahai'ula) lies more than 3 miles further to the north.

A survey of recreational activity in the general area, north of Keahole Point was conducted prior to the farm installation, from August to September, 2001, in

conjunction with the original farm site environmental assessment [14]. The survey covered 2 months of summer conditions, which was considered the best means of ensuring that the data represented the heaviest use of the area. The overarching finding of the survey was that the area is only used for transit: of the 150 observations made over the 61 consecutive days of the survey, only one boat was seen within the farm site – a boat transiting through the area. Most activity in the general Keahole-to-Unualoha area was recreational dive boats and commercial dive tour operations along the reef and shoreline south of Unualoha Point (directly inshore from the proposed farm site), and in Makako Bay itself.

Observations by the Kona Blue staff on the farm site suggest that this trend continues – the use of the farm lease area is merely for transit. Fishing boats now occasionally troll lines close to the central area, to try to take advantage of the aggregative effects of the net pens. There are no records of catch rates around the farm, but anecdotal evidence indicates that catches are primarily ono (wahoo, *Acanthocybium solandri*), with infrequent catches of ahi (yellow-tuna, *Thunnus alalunga*).

Kona Blue's permit allows restricted public activities in the lease area, precluding anchoring, scuba diving, spearfishing, or swimming within the 90 acres. These limits are considered the minimum needed to protect the company's investment, to limit their liability (and retain insurance coverage), and to assure public safety. Fishing by the public from unanchored boats (trolling, or linefishing from drifting boats) is still permitted, but with the caveat that any fishing lines that become entangled in the net pen mooring lines must be left in place and cannot be retrieved by divers. The company also requests that fishermen not troll through the center of the farm site because of the potential for fishing lines to entangle divers, or for lures to hook into mooring lines or nets. Boats transiting the net pen area are also requested to observe a slow "no-wake" boat-speed to maximize safety for divers. Unguided recreational scuba diving or unauthorized commercial scuba dive tours are not permitted within the lease area because of liability, safety, and security concerns.

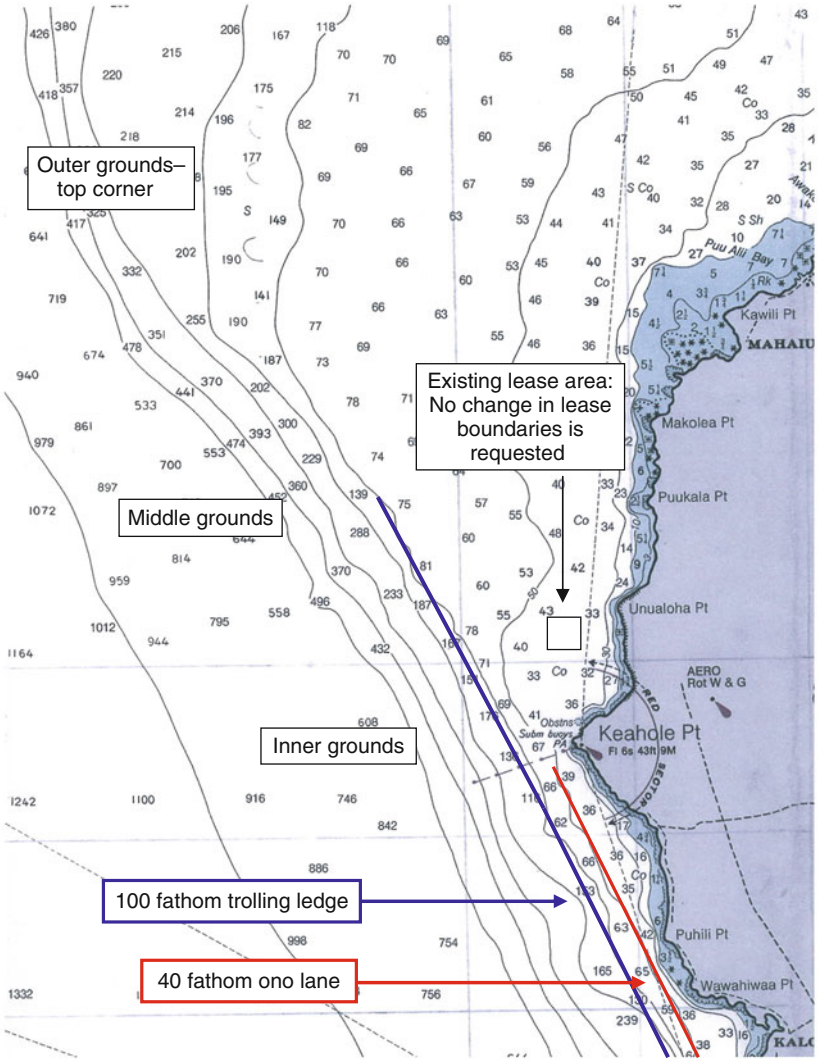
The loss of access to recreational activities within this relatively small area of ocean space is not considered significant. Kona Blue's ongoing observations affirm that there is virtually no fishing or other recreational use of the lease area, or the areas adjacent to the lease area, beyond trolling, which is probably enhanced by the farm's presence.

Viewplane Aesthetics Community value judgments and perceptions of how the oceans should be used largely govern the impact of the project on the community's aesthetic enjoyment of the area. In community meetings, Kona Blue generally enjoys strong support for the broad goals of the company. There is wide recognition of the severely depleted status of bottom fish species in Hawaii. The awareness of the global fisheries crisis has recently been amplified by several scientific studies, such as that of Worm et al. [15], which projected a collapse of world fish stocks by 2048, unless significant remedial changes are made to fisheries and marine ecosystem management.

The visual impact of the project is minor, compared with the adjoining properties of Kona International Airport and the aquaculture operations at the Natural Energy Laboratory of Hawaii Authority (NELHA). The major visual impact from the farm operation is from the experimental surface pens and the feed barge. There is also the additional presence of work and dive boats, and harvest boats, on some days. However, the impacts of these structures and activities are not significant, given the distance from the nearest residences, more than 3 miles away.

There is general community acceptance that the project fits in well with the overall ambience of innovative aquaculture at NELHA, and the need for Kona to develop alternative industries beyond tourism. Fisherfolk and other mariners recognize the validity of the criteria that Kona Blue has used to select this site (c.f. deeper or shallower sites), and have not expressed a strong preference for the project to be located elsewhere. Applicants for farm permits in other areas of the Kona Coast (around Kawaihae) have, on occasion, been told that their project would more appropriately be located "down near NELHA and Kona Blue."

Cultural Resources, Practices, and Mechanisms for Impact Prior to the 1801 lava flow that inundated the area, Keahole was the site of the largest fish pond in the Hawaiian islands. The Pai'ea pond (reputedly



Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Figure 10
Kona Blue's offshore fish farm site in relation to primary fishing areas. The farm site is well inside of both the 100 fathom (200 m) trolling ledge along the "grounds" offshore of Keahole Point and the 40 fathom (80 m) ono lane. Reef fishing and opelu ko'a are found well inshore of the proposed site, along the edge of the reef, in waters up to 120 ft deep (40 m). Fishing grounds for opelu at night are usually deeper than 40 fathoms (80 m)

King Kamehameha's favorite pond) was approximately 3 miles long and 1/2 mile wide; canoes were used to traverse from one side to the other. The farm site is directly offshore from where Pai'ea once stood. Fish farming could therefore be considered historically and traditionally appropriate to the area.

The farm lease area is over a mile from the traditional marlin, tuna, and wahoo fishing grounds (Fig. 10). The site is too deep for free-diving or scuba diving activity but suitable for "blue-water" spearfishing. Usually, however, blue-water spearfishing is practiced close to a point or drop-off, rather than over bare sand substrate around 200 ft deep. There are no significant benthic plant or animal populations in the farm lease area, and there are virtually no benthic or pelagic fishing activities in this depth range. Kona crabs and nabeta (*Xyrichtys pavo*) are the only benthic resources that occur on sand bottom at this depth,

but informants suggest that the currents are too strong for any significant fishing effort this close to Keahole Point [16].

The only potentially impacted cultural resource that was cited during extensive discussions with community and kupuna (elder) groups for the original farm site was the several opelu ko'a ("holes" or schooling places for mackerel scad – *Decapterus macarellus*) that occur in the general region. The locations of these ko'a are considered to be part of traditional marine lore, and are considered inappropriate for publication, or for sharing outside of the families or community groups who have traditionally fished these ko'a. However, in private meetings with the most knowledgeable kupuna, the locations of the traditional opelu ko'a were determined to be outside of the proposed project location [17]. Opelu aggregations usually occur in water around 120 ft deep, close to reef drop-offs, and well shoreward of the farm area.

Access to, or practice of any other customary activities has not been significantly constrained by the farm array or operations. The exclusive control over the waters (and the fish) inside the net pens is consistent with traditional and cultural practices that identified fish traps or lobster traps – and the animals therein – as the private property of the trap owner. The same principles apply here.

Global Impacts: Fish Meal and Fish Oil Usage

Fish Meal and Fish Oil Usage

Fish such as *Seriola rivoliana* (Kona Kampachi®) usually feed toward the top of the trophic chain in the wild. They therefore possess digestive systems and nutritional requirements that are adapted for feeds with high protein and lipid levels, and low levels of carbohydrates.

Fish meal and fish oil usage in fish feeds can be considered a valid use of a natural, sustainable, renewable resource, so long as the fishery from where the fish meal and fish is sourced is responsibly managed. Although stocks such as the Peruvian anchovetta fishery are sustainable in the sense that they are very well managed, they are not scalable. If mariculture is going to fulfill its potential for increasing seafood consumption to meet growing demands, then some alternative sources of proteins and oils will be required.

Kona Blue has therefore been focused on reducing the inclusion rate of fish meal and fish oil, such as Peruvian anchovies, from targeted reduction fisheries and increasing the use of agricultural oils and proteins, such as soy, canola, wheat, corn and poultry meal, and oil.

Improving Feed Conversion Efficiencies: An Evolutionary Approach Though efficient use of fish meal and fish oil from targeted reduction fisheries is both rational and justifiable, this by no means suggests that these resources are unlimited, or that alternatives should not be searched for. If open-ocean mariculture is to develop into a food production system that can provide a significant proportion of the nutritional needs of a growing planet, then additional sources of sustainable proteins and oils for feedstuff must be found for this industry. The arc of Kona Blue's feed development strategies is perhaps instructive of directions that open-ocean mariculture, as a global industry, might follow to achieve such scalable sustainability.

Initially, Kona Blue Water Farms fed the Kona Kampachi® with a diet that was considered "organic" by European standards. At the time, USDA did not have (and still does not have) Organic standards for aquaculture feeds. In the EU, however, Organic fish food was considered to be that which was most similar to the animal's diet in the wild. This feed, therefore comprised largely comprised fish meal and fish oil derived from Peruvian anchovies.

With the recognition of the need for more scalable feedstuff alternatives, however, Kona Blue worked with the feed vendor to develop a new diet that lowered the inclusion rate of fish meal and fish oil from Peruvian anchovies to a combined total of 50%. This diet included soybean meal, wheat gluten, canola, and other grain proteins and oils. The biological efficiency for this diet, however, was still suboptimal, with a fish-in:fish-out ratio (FIFO) of over 2:1. (i.e., an input of more than 2 lb of anchovies for each pound of Kona Kampachi® produced).

The inclusion rate of agricultural proteins in diets for marine piscivorous fish is limited by the presence of a range of "anti-nutritional factors" in the grains and less-purified meals. (Aside: although often described as carnivorous, most marine fish such as groupers, snappers, jacks, and bream, are perhaps more accurately

described as "carbohydrate intolerant." They require diets that are high in protein and lipid, and low in carbohydrate. There is no specific nutritional requirement that these fish eat meat). For this reason, soybean meal is restricted to about 20% of the diet for most marine fish. To reduce the fish meal and fish oil inclusion rate further, and to further lower the FIFO, would therefore require proteins and oils from other sources. By-products from both edible fishery processing and poultry processing were therefore included in the revised Kona Blue diet, allowing the Peruvian anchovy inclusion rate to be further reduced to 30% of the ration: 20% fish meal and 10% fish oil.

Inclusion of poultry processing by-products, however, meant that some customers, such as Whole Foods Markets (WFM), a high-end organic and natural foods retailer, would no longer carry Kona Kampachi®, even if the poultry used for the by-products was of Organic origin. This position by WFM was out of consideration for those of their customers that were vegetarians, but still wanted to eat fish. WFM asserted that these customers would not want to eat that fish if fish had eaten a pellet that contained proteins or oils that were derived from mammals or birds. Kona Blue appealed to WFM to review their position, given the importance of reducing mankind's global footprint on the oceans, that is, the reliance of humans on natural marine resources – but as of 2010, there has been no change in this policy.

Kona Blue has recently tested two diets that completely eliminate from the Kona Kampachi® diet any fish meal and fish oil sourced from targeted reduction fisheries, and any land animal processing by-products. These innovative diets use processed by-products from sustainably managed fisheries intended for human consumption. As the trimmings from these sources would otherwise have been discarded, used as fertilizer, or burnt as fuel, the use of these fish meal and fish oil products in the Kona Kampachi® diet represents an ideal reuse of natural resources. These diets therefore would result in a zero FIFO ratio, that is, no targeted reduction fishery by-products included in the diet of the end product.

Alternative Feedstuffs for Open-Ocean Mariculture

Kona Blue is involved in testing a range of alternative feedstuffs for Kona Kampachi® diets, which also offer potential for other species of marine fish. Alternative soy products, other agricultural grain concentrates, yeast, and other single cell proteins, edible fishery by-products and – more recently, with the boom in microalgae culture for biodiesel production – defatted microalgae by-products, have all either been tested, or are under development for Kona Kampachi® feed trials.

Kona Blue has tested a range of soy-based diets, with soy protein concentrates and omega-3 oil rich strains of soybeans. These trials suggest that the inclusion rate of soy protein concentrates cannot, by itself, exceed the same 20% threshold that limits soybean meal. Above this level, growth rates and feed conversion ratios are depressed. With the inclusion of taurine in the formula, however, soy protein concentrates could replace fish meal as the source of protein up to 40% of the diet with no detriment to fish growth rates or feeding efficiencies.

There is a diverse array of edible fishery processing by-products that are available for use in aquaculture diets, and this direction offers tremendous potential for further development. The processing by-products from most wild salmon runs, for example, are woefully underutilized, and are often disposed of directly back into the rivers from which the fish are taken. Logistical and economic constraints limit the use of these trimmings, however, as the processing plants are usually small and isolated, the salmon runs are only of short duration, and storage and transport of fish meal or fish oil by-products from these villages to reduction facilities and feed mills is a challenge. Development of fish silage systems offers one potential, partial solution.

However, even the less-seasonal, larger-scale processing of farmed salmon in more centralized plants presents difficulties for the utilization of by-products. For biosecurity reasons, most fish feed plants will not run salmon-derived feed stuffs through their machinery because of the potential for contamination of feeds from viruses, bacteria, or other pathogenic vectors that may be found in the by-products. Screening for known pathogens is not an adequate solution: even though the chance could be considered very slim that some unknown pathogen may be unwittingly dispersed via extruded feed, the potential catastrophic consequences of such widespread and rapid disease dissemination are sufficient to ensure that no such chance be offered. This

therefore excludes almost all large feed mills in salmon-farming regions from using salmon by-products.

Similar inefficiencies are found in the reuse of trimmings from the pollock fishery (*Theragra chalcogramma*) in the northern Pacific. This fishery primarily processes most of the catch at sea, into surimi. Trimmings from these fish constitute around 65% of the wet weight of the catch. For a fishery that has averaged around 1.3 million metric tonnes, this then represents around 850,000 tonnes of wet weight by-product annually that could be converted into fish meal and fish oil. For many years, much of this by-product was discarded back into the ocean, or the rendered fish oil was burnt in the diesel generators of the processing vessels. Some 8 million gallons of fish oil in Alaska is largely disposed of as biodiesel [18]. More recently, some proportion of these trimmings have been used to make a high-quality white fish meal that is largely exported to Asia, where it is valued in feeds for farmed eels. However, the proportion of by-product that is reused or recycled is not reported. Again, economics and logistics conspire against development of a rational supplement to targeted reduction fisheries. The increasing prices of fish meal and fish oil, however, driven by the growing demand for animal feeds from developing economies (notably China and India) may be a greater incentive to resolve these constraints. Edible fishery by-products may yet play a significant role in aquacultue feedstuff sourcing.

There is ample evidence that some or all of these innovative feedstuffs could help to reduce the demand for fish meal and fish oil from clupeids in the medium- to long-term.

Is Aquaculture "Fishing down the Food Chain"?

Much interest has been recently focused on the problem of "fishing down the food chain," beginning with Pauly et al.[19], and Taylor et al.[20]. This is the trend over time for commercial fisheries – driven by serial stock depletion – to shift their target species to those lower on the trophic pyramid. Fishermen first start out exploiting the high-value, top-end predators, then move on to mid-level predators, and then down toward herbivores and detritivores – what was previously considered bycatch. Fisheries generally start out targeting the larger, sweeter-tasting species – tunas, snappers, groupers, and such. As these become increasingly scarce, fishermen apply greater fishing power, and fish longer and deeper, retaining or targeting what was previously considered "trash." The argument portends that at some stage, the food web is reduced to an ocean full of jellyfish. "Fishing down the food chain" is a condemnation of the inherent unsustainability of most commercial fisheries management – or rather, mismanagement.

Fish farming has somehow been implicated in this practice on the basis of farmed fish being fed pellets that are partly made up of fish meal and fish oil derived from anchovies, menhaden, sardines, or the like. These fish (collectively, the clupeiforms) usually form the first step in the ocean food chain beyond primary production. Some scientists and anti-aquaculture advocates misconstrue or deliberately misinterpret the complexities of ecological and economic cause-and-effect, and represent the use of clupeiforms as feed for farmed fish as wanton. This has been led by respected institutions such as the Monterey Bay Aquarium [21, 22], but has also spilled into mainstream media, such as the *NY Times* [23], *Conservation Magazine*'s article on "10 Solutions to Save the Ocean" [24], *The Ecologist* [25], and *The Economist* [26]. The notion that aquaculture is guilty of "fishing down the food chain" is now lodged within the public consciousness.

The bottom of the food chain, however, is where fishing should preferentially be done. It makes far more sense to use herbivores or planktivores from the base of the trophic pyramid as either human food or feed for farmed fish, than to be targeting top-end predators. This makes economic sense, but it also makes sense from other perspectives: it is better for the ocean's ecosystems, it is better from the viewpoint of bioenergetics transfer through the trophic pyramid, it is better for consumer health, and it makes for better fisheries management.

The economics are simple: Peruvian anchovies and menhaden are not highly valued in the market, so they are cheap. Maybe this will change in time, and prices for anchovies and sardines will increase, as more people develop a taste for oily baitfish, but it is more likely that most consumers will still prefer larger piscivorous marine fish as sashimi or fillets.

The ocean's ecosystem offers several reasons why the bottom of the trophic pyramid is a better place

for humans to extract nutrition from the sea. It is, most simply, a matter of mass and mathematics. Herbivorous fish are more abundant, with greater biomass. Catching 1,000 t of Peruvian anchovies has little impact on the 6 million ton spawning biomass (around 0.025%). By contrast, 1,000 t of tuna represents around 10% of the Bluefin Tuna spawning stock in the Western Atlantic, the biomass of which is currently estimated at less than 10,000 t [27].

Moreover, Clupeiforms are classic "r-selected" species, with their smaller body size, faster maturing, and shorter life spans [28]. They are highly opportunistic: a decrease in population size in Peruvian anchovies often results in increased recruitment from the next spawning. From an ecological perspective, these species are precisely where fishing effort should be targeted, not the larger, more vulnerable, slower-growing "K-selected" species at the top of the food chain. In agricultural terms, most of the crops that humans raise are strongly "r-selected" – wheat, corn, barley, rice, while targeting a "K-selected" species in agriculture might be the equivalent of chopping down oak trees to eat the acorns.

Herbivorous clupeiforms also grow and reproduce faster. Menhaden stock resilience to fishing pressure is "high" [29], with a population doubling time of only 15 months. Northern Bluefin Tuna, by contrast, have "low" stock resilience, and a minimum population doubling time of 4.5–14 years [30]. Therfore, if half the menhaden were harvested, it would take 15 months for the stock to recover. However, if half the tuna population was taken, it would take, at a minimum, between 4.5 and 14 years to recover. Southern bluefin tuna also do not begin to spawn until they are perhaps 11 years old, and may live to "at least 40 years of age" [30]. However, Peruvian anchovies are sexually mature within 1 year, and only live for around 3 years. The 3-year old anchovies then die and fall to the ocean floor.

The public health imperative should also provide impetus to source fish meal and fish oil from lower down the food chain. Menhaden and anchovies filter algae and zooplankton directly from the water. They are therefore high in heart-healthy omega-3 oils, yet low in the persistent organic pollutants, such as mercury and PCBs. These pollutants, however, are concentrated as they move further up the food chain. It is primarily top-level predators – sharks and tuna – that are on FDA advisories for pregnant and nursing mothers, and children. By contrast, an aquaculture species that can achieve a feed conversion efficiency of close to 1:1 (FIFO, or Fish In : Fish Out) contain essentially the same contaminant loading as the clupeiforms at the base of the food chain.

Clupeiform fisheries are also more readily managed, with relatively simple stock dynamics and ecosystem interactions. The major inputs to clupeiform stocks are the spawning biomass and primary productivity, which is usually driven by the strength of the nutrient-rich upwelling. Most of the fisheries occur within the EEZ of a single nation, where there are direct incentives for sound management and enforcement, and where access can be regulated. Tuna and swordfish, by contrast, are highly migratory species. Donut-holes of high-seas waters, beyond any country's 200-mile zone, provide opportunities for distant-water fishing nations to concentrate their boats and effort. Attempts at managing tuna stocks are typified by the International Commission for the Conservation of Atlantic Tunas (ICCAT), which has 46 members, and almost no enforcement capabilities. And while Hawaii's long-line fishery targeting big-eye tuna may be very well managed, for example, heavily subsidized European or Asian purse-seiner fleets target the juveniles of the same stock in the South-Western Pacific.

Moreover, the carbon footprint of clupeiform fisheries is minimal. These fish are usually taken by purse-seiners, working close to the coast, encircling schools containing hundreds of tons at a time. The carbon footprint for species higher on the foodchain is much higher, with fish being caught by diesel-powered trawlers or trollers, or – for bluefin tuna and swordfish – by harpooning the fish, one at a time.

Most importantly, however, it is far better from a bioenergetic perspective to target fish closer to the bottom of the food chain. Applying the 10% trophic transfer rule means that the 1 lb of wild tuna sashimi on a consumer's plate needed to eat 10 lb of anchovies – or its equivalent in fish meal and fish oil. Or maybe, if there were two steps in the trophic pyramid, each pound of wild tuna required 100 lb of anchovies to first be converted into 10 lb of mackerel.

Aquaculture, by contrast, is always a single step – clupeiforms-to-crop. But aquaculture can also use alternative agricultural proteins and oils, such as corn

and wheat gluten, soy proteins and oils, canola, and other animal processing by-products. These other proteins and oils reduce the fish meal and fish oil inputs to the extent that some of the purported "carnivores" can thrive on a diet that is around 20% fish meal and fish oil. On the "sustainability quotient" – the number of pounds of fish-in to produce 1 lb of fish-out (the FIFO) can then attain the perfectly efficient goal of parity, or 1:1 (i.e., every pound of Peruvian anchovies in the farmed fish diet produces 1 lb of product). The result is efficient conversion of a low-value anchovy into a high-value marine fish, without disrupting the fragile top of the trophic pyramid.

Larger, wild fish are more bioenergetically wanton. Wild fish lose energy through inefficient digestion, in hunting prey, trying to avoid predation, spawning, and succumbing to natural mortality. As wild fish grow larger, they also become increasingly inefficient – a greater proportion of energy is needed to maintain the animal's metabolism. Any bycatch will compound these inefficiencies further. The global bycatch ratio is around 0.28 lb of discard for every pound of target species.

Earlier estimates [20] suggested that farmed fish might be more efficient than wild fish, based on a single trophic step, by a factor ranging from 2 to 5. Combining the life cycle inefficiencies, trophic inefficiencies, and bycatch inefficiencies of wild fish, however, means that farmed fish may be more efficient than wild fish by a factor of around 60 (Table 1, and [31]).

This reasoning does not advocate for greater fishing effort on anchovies. To the contrary – caution is called for. While most of these stocks are sustainably managed at current levels, they could not withstand any greater pressure. These clupeiform stocks should continue to be very closely monitored and highly regulated. Large marine protected areas should also be established to allow some clupeiform-based ecosystems to flourish in their natural state (rather than attempting ecosystem based management). But it is imperative that the fisheries at the base of the food chain be better managed, and environmentally sound aquaculture endorsed so that pressure can be taken safely off the top of the food chain.

Fishing at the bottom of the food chain should therefore be encouraged preferentially over any other kind of fishing. This is not a function of recent overfishing: even 100 years ago, this principle would have still held true. People always *should have been* fishing at the bottom of the food chain. To continue to accuse aquaculture as being part of the problem of "fishing down the food chain" is therefore disingenuous. Aquaculture is an important part of the solution to the feeding of the growing humanity. To assert otherwise confuses the consumer and discourages the policy shifts needed toward more sustainable aquaculture, and healthier oceans.

Future Directions

The Challenge

Open-ocean mariculture must expand, but it must expand in an environmentally responsible manner. While opponents may cite the precautionary principle as reason to not move forward, or to do so only cautiously, with experimental site permits or restrictive legislation, there is an imperative for action. Extractive pressures cannot be increased on already depleted wild fishery resources, and the public health costs of limited seafood consumption cannot be accepted any more. These two trends must be turned around by finding alternative sources of healthful seafood. Open-ocean mariculture is the only practical means of achieving this. To urge inaction, then, is in effect advocating for either a greater fishing pressure on wild stocks, or the increasing human mortality and suffering from heart disease and stroke. If the consequences of inaction are so inevitable and so severe, and the potential consequences of action are only slight and temporary, then the precautionary principle insists that action must be taken. Therefore, the requisite regulatory framework for industry growth must expediently be set in place and the needed technologies and support industries developed to proceed forward.

The single greatest challenge will remain that of effecting a change in the mind-set of the environmental community to the extent that there is broad acceptance of the need for expansion of open-ocean mariculture as a conservation tool, that is, as an alternative to targeting larger wild fish. Until this is accomplished, then, this industry will continue to be smeared with irrational fears, community prejudice and consumer bias, and the promise it offers will be left woefully unfulfilled.

Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii. Table 1 Relative ecological efficiencies of farmed and Wild-Caught fish. The table shows the compounded cost in terms of anchovy-equivalents for farmed and wild-caught fish. Low-end estimates and high-end estimates are provided for each type of fish and compared cross-ways to obtain a lowest relative rate and highest relative rate

	Farmed fish		Wild-Caught fish		Global mean
	Low-end estimate	High-end estimate	Low-end estimate	High-end estimate	Ratio of wild to farmed
Life cycle efficiency[a]	1	1	3	10	6
Trophic transfer efficiency[b]	1	8	10	100	7.3[c]
"Bycatch" efficiency	1	1	1[(4)]	11[e]	1.3[d]
Compounded "cost"	1	8	30	11,000	57

The lowest relative rate extrapolated from this table is that the least-sustainably farmed fish are around 4× more ecologically efficient than the most sustainably harvested wild fish (i.e., 30:8). The highest relative rate is that the most sustainably farmed fish could be 11,000× more ecologically efficient than the least-sustainably harvested wild fish (i.e., 11,000:1). The Global Mean of wild fish efficiency to farmed fish efficiency is around 57×

[a]There are no published estimates of the relative life cycle efficiencies of farmed versus wild fish. However, fish that reach reproductive age in captivity can see Feed Conversion Ratios increase by factors of 5 or 10 over juvenile and sub-adult fish. Natural mortality and the nutritional cost of maintenance of basal metabolic processes during periods of food depravation also increase the "Economic" Feed Conversion Ratio for wild fish populations

[b]In 1997, food conversion efficiencies (FCE) for farmed marine fish and farmed salmon were around 5:1 and 3:1, respectively (Naylor et al. 2000). By 2010, however, FCEs are projected to reach 1.5:1 for farmed marine fish, and as low as 1.2:1 for farmed salmon (Tacon 2005). Kona Blue has been able to culture Kona Kampachi® on a diet that equates to a 1:1 ratio of wet-fish-in to wet-fish-out. However, if a less-sustainably farmed fish is fed a pellet high in fish meal and fish oil (say, to meet the Scottish Soils Association's Organic standards, with around 80% fish meal and fish oil), this diet could equate to around 4 lb of wet anchovy-equivalents for every 1 lb of dry pellet (a wet-fish to fish-meal ratio of 5:1 is considered standard). On this diet, most commercially farmed species might have food conversion ratios of around 2:1 (dry pellet to wet fish), implying an FCE of 8 lb of wet-fish-in for every 1 lb of wet-fish-out

[c]Tacon's (ibid) estimate of FCEs for farmed salmon and farmed marine fish might be conservatively pooled at, say, 1.5:1, that is, 1.5 lb of anchovy-equivalents for every pound of farmed fish produced worldwide. There is a differential of around 1.1 trophic levels between global fishery landings (with a mean trophic level of around 3.3) and the Peruvian anchovetta fishery (with a trophic level of around 2.2: Pauly et al, [19]). At a presumed 10% biomass transfer efficiency up each trophic level, this implies 11 lb of anchovy-equivalents to produce a pound of harvested wild fish. The median ratio of wild to farmed trophic transfer efficiencies can therefore be estimated at 11:1.5, or 7.3:1 overall

[d]Harrington et al. (2005), report a "nationwide discard-to-landings ratio of 0.28" (i.e., for 3.7 million tons landed, some 1.06 million tons were discarded). However, for highly selective fishing methods, such as harpooning, bycatch is effectively zero, as for farmed fish

[e]For finfish, the ratio of bycatch to target fish (in the Northern Pacific) can be as high as 11:1 because the bycatch is either too young, out of season, or the vessel has no permit to keep it (Alverson 1998)

Further! Deeper!

The Kona Blue operations to date have produced annual harvests of around 500 T, and have demonstrated clearly that this can be achieved without any significant impact on ocean ecosystems or resources. The key now is to ensure that these impacts remain insignificant, as the industry scales. Expansion opportunities, in terms of broader acceptance and greater access, will be conditional on future farm sites being located further offshore and in increasingly deeper waters. The industry therefore needs to develop the technologies to support these trends and to ensure that carrying capacities and prudent biosecurity practices are adhered to as the industry grows.

There are economic and environmental drivers for larger-scale open-ocean mariculture operations in

deeper water, further offshore. As the automation systems for net pen management become increasingly sophisticated [32], sea state becomes less of an impediment to more remote, more exposed sites. The major disincentive then becomes travel time to and from a site. Beyond about a 20 mile distance, it is increasingly difficult to maintain a farm site with day-workers, and some on-site residence becomes necessary. This, then, argues for further scaling: once a farm site is manned 24 h, with staff changes twice-weekly or weekly and periodic delivery of feed and other supplies, there are strong commercial reasons for the operation to grow in scale to support the inherent fixed costs.

Larger scale net pens present some challenges in the open ocean, particularly with fish handling, but there are dvantages as well. Larger pens are more cost-effective in terms of cost per unit of volume (i.e., cost per cubic meter) and in terms of managing the stock. A farmer manages a net pen, rather than a fish: a net pen of 3,000 m^3 and 50,000 fish requires roughly the same level of management as a net pen of 24,000 m^3 and 400,000 fish. Similarly, where there is a significant cost to the netting material, or cost to maintaining the netting, then the lower surface area to volume ratios of larger net pens are increasingly attractive. Larger net pens also require larger hatcheries, to produce sufficient fish in one cohort to stock the pen, and this results in greater hatchery efficiencies.

New netting materials under development could revolutionize the net pen designs. Kikkonet™ appears to offer significant advantages over multi-strand nylon or cotton webbing, with greater rigidity and predator-breach resistance reducing the risk of escapes and easier cleaning capabilities with the single monofilament material. Similar advantages may be offered by new brass alloy materials under development, which almost completely eliminate biofouling and the attendant fish health concerns, as well as offering improved resistance to breaches from predators or mechanical tearing. Both Kikkonet™ and brass webbing, however, require rigid frames in open-ocean environments: constant wave and current movement can quickly destroy netting material if the net pen frame allows any excess movement. Each of these materials represents a significantly higher capital cost than nylon or cotton netting, but there are tremendous operational savings to be gained from reduced risk of failure and reduced labor for net changing, net washing, antifoulant dipping, and net loft work.

Although much discussion has focused on the advantages of surface and submerged net pens and the relative operational efficiencies of each, this may be the single most important deciding factor for pen design: rigid-framed nets such as the submersible Sea Station® and Aquapod® can support more robust, rigid netting materials. Flexible framed nets such as PolarCirkel®, or those that rely on gravity to maintain net shape, such as Wavemaster® can only support pliable nets of nylon and cotton in the higher wave and current conditions offshore. Where sea conditions or predator prevalence demand the more robust, rigid mesh forms of brass or Kikkonet, then submersible rigid-framed pens would appear to have operational advantages.

Letting Go

The potential for untethered "trans-ocean drifter net pen" fish farms has, up to now, been the stuff of science fiction [3]. However, the concept of untethered pens offers the potential for far larger scaling of operations, with almost negligible potential for any environmental impact; a net pen in very deep water is a nonpoint source for effluent impacts on water quality and substrate, and there is almost no potential for interaction with wild fish stocks (apart from aggregative effects). There are also advantages for fish health management on the farm – a drifter pen is essentially perpetually fallow. Drifter pens that ride regional ocean eddies or powered pens (either towed by a surface vessel, or where the fish containment is integrated into the vessel hull) are already moving from concept to prototype; within 5–10 years, some form of untethered net pen may become a commercial reality. Several examples are worth noting:

In June, 2008, a trial in Puerto Rico tested a submerged, self-propelled, 3,200 m^3. Aquapod as a demonstration of untethered aquaculture operations. The system was able to achieve 30 cm/s forward speed by using two electrically driven 2.5 m diameter propellers powered by a diesel generator set onboard a small towed vessel. The Aquapod was able to hold position in 25 cm/s currents and was easily maneuverable. The concept of remote-controlled or

autonomous operation was judged feasible using cage-mounted sensors and buoy-mounted GPS and communications.

In October 2009, the State of Hawaii approved a tuna farm permit for a 247 acre site offshore of the Kohala Coast (north of the Kona Blue farm site) in water over 1,320 ft deep, for up to 12 untethered Oceanspheres. With no anchors, it is proposed that these fish farming platforms will be held in position by self-propulsive forces powered by a hybrid Solar Ocean Thermal Energy Generator. Hawaii Oceanic Technology, Inc. plans to deploy the first of these net pens in 2011.

A regional drifter pen concept under development by Kona Blue – christened the *Velella* system – is projected to remain within a prescribed geographical area without anchoring, by riding the eddies in the lee of oceanic islands such as Hawaii, or by riding the gyres found in semi-enclosed seas, such as the Sea of Cortez or the Gulf of Mexico. The advantages of this concept are that it then enables the operation to consistently stock, feed, tend, and harvest the fish in the pen, as well as changing crew and other maintenance tasks. By being untethered, the *Velella* can be located in very deep water, beyond normal mooring depths, and any potential environmental impacts are reduced to de minimis levels.

As untethered net pens do not occupy a fixed geographical space, they do not require a lease but rather are more properly regulated as non-powered vessels. Where there is no point source effluent from a motile pen, the monitoring and regulatory requirements may also be those applied to vessels, rather than fixed farm locations. Projects such as *Velella* might also pass through or be deliberately sited in international waters, where no permits are presently required and where no regulatory framework is currently in place. This regulatory vacuum should be remedied, to both govern its growth but also encourage the development of this technology.

IMTA

Much interest is often focused on Integrated Multi-trophic Aquaculture (IMTA) as a means of improving the sustainability of open-ocean fish farming, by mitigating effluent impacts and more fully utilizing the nutritional inputs. In nearshore systems, the siting of filter-feeding bivalves around fish farm net pens in IMTA systems has been shown to reduce particulates in the water column. Similarly, siting macroalgal culture systems downcurrent of the fish farm can also reduce dissolved organics. These polyculture systems also offer secondary products (such as edible seaweeds and mussels) that are themselves marketable.

In open-ocean mariculture systems, however, the applicability and utility of IMTA is somewhat dubious. The priority for siting open-ocean mariculture operations should be to strive for greater water clarity, depth, and current movement to optimize fish health and minimize any potential impacts on the effluent. These criteria are contrary to the desired characteristics of an IMTA operation, which presumes some entrainment of waters after it passes through the fish pen, with enrichment of the effluent providing benefits to the filter feeders and phototrophs. If open-ocean mariculture operations do indeed have no measureable impact on water quality, then presumably there is no benefit to be gained from co-siting the fish with filter feeders and plants.

Furthermore, the capital and maintenance costs for the secondary production systems would be prohibitive. Open-ocean mariculture, in the foreseeable future, will need to focus on high-value species such as tuna, yellowtails, snappers, and groupers to provide a reasonable return. Only with the development of far greater operational efficiencies, in later years, will lower-value finfish species become economically viable. The commercial returns from deploying and maintaining even lower-valued macroalgae and bivalve culture lines around an offshore site would be even further deferred.

In addition, the co-siting of mussels and seaweed, say, around an open-ocean site represents engineering and biological risks to the fish farm operation. The additional drag from the long-lines would add tremendous strain on the mooring system for the net pens. If moored separately, there would still be concerns that the secondary producer moorings could fail under load and entangle with the fish's net pen or moorings. The resting stages of pathogens or parasites might also be

harbored in the cultured species or ecosystem that surrounds the long-lines, resulting in a proliferative feedback loop and elevated levels of these pests in the cultured fish.

The overarching goal of open-ocean mariculture is growing high-value species in near-pristine waters, with little potential for their interaction with surrounding biologically active substrates. The notion of burdening an offshore operation with lower-value production systems is antithetical to this goal. The fish carrying capacity should allow the natural assimilative capacities of the surrounding ecosystem to fully absorb the farm's ecological footprint. The nutritional by-products, that is, the nutrients in the effluent from open-ocean mariculture systems can still promote productivity on a broader scale, especially in oligotrophic tropical waters. However, a deeper ecological perspective would suggest that there is no need for man to capture this productivity for commercial gain. The oceans can use these inputs in other ways; they are not necessarily "lost."

Bibliography

Primary Literature

1. FAO (2009) The state of world fisheries and aquaculture. FAO, Rome. www.fao.org/docrep/011/i0250e/i0250e00.HTM
2. Clarke AC (1957) The deep range. Harcourt, New York, p 238
3. Mann CC (2004) The bluewater revolution. Wired Magazine, Issue 12.05 – May 2004
4. Environmental monitoring data for Kona Blue. www.kona-blue.com/emonitoring.php
5. See 2003 Final EA. www.kona-blue.com/permitapplication.php
6. Tagawa AW, Tam CKM (2006) Hawaii's Ulua and Papio tagging project 2000 to 2004. DAR Technical Report 06–01. DLNR, Honolulu, p 75
7. Meyer C (Personal communication, 2/17/2010)
8. Balazs GH (1980) Synopsis of biological data on the green turtle in the Hawaiian Islands. U.S. Dep. Commer., NOAA Tech. Memo. NOAA-TM-NMFS-SWFC-7, p 141
9. Viezbieke J (Personal communication)
10. 2009 Draft EA Appendix 2: Marine Mammal Report from Kona Blue to NOAA, dated 11/26/08, www.kona-blue.com/download/REVISED_DraftEnvironmentalAssessment2.pdf
11. Nitta ET, Henderson JR (1993) A review of interactions between Hawaii's fisheries and protected species. Mar Fish Rev 55(2):83–92
12. Celikkol B (1999) Biological assessment of the University of New Hampshire open ocean aquaculture demonstration project finfish component. Prepared for U.S. Army Corps of Engineers, New England Division, Concord, p 60, plus appendices
13. Wursig B, Gailey GA (2002) Marine mammals and aquaculture: conflicts and potential resolutions. In: Stickney RR, McVey JP (eds) Responsible marine aquaculture. CAB International, Wellington, pp 45–59
14. See Appendix III, Black Pearls, Inc., Final Environmental Assessment, 2003, http://www.kona-blue.com/permitapplication.php
15. Worm B, Barbier EB, Beaumont N, EmmetDuffy J, Folke C, Halpern BS, Jackson JBC, Lotze HK, Micheli F, Palumbi SR, Sala E, Sekoe KA, Stachowicz JJ, Watson R (2006) Impacts of biodiversity loss on ocean ecosystem services. Science 314(5800):787–790
16. Punihaole R (2003) Personal communication
17. Maly K, in litt, 8/26/02
18. Alaska Energy Authority, undated brochure
19. Pauly D, Christensen V, Dalsgaard J, Froese R, Torres F Jr (1998) Fishing down marine food webs. Science 279:860–863
20. Taylor R, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MCM, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405:1017–1024
21. O'Neill B (2006) Seafood Watch®Seafood Report: U.S. Farmed Rainbow Trout, *Oncorhynchus mykiss*. Final Report. June 23, 2006. Monterey Bay Aquarium, p 26. www.montereybayaquarium.org/cr/cr_seafoodwatch/content/media/MBA_SeafoodWatch_FarmedTroutReport.pdf
22. Miranda IT, Peet C (2008) Seafood Watch®Seafood Report: Farmed Yellowtail, *Seriola* spp. Final Report. 22 Oct 2008, Monterey Bay Aquarium, p 73. www.montereybayaquarium.org/cr/cr_seafoodwatch/content/media/MBA_SeafoodWatch_FarmedYellowtailReport.pdf
23. Bittman M (2008) A seafood snob ponders the future of fish. The New York Times, 16 Nov 2008, p. WK1. http://www.nytimes.com/2008/11/16/weekinreview/16bittman.html
24. Simpson S (ed) (2007) 10 solutions to save the ocean. Conservation Magazine 8(3)
25. Wasley A, Wickens J (2008) Fishy business. The Ecologist, Dec 2008–Jan 2009, pp 24–29
26. The Economist (2009) Come, friends, and plough the sea – but make sure the farmed fish are veggies. The Economist: A Special Report on the Sea. 3 Jan 2009, p 11
27. ICCAT (2008) Report of the Standing Committee on Research and Statistics (SCRS); Madrid, Spain, September 29 to October 3, 2008. BFTW-Figure 2., p 89. International Commission for the Conservation of Atlantic Tuna, Madrid, Spain. http://www.iccat.int/Documents/Meetings/Docs/2008_SCRS_ENG.pdf
28. Pianka ER (1970) On r and K selection. Am Nat 104:592–597
29. Fishbase (2009a) *Brevoortia patronus,* Gulf menhaden. http://www.fishbase.org/Summary/SpeciesSummary.php?id=1589 (1/30/2009)
30. Fishbase (2009b) *Thunnus thynnus,* Northern bluefin tuna. http://www.fishbase.org/Summary/SpeciesSummary.php?id=147 (1/30/2009)

31. Pauly D, Christensen V (1995) Primary production required to sustain global fisheries. Nature 374:255–257
32. Langan R (2010) Technology needs for improved operational efficiency of open Ocean Cage Culture. J Marine Tech Soc 44(3):47–55

Books and Reviews

Bridger CJ, Costa-Pierce BP (2003) Open ocean aquaculture – from research to commercial reality. The World Aquaculture Society, Baton Rouge, p 351

ICES (2006) Report of the Working Group on Environmental Interactions of Mariculture (WGEIM). Narrangansett, Rhode Island, U.S.A. ICES CM 2006/MCC:03, p 195

IUCN (2004) Mediterranean marine aquaculture and environment; Identification of issues. IUCN Center for Mediterranean Cooperation, p 34

Halwart M, Soto D, Arthur JR (eds) (2007) Cage aquaculture. Regional reviews and global overview. FAO Fisheries Technical Paper No. 498. UN-FAO, Rome, p 254

Lee CS, O'Bryen PJ (eds) (2007) Open ocean aquaculture – moving forward. Oceanic Institute, Waimanalo, p 78

Marine Aquaculture Task Force (2007) Sustainable marine aquaculture: fulfilling the promise; managing the risks. Woods Hole Oceanographic Institute, Woods Hole, p 128

NOAA (2008) Offshore aquaculture in the United States: economic considerations, implications and opportunities. NOAA, Silver Spring, p 272. http://aquaculture.noaa.gov

Fertilizer Science and Technology

J. F. Angus
CSIRO Plant Industry, Canberra, ACT, Australia

Article Outline

Glossary

Essential nutrients There are 16 mineral elements that are essential for plant growth. They are referred to here by their symbols: nitrogen, N; phosphorus, P; potassium, K; calcium, Ca; magnesium, Mg; sulfur, S; boron, B; iron, Fe; manganese, Mn; copper, Cu; zinc, Zn; molybdenum, Mo; sodium, Na; chlorine, Cl; silicon, Si; nickel, Ni.

Fertilizer efficiency This refers to the additional grain or other agricultural product produced per unit of additional nutrient applied in fertilizer. A related concept is Fertilizer Recovery, which is the additional mass of a nutrient in the aboveground parts of a crop expressed as a proportion of the additional nutrient applied in fertilizer.

Macronutrients N, P, and K.

Micronutrients Cl, Fe, Mn, B, Zn, Cu, Mo, and Ni.

Soil microbial processes These include mineralization, which is oxidation of organic matter and the release of mineral nutrients; immobilization, which is the reverse process of incorporating mineral nutrients into organic matter; and nitrification, which is conversion of ammonium to nitrate.

Definition of the Subject

Fertilizers are compounds or mixtures delivered as solids, liquids or gases, that supply essential nutrients to crops in soluble forms that are convenient and safe to handle. Fertilizers may be applied to the soil or directly to foliage. All nutrients except N are manufactured by concentrating and refining ores extracted from mines. N fertilizers are manufactured from ammonia, which is synthesized from N_2 and H_2. Science contributes to fertilizer use with improved products and methods to increase fertilizer efficiency, profitability of nutrient used, and reducing adverse environmental effects. Technology contributes to fertilizer use by improving the efficiency of manufacture and the complex logistics of delivering hundreds of million tons of products to farms safely, economically, and on time. Fertilizers can be both inorganic and organic, but this contribution refers mostly to inorganic or manufactured fertilizers, since these provide most of the nutrients now added to soil and crops.

Introduction

Fertilizers are indispensable because nutrient supplies from the soil are normally inadequate for the high-yielding crops needed to supply food and fiber for the growing human population. Fertilizers replenish nutrients removed by previous crops and supplement the supply of nutrients from the soil to a level that will produce the farmer's target yield. Fertilizer may also improve the quality of human food, stockfeed, and fiber. The nutrients in fertilizer are a nonrenewable resource and, if they escape from the field, can cause environmental damage. This article discusses the sustainability of fertilizer in relation to these issues.

Before the invention of fertilizers, the only ways that farmers could accumulate nutrients on a field was to

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Fertilizer Science and Technology. Table 1 Growth in world population, cereal production, and consumption of nutrients in fertilizer during the second half of the twentieth century

	Annual growth (%)
Human population[a]	1.8
Cereals[a]	
Maize	2.7
Rice	2.4
Wheat	2.3
Fertilizer[b]	
N	7.1
P	4.6
K	3.7

[a]1961–2001 [3]
[b]1960–1988 [4]

fallow before growing a crop, or to transfer animal manure from grasslands and barns to cropped land, either directly or by grazing livestock on grassland by day and enclosing them by night in a field prior to cropping. An additional source of N has been, and still is, the residues of previous legume crops, pastures, and green manures. With these natural nutrient sources, land is temporarily taken out of crop production. Fertilizer allows land to be used for continuous production.

The consumption of the macronutrients in fertilizer, N, P and, K has grown rapidly since the middle of the twentieth century and has increased the food supply more rapidly than the growth of the human population (Table 1). The increase in fertilizer supply has been even more rapid than the food supply. It is difficult to overstate the importance of fertilizers in sustaining humanity: Stewart et al. [1] concluded from the results of experiments that production of 30–50% of the world's food is a result of commercial fertilizers and Smil [2] calculated from nutrient balances that that nourishment of 40% of the world's population depended on N fertilizers.

The use of the macronutrients is uneven around the world, as shown in Fig. 1. On all continents, fertilizer provides relatively more N than P or K. The usage of all three nutrients in Europe and North America most closely approaches crop requirement while usage in Asia and Africa is relatively high in N. The K applications are negligible in Oceania and Africa probably because soil levels of K tend to be relatively high in dry regions.

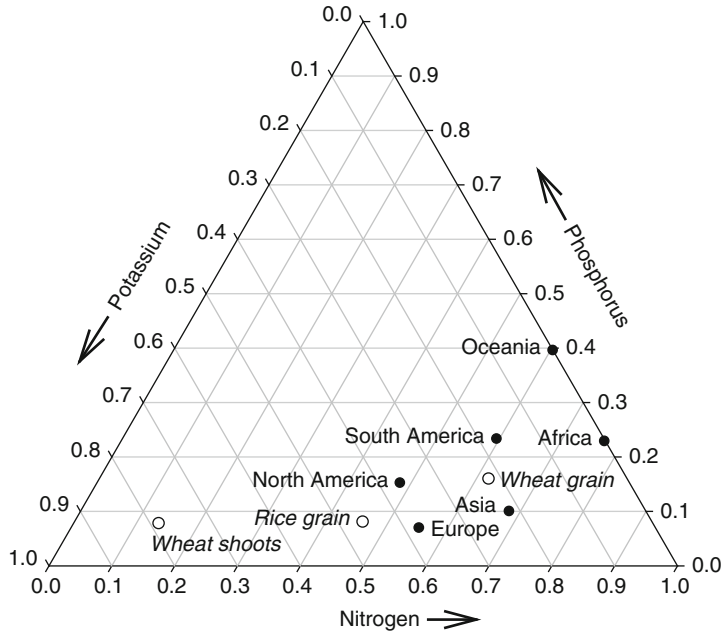
This article discusses the sustainability of fertilizers from the perspectives of resources and logistics involved in supplying fertilizer to the farm, the on-farm effects on productivity, and on-site and off-site environmental effects.

Mining and Manufacture of Fertilizers

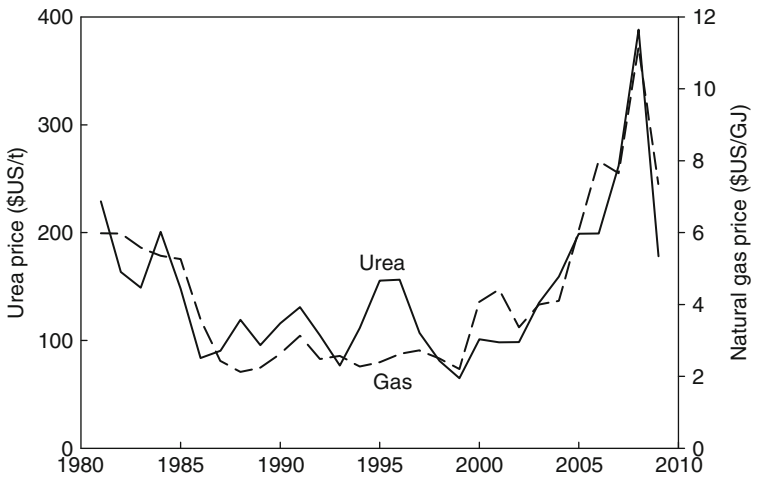
The raw materials for fertilizer manufacture, apart from N, are mined from ore deposits. In the century before 1920, most fertilizer N was also extracted from mines and, before that, from accumulations of nitrate in soil where farm animals had been housed for long periods. Much of this nitrate was used to manufacture gunpowder and other explosives as well as for fertilizer. The increasing demand for explosives and N fertilizer in the nineteenth century led to brutal mining industries that extracted guano (vitrified fecal deposits from sea birds, mostly on tropical islands) and nitrates, a sorry history told by Bown [7].

In modern mining industries, the nutrient compounds in the ore are concentrated by chemical or physical processes into fertilizers that are stable and available for plant uptake. In some cases, two or more nutrient elements are combined, by chemical reaction or physical mixing, into compound fertilizers. Most fertilizers are applied as solids, and single-element and compound fertilizers are normally manufactured as relatively homogeneous granules, which flow freely, minimize water absorption, produce little dust, and which can be transferred efficiently between storages and applied to the soil using machinery or by hand. Most nutrients are available as several different chemical compounds. Examples of these compounds and the manufacturing processes are described by Tisdale et al. [8].

Nitrogen fertilizers are produced from ammonia, which is synthesized by the Haber-Bosch process under conditions of high temperature and pressure using N_2 and H_2 [2]. The usual source of H_2 is natural gas, and the price of N fertilizer is closely linked to the natural gas price (Fig. 2).



Fertilizer Science and Technology. Figure 1
Variation between continents in the proportions of N, P, and K used in fertilizer, and some examples of the proportions of these nutrients contained in whole crops (fertilizer data from FAOstats, rice data from Dobermann and Fairhurst [5], and wheat data from Bar-Tal et al. [6])



Fertilizer Science and Technology. Figure 2
The price of urea generally follows the price of natural gas, which represents the main production cost [9]

Many highly productive farming systems directly inject anhydrous ammonia into the soil. The advantage of ammonia is its high N concentration (82%) and hence low transport cost. Offsetting this is the need for expensive containment vessels, typically made from thick steel, and other costs of ensuring safety. The use of ammonia is confined to application at or before the time of sowing because of toxicity to growing plants.

Applications of a large N amount at these times can cause yield reductions, as discussed below.

Ammonia is used to produce many forms of N fertilizer. The most common is urea, which has the advantages that it contains a relatively large percentage of N (46% N) and is safe to handle, unlike ammonium nitrate (34% N), which can be made to explode, and anhydrous ammonia, which is toxic to humans and animals at low concentrations in the atmosphere. Other common N-containing fertilizers are the solid ammonium phosphates (10–20% N) and the solution of urea-ammonium nitrate (28–32% N).

Fertilizer Distribution, Marketing, and Price

Most fertilizer in developing countries is granular and is sold in 40–50 kg bags and broadcast by hand, apart from a small but increasing amount applied by machine at the time of sowing. In developed countries, most of the solid fertilizer is supplied in bulk and increasing amounts of liquid fertilizer are used because of the convenience of pumping the product between vessels during transport and application. These advantages can compensate for the additional cost of transporting the water in which the nutrients are dissolved. Most of the advantages of liquid are for on-farm handling, so an efficient system is for fertilizers to be delivered as solids from the manufacturer to the reseller who then dissolves the solids just before delivery to the farm. The advantages of liquid fertilizer (and gaseous anhydrous ammonia) are greatest for highly productive regions where farms are close to resellers and where it is possible to grow two or more crops each year and spread the cost of specialized equipment for mixing and applying fertilizer over a large crop area. Resellers extend the time of distribution by offering discounts for early use, but this can be at the expense of reduced fertilizer efficiency. One of the hazards of liquid fertilizer is soil compaction due to the mass of water and nutrients contained in equipment at the time of sowing or in spray carts for post-sowing applications.

Extensive dryland farming systems in developed countries use solid fertilizers where the transport costs are high. Solid fertilizer has an advantage for topdressing because granules can typically be mechanically thrown up to 10–15 m on each side of an implement, which is consistent with the 10–15-m width of seeders and 20–30-m width of boom sprays used for controlled traffic. The maximum spreading distance of granules may impose limitations as seeders and spray booms continue to get wider.

Some of the largest financial and environmental costs of fertilizer are related to transport. Products with low concentrations of nutrients are necessarily expensive to transport and their use is confined to specialized applications, for example calcium nitrate, which contains only 16% N, for some vegetables. During the second half of the twentieth century, there has been a general increase in nutrient concentrations leading to reduced cost of transport to the farm and handling on the farm. Nevertheless, the weight of fertilizer means that transport is expensive and low-cost transport methods such as ship, barge, and rail are preferable to road transport. Transport costs are minimized when fertilizer is back-loaded on vehicles carrying agricultural produce to cities. Transport makes up a large part of the on-farm cost of fertilizer in rural areas of sub-Saharan Africa and presents a major obstacle to increasing crop yields [10].

The transport cost is even greater for manures and compost than they are for inorganic fertilizer. Dry animal manures typically contain less than 2% N and 0.4% P, so they cannot be economically carried far from the source. Instead, manures tend to be applied in a radius up to tens of kilometers. Where there is a large source of manure, such as a feedlot or large dairy, the available nutrients can overload the capacity for crop uptake, so that nutrients may accumulate in the soil within a few years. Where the manure contains potentially toxic material such as Cu contained in pig manure, nutrient loading of the soil needs to be closely monitored.

Fertilizer represents the main demand for N, P, and K, but for the micronutrients, usage for fertilizer manufacture is much less than for manufacturing industry. In the case of Zn, annual world production is about 20 million tons, but the amount of Zn contained in all cereal grains is less than 0.1 million tons per year.

The use of compound fertilizers has advantages in ease of handling and may be cheaper than single-element fertilizers. For example, the nutrients in ammonium phosphates are generally cheaper to the farmer than when they are purchased separately, for

example as urea and triple superphosphate. In such situations, compound fertilizers can be profitable for the farmer, even if one of the nutrients is not particularly deficient. However with some NPK blends, there may be situations where crops do not respond to all nutrients contained in the fertilizer, and where the compound fertilizer is expensive, the whole product may be unprofitable to use. Such a situation applied to many farms in the Philippines where yield of rice did not respond to either P or K in a NPK compound fertilizer [11]. In this situation, application of the NPK was unprofitable on one third of farms and there was no simple way of determining which fields were deficient in which nutrient element. In such situations, the simplest solution is to make single-element fertilizers available so that strip trials conducted by farmers can show which nutrients give profitable responses [12].

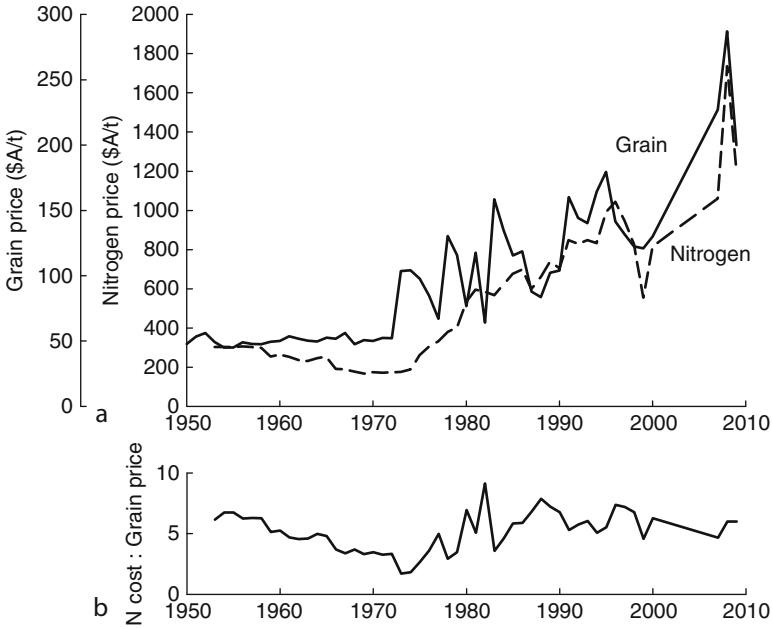
The main fertilizers such as urea, MAP (monoammonium phosphate), and DAP (diammonium phosphate) can be regarded as generic and unspecialized commodities that are traded freely. They are sold by manufacturers to distributors on world markets that are generally open and transparent. An insight to the wholesale market is available online [13]. There is little opportunity for manufacturers or distributors to compete other than through price and service. Intense price competition does not support the extension services offered by many fertilizer companies until the 1980s or 1990s.

Marketing is generally in the hands of agribusiness employees and media campaigns. Increasingly, advice about fertilizers is supplied to farmers at or near the point of sale by fee-for-service agronomists employed by fertilizer vendors. This represents a conflict of interest between the need for agribusiness to maximize sales and minimize carryover of fertilizer inventory, and the need of farmers to apply the fertilizer at an optimum amount and timing. Independent advice to farmers is important since fertilizer usually represents the single greatest cost of crop production.

Since generic fertilizers do not provide large profits, manufacturers or distributors aim to develop unique products that are easy to use or contain additives that increase fertilizer-use efficiency. Examples are additives to N fertilizers that inhibit urea hydrolysis and so limit the potential for ammonia volatilization, or nitrification inhibitors that restrict the production of nitrate and hence loss by leaching and denitrification [14]. The cost of these additives may not necessarily be justified by additional profit, and they need to be assessed on a case-by-case basis [15]. Some urease and nitrification inhibitors are powerful biocides and have effects other than on N relations, for example, the nitrification inhibitor nitrapyrin has fungicidal activity, so any yield benefits may not be due only to effects on N supply to crops [16]. Slowing the release of nutrients from fertilizer by coating granules with a polymer is another possible means of maintaining adequate nutrient levels near the roots [17]. The cost-effectiveness of these products also needs to be assessed on a case-by-case basis. The most promising markets for fertilizer additives are where subsidies are offered to minimize N release into the environment.

World fertilizer prices fluctuate, depending on variations in supply and demand. The fluctuations for particular products can be massive, for example, the urea price spike in the mid-1990s when Chinese fertilizer companies suddenly increased urea imports (Fig. 2). The cost of fertilizers generally can also fluctuate strongly, as in 2008 when fertilizer price increased in tandem with a rise in world grain prices (Fig. 3). It is clear from Fig. 3 that the price of grain and cost of fertilizer tend to readjust to maintain a reasonably constant cost:price ratio of about 6, even when grain price or fertilizer cost are disrupted by an external shock.

The price of fertilizer in many markets has been subsidized by governments, generally leading to fertilizer overuse, inefficient methods of application and, in some cases, excess agricultural production that is dumped on world markets. In some countries, the subsidy is through supply of natural gas at less than world price for ammonia production. Fertilizer subsidies have similar effects to subsidies on the price of agricultural production, in that they lead to overproduction because the optimum application rate of fertilizer is affected by the ratio of product price to fertilizer cost. Subsidies on agricultural products and fertilizers have long been criticized because they depress the price of agricultural products in world markets and so reduce the incomes of unsubsidized producers. Criticism is also justified because the overfertilization promoted by subsidies is a major cause of environmental damage [19, 20].



Fertilizer Science and Technology. Figure 3

(**a**) Farm-gate cost of fertilizer N and price of wheat, both expressed in $A and (**b**) the cost to price ratio for N and wheat grain (updated from Angus [18]). Costs and prices are expressed in Australian dollars ($A) since these represent world prices undistorted by subsidies

Fertilizer Effects on Agricultural Productivity

In their textbook on plant nutrition, Mengel and Kirkby [21] identify 16 mineral elements as essential for plants. Three of these, Na, Cl, and Si, are normally present in plant tissue at concentrations greater than needed for optimum growth and Ni is rarely or never included in fertilizers. Table 2 lists the remaining 12. A further two, cobalt and selenium, are not essential for plants but are essential animal (and human) nutrients and can be supplied in fertilizer or administered directly to farm animals. The other essential animal and human nutrient, iodine, is not normally supplied through fertilizer.

The concentration of the nutrients in plant tissue varies enormously; for example, N is 100,000 times more concentrated than Mo in cereal grain. The potential yield response to a nutrient, when it is deficient, can be estimated from the inverse of the nutrient concentration. So, for example, if the N concentration in grain is 0.02, as shown in Table 2, a first approximation of potential grain response is 50 kg grain per kg of additional N, assuming that the N concentration of the grain is unchanged by the fertilizer. In reality, not all the fertilizer N is present in the grain. In the case of wheat, 0.7 is a reasonable estimate of the proportion of aboveground N contained in mature grain. A better approximation of the maximum grain response to applied N is then $50 \times 0.7 = 35$ kg grain per kg N. Table 2 shows the equivalent calculation for all nutrients. This approach applies only to the first small application of a nutrient and does not account for the diminishing returns of yield to additional applications.

Table 2 also presents estimates of the maximum net financial return calculated from the maximum grain response, the cost of nutrients in fertilizer, and the price of grain. Note again that these are the maximum responses and returns, and real returns are normally much less because not all the fertilizer is recovered by crops. The costs and prices are necessarily assumptions and will vary greatly between markets and between years. However, the differences between nutrients are so great that even with approximations they serve to show some important points.

The maximum responses of additional grain per unit of nutrient applied in fertilizer in Table 2 can

Fertilizer Science and Technology. Table 2 Typical concentrations of nutrients in cereal grain, *C*, and the proportion of the aboveground nutrient in the grain [21], called the harvest index for the nutrient (HI). The maximum grain response to the nutrient is given by HI/C. The maximum net financial return is given by [*G*(HI/*C*) − F]/F where *F* is the cost of nutrient in fertilizer and *G* is grain price, assumed here to be $US 0.2/kg

Nutrient	Concentration in grain (*C*)	Proportion in grain (HI)	Maximum fertilizer efficiency (HI/*C*) (kg grain/kg fertilizer)	Nutrient cost ($US/kg)	Maximum net returns ($/$)
N	0.02	0.7	35	1.0	6
P	0.003	0.7	233	5.0	9
K	0.004	0.2	50	0.8	12
Ca	0.005	0.7	140	0.2	140
Mg	0.003	0.5	167	1.1	30
S	0.003	0.4	133	0.2	130
B	0.0001	0.1	1,000	12	15
Fe	0.0002	0.3	1,500	3	100
Mn	0.00002	0.5	25,000	5	1,000
Cu	0.00002	0.5	25,000	5	1,000
Zn	0.00002	0.5	25,000	5	1,000
Mo	0.0000002	0.5	25,000,000	35	150,000

serve as benchmarks. When a crop is growing on soil that is believed to be deficient in a nutrient, it should give a yield response comparable with these benchmarks. If it does not it is likely that the nutrient is not seriously deficient or there is a limitation other than the deficiency that is limiting efficient utilization of the nutrient.

The maximum financial crop responses to the micronutrients such as Zn, Cu, and Mo are so large that they are normally applied if there is even a suspicion of deficiency. There are many farming systems where micronutrients still give major grain responses, for example in central Turkey, where widespread Zn deficiency was identified in the 1990s [22]. Continued vigilance is needed in all farming systems in case micronutrients become deficient.

Even for N and P fertilizer, the maximum financial responses are large enough to provide a profit even when the efficiency of recovery is low. In these examples crop recovery of only one sixth of the applied N or one ninth of the applied P gives a break-even return on cost of the fertilizer.

The grain-yield responses to fertilizers are usually much less than these maxima and a great deal of research goes into increasing fertilizer efficiency. Where deficiencies are severe and there is only one nutrient lacking, visual symptoms can be compared with standard illustrations [23]. For less acute deficiencies, a traditional method is to calibrate yield against a soil test and identify the critical nutrient concentration that gives near-maximum yield [8]. The process of soil testing consists of sampling the soil in a field with many cores, combining the samples, and analyzing for nutrient concentration.

Soil tests are not the only methods for determining nutrient status and Table 3 shows examples of methods to estimate N status, including tests related to the nutrient status of vegetative plants. Tests of plant tissue are generally more reliable than soil tests because the volume of soil sampled by plant roots is normally more than the soil volume explored by coring [24, 25].

There are limitations to basing optimum fertilizer application only on tests of soil or plant nutrient status. The approach is suitable for a cropping system in which prices and yields do not vary greatly from year to year, but is less useful where the variation is large. The optimum fertilizer application varies with the ratio of

Fertilizer Science and Technology. Table 3 Tests of N status

Pre-sowing tests	Tests during crop growth
Field history	Tissue N concentration
Total N in the topsoil	Tissue nitrate concentration
Mineral N in the topsoil	Sap nitrate concentration
N released during soil incubation	Shoot density
Previous grain protein	Leaf chlorophyll concentration
	Leaf color compared with standards
	Lower leaf senescence
	Strip trials

Fertilizer Science and Technology. Table 4 Example of a nutrient budget for N fertilizer applied to wheat

Crop N demand	
Target yield	4 t/ha
Target grain protein	12%
N in grain[a]	84 kg/ha
N in aboveground crop[b]	120 kg/ha
Soil N supply	
Soil mineral N at sowing[c]	50 kg/ha
N mineralization during crop growth	80 kg/ha
N available for crop uptake (50%)[d]	65 kg/ha
Fertilizer N requirement	
Additional N required in the crop[e]	55 kg/ha
Fertilizer N required[d]	110 kg/ha

[a]Grain N is the product of yield and grain N, assuming that 1 kg of N = 5.7 kg of grain protein
[b]Assuming grain N is 70% of aboveground N
[c]Soil mineral N sampled to a depth of at least 60 cm
[d]Assuming 50% of soil and fertilizer mineral N is available for crop uptake
[e]Difference between crop demand and soil supply

grain price to fertilizer cost, and the optimum fertilizer rate is high when the ratio of grain price is high relative to fertilizer cost.

The optimum fertilizer rate also depends on yield potential, and higher rates are justified by increasing yield potential. Soil and plant tests emphasize the supply of nutrients but it is important to place equal emphasis on crop nutrient demand. Table 4 shows an example of a nutrient budget, based on a method developed by Myers [26], to illustrate the importance of N supply and demand.

When the quality of the grain is affected by fertilizer, it is necessary to consider the responses of both yield and quality and the ratios of fertilizer cost to the grain price and quality premiums. Angus [27] presents a method to estimate the economic optimum fertilizer for wheat in relation to responses by grain yield and protein.

A well-known but generally erroneous notion about nutrients is the Law of the Minimum, which claims that only one nutrient can limit yield of a particular crop. Where one nutrient is drastically deficient, the Law of the Minimum is a fairly good rule of thumb, for example when a micronutrient such as Zn gives a spectacular yield response. More generally, this Law is deceptive, and crops and cropping systems tend to regulate the quantity of nutrients in the soil by luxury extraction of those that are adequate or surplus, and poor extraction of those that are deficient. The different rates of extraction continue until many nutrients become co-limiting and are required for the yield to reach the biological potential [28].

The weakness of the Law of the Minimum can be seen from the many experiments where fertilization with any one of several nutrients can increase yield (e.g., [11]). Fertilizer management therefore usually needs to consider more than one nutrient. Where several nutrients simultaneously limit yield, the combined effect may be additive, meaning that the yield response of the combined nutrients is the same as the sum of yield responses of the nutrients applied singly. In other situations there may be a positive (or negative) interaction, meaning that the effect of the nutrients when applied together is greater (or less) than the sum of the effects of the individual nutrients. As with many aspects of fertilizer management, there is no alternative to conducting experiments on farms to quantify crop responses to nutrients and their interactions. This rest of this section discusses the macronutrients, N, P, and K, and, as examples of minor and micronutrients, S and Zn.

Nitrogen

Nitrogen is used in greater quantity than other nutrients but the financial returns are often less (Table 1). Nitrogen fertilizer has a reputation for low efficiency and many studies show that a crop typically recovers about half the fertilizer N applied, but the proportion can vary from zero to almost complete recovery [24]. The reasons for incomplete recovery are numerous but often the soil can supply as much N as the crop can take up, and any additional N remains in the soil where it may be lost in several processes. Nitrogen losses include leached nitrate, gaseous ammonia volatilization from urea and ammonia-based fertilizer, and nitrous oxide (N_2O) and N_2 from denitrification. Urea is at particular risk of loss, mainly because the soil becomes alkaline around a dissolving granule, so that any ammonium formed is rapidly converted to ammonia, which volatilizes.

Another cause of low crop recovery is immobilization of fertilizer N by soil microbes. This process is not necessarily a loss, since the N can be present in the soil organic matter (SOM) for one or more seasons, during which it is normally mineralized and taken up by later crops.

Nitrogen is unusual among the nutrients in that excess application often causes a reduction in crop yield. In wet and high-yielding environments the reasons for yield reductions are lodging, because the crop becomes tall and top-heavy, and because of disease in thick foliage. In dry conditions, excess N stimulates growth, which can lead to exhaustion of soil water before maturity. Another and often more important reason is that high N levels reduce the concentration of water-soluble carbohydrates in the stems, which is an important source of assimilates for grain growth [29]. The time of application of N fertilizer is important in methods to avoid losses. Responses to topdressing N on wheat during the stem elongation phase are comparable to the responses to the same amount of N applied at sowing in both highly productive environments in Europe [30] and in water-limited environments in Australia [31]. Fertilizer N applied at sowing promotes rapid seedling growth, which leads to more risk of lodging, foliar disease, and haying-off than the same amount of N applied at stem elongation or later. Even when growing rapidly, small plants cannot take up large amounts of N, so mineral N in the soil at the time of sowing is at risk of loss from leaching and denitrification.

Mid-season application of N is effective because it coincides with the period of maximum crop uptake and so closely matches supply with demand. Application of N during or after the stem elongation phase in cereals usually increases grain protein more than application at sowing.

The N losses from irrigated rice can be particularly high. De Datta [32] reported that the average recovery of N fertilizer in rice experiments was 35–40%. Extensive research showed that the main pathways for N loss from lowland rice are ammonia volatilization and denitrification when urea is broadcast onto wet soil or shallow water. This research did not show ways to minimize losses and Fujisaka [33] suggested that research should focus more on methods to increase N efficiency rather than quantify the loss pathways. Subsequent agronomic research showed that with suitable methods of application the recovery of N, supplied as urea, can be as high as 75% for tropical rice [34] and 90% for temperate rice at a commercial scale [24].

The most efficient ways to increase N use efficiency (NUE) are different in each region and with each cropping system. Practices that are often effective are injecting fertilizer into the soil rather than broadcasting it on the surface and delaying application of some or all of the fertilizer so that the supply of N is well synchronized with the crop demand. Other ways to increase NUE are to ensure that other factors are not limiting. These limitations can include deficiency of other nutrients, weeds, herbicide damage, and root disease. An example of the importance of overcoming such limitations is apparent in a 40% increase in dryland wheat yield in Australia during the 1990s, which coincided with the adoption of canola break crops and higher applications of N fertilizer. Angus [18] suggested that break crops controlled widespread root disease and provided suitable conditions for a large yield response to N fertilizer.

There have also been reports of improvements of NUE in a single season by classical plant breeding [35] or modification of single genes [36]. Genetic improvements in N use efficiency are promising but it is not yet clear whether they are consistent across environments and seasons, and whether they are as cost effective as the established and reliable improvements in crop management.

There is a widespread and recurring problem of farmers applying more than the economically optimum rate of N fertilizer. The problem was apparent in Europe and the USA from at least the 1970s [20] and the same pattern of overuse emerged in many other countries, including China since the 1980s [37]. N fertilizer applied in excess of crop requirements is sometimes called "insurance nitrogen" [38], implying that farmers are prepared to pay for the excess provided that the yield is maximized. Another explanation lies in the concept of "farming styles" of van der Ploeg [39], which interprets farming practices in relation to farmers' goals. Farmers who want to boast of the highest district yields apply heavy inputs with little regard for input costs and net profit. These competitive individuals are uninterested in the profit-maximizing paradigm of agricultural science. To reverse the pattern of fertilizer overuse, it is important to redirect their competitiveness to efficiency rather than yield.

The reason that overfertilization is more common with N than other nutrients is not clear, but may be related to the rapid and visible response by crops. Within 5–10 days of N application, crops usually become greener and taller, which gratifies farmers, at least when they first observe the response. To this extent, N fertilizer provides its own advertising. The greener and taller fertilized crops may or may not yield more than those that receive no additional fertilizer. Reasons for a lack of yield response may be that the real yield limitation is not N but some other limitation such as water deficit, disease, deficiency of another nutrient, frost, weeds, or herbicide damage. Since the 1990s, the application rates of N fertilizer have been static or falling in Europe and North America but crop yields have continued to rise [40]. Apparently, farmers and advisers in these regions are learning the lesson that the previous rates were excessive and the time of application was too early.

Phosphorus

Soil contains P as both soluble and insoluble inorganic phosphates and in organic forms including phytates, which make up more than 50% of the soil P. Fertilizer P adds to the soluble pool that can be taken up by crops, but the longer it remains in the soil the more is precipitated as insoluble iron and aluminum compounds in acid soil, or as insoluble calcium compounds in alkaline soil. Soils vary in the speed with which they precipitate soluble P, so soil tests are needed to measure plant-available P and the optimum rate of P fertilizer [8]. Tests of soil P and other nutrients based on ion exchange resins often give closer relations with plant uptake than other extraction methods [41]. Plant P tests are not as useful as plant N tests because P is applied at or before sowing.

Fertilizer management is designed to maximize and prolong P availability to the crop. The most powerful method is to include all the P in a band near the seed so as to maximize plant access before the P becomes unavailable due to precipitation. As with N fertilization, it is important to supply only enough for the crop since some of the excess P is likely to be precipitated. This applies whether the fertilizer is in the solid or liquid form. Liquid forms of P fertilizer are relatively more effective than solid forms on alkaline soils because the precipitated P is still partly available [42].

Most P in the soil is attached to clay particles and is not readily leached from any but the sandiest soils. Because most soil P is close to the surface it is unavailable when the topsoil dries out, so fertilizer injection below the surface can improve the efficiency of uptake in dry environments [43]. Because of the advantages of banding and deep placement, all the P fertilizer needs to be applied at the time of sowing.

Potassium

Crops require a large supply of K but remove little in grain. Most of the K taken up by plants remains in the straw and returns to the soil when the straw decomposes or is burnt, provided the ash that remains after burning is not blown away in the wind. Once in the soil, K is not readily leached because of its positive charge and tends to be used by crops with greater efficiency than N or P. An exception is on soils that contain a large proportion of 2:1 clay minerals, where K is strongly fixed and becomes unavailable to roots.

Other Nutrients

The other nine nutrients commonly applied as fertilizers are not all discussed individually and the reader is

referred to nutrient aspects by Mengel and Kirkby [21] and fertilizer aspects by Tisdale et al. [8]. This section discusses S and Zn because of their increasing importance.

Sulfur is present in the soil as sulfate, which as an anion is subject to leaching. Until the later part of the twentieth century, it was inadvertently applied in many regions. One form was in superphosphate, which was used for its P content (9%) but supplied S (11%) as well. The "high analysis" fertilizers, which largely replaced superphosphate, contain relatively small amounts of S. Another source of S for plants was atmospheric deposition as oxides of S from smokestacks, particularly on smelters of metal sulfides. This source of S is also decreasing because smokestacks are being fitted with "scrubbers" to reduce air pollution. Fertilizer S will be increasingly needed to replace these sources.

Zinc is important for not only increasing crop growth, but also as an essential human nutrient that is becoming seriously deficient. Zinc deficiency is widespread in soils and plants, particularly in west and south Asia [44], and methods and products are available in some countries to correct Zn deficiency [23]. Some, but not enough, compound fertilizers in developed countries include Zn but in many developing countries, Zn is available only as zinc sulfate. Sometimes this expensive compound is diluted by unscrupulous retailers with salts that look similar, such as ammonium sulfate, so the product is an ineffective source of Zn (R.J. Buresh, 2011, personal communication).

Much of the Zn content of plants is transferred from the soil to plant roots by arbuscular mycorrhizal fungi (AMF). The large surface area of AMF filaments increases the ability of plants to explore the soil for immobile nutrients such as Zn. AMF are highly effective in soils with low nutrient status, but the modern farming practices of high levels of P fertilizer and growing brassica break crops tends to reduce AMF activity and increase the need for Zn fertilizer. Breeding plants for increased Zn uptake from soil can be effective in the short term but without supply of Zn fertilizer will exhaust soil reserves and hasten the onset of severe deficiency [45].

Fertilizer Recommendations and Decision Support

Methods for prescribing the fertilizer amount evolved from blanket recommendation provided by advisers, meaning the same practice irrespective of fields or seasons. Soil tests and nutrient budgets, as described earlier, give more differentiated prognoses of response can be used to develop "rules of thumb," which are usually accepted by busy farmers. Computer-based decision support systems can take account of many factors that are known to affect nutrient response but are not as widely adopted as simple systems and rules of thumb. McCown [46] concluded that adoption of decision support systems had not met the promise they offered.

Fertilizers and Precision Agriculture

Precision agriculture is a set of principles that has developed since the 1990s, aimed at managing crops at a scale of several meters rather than as a whole field. It relies on a global positioning system (GPS) that estimates location with precision ranging from 4 to 0.02 m, spatial data about crop production such as maps from a yield monitor, biomass images from a satellite or aircraft, or spatial data about soil conditions such as electrical conductivity [47]. Fertilizer can be managed by precision agriculture by varying the application rate according to the demand by the crop and/or the supply from the soil in different zones in a field. The farmer or adviser first prepares a prescription specifying the fertilizer rate for each zone and programs this in a computer carried in a tractor. This computer continuously determines where the tractor is located relative to the zones in the field, based on signals from the GPS receiver, and controls an actuator on the fertilizer implement that delivers the appropriate rate of fertilizer in each zone.

Yield maps provide useful data for deciding on fertilizer strategies and tactics. If one part of a field is consistently low yielding and it has previously received the same amount of fertilizer as the rest of the field, then it is likely that soil nutrient levels have accumulated. This pattern is common in the case of P fertilizer, and provides evidence to reduce the application rate on low-yielding zones. Variable applications of N fertilizer

are a common use of yield maps. An example is in response to natural variation in soil organic matter, with additional N supplied to low-fertility zones [48]. Another example is in response to "unnatural" soil variation due to land leveling, where "cuts" expose subsoil containing low nutrient levels and "fills" consist of additional amounts of topsoil that is relatively fertile.

In these examples, additional fertilizer is designed to compensate for low soil nutrient status. If yield variation is due to soil properties other than nutrient status, then it may be inappropriate to compensate for low yield with more fertilizer and the best strategy is the exact opposite, that is, to apply more fertilizer to zones that consistently give the highest yields. An example of this situation is when spatial yield variation is due to the differences in the available soil water supply. Angus et al. [49] investigated yield and crop response to N across fields that varied in subsoil constraints of salinity, sodicity, and high concentration of boron. They showed that wheat yield and the crop response to N fertilizer were greatest on parts of a field with the least subsoil constraints, and yield responses to applied N were least on zones with the most severe constraints. Even in the absence of subsoil constraints, the soil water-holding capacity can control yield of dryland crops, and the highest rates of N fertilizer should be applied to zones with large water-holding capacity [50]. Spatial soil variation is often best measured by electromagnetic induction, which directly measures apparent electrical conductivity, which is a proxy for other properties [51].

Where the response of a crop to fertilizer depends on its N status rather than other soil conditions, an optical sensor can provide useful information about a static or on-the-go estimate of crop N status. Optical systems using infrared sensors, such as the Greenseeker [52] and N-Sensor [53], can estimate N status of crops at a scale of about 1 m^2 while mounted on a tractor or fertilizer distributor, and can regulate the fertilizer rate on-the-go. A simple system using a domestic digital camera can provide equivalent information for a single scene [54].

The previous examples applied when the spatial yield patterns are consistent between years. A more complex approach is needed when the fertilizer response is inconsistent between zones and between years. For example in a dry season, N fertilizer may increase yield in low-lying, wet zones of a field but reduce yield in hilly, dry zones. Conversely, in a wet season, N fertilizer may be most effective in the hilly zones. The best response to this situation is not clear. One possibility is to examine yield maps for several years and manage fertilizer for an average season. Another is to delay the decision to apply fertilizer until the seasonal pattern is apparent. It may also be possible to minimize yield variation due to water movement by land-forming and drainage.

Fertilizers in Organic Farming

Many consumers are prepared to pay premiums for organically grown produce and a small proportion of farmers want to supply food produced by one of the organic farming systems. For certification of organic produce, the several organic farming systems generally require nutrients to be applied as manure and unprocessed minerals but not as processed fertilizer. Application of sufficient manure can provide optimum crop nutrition and until recently it was assumed that there were no disadvantages to the nutrient relations of organic farming. Research compiled by Kirchmann and Bergström [55] shows that this assumption is invalid because there is consistently more N leaching under organic than conventional systems, apparently because the soil N supply is not well synchronized with crop N demand. These results suggest that the nutrient relations of organic farms may be less sustainable than conventional farms. Many governments subsidize organic farming systems and thus support unsustainable nutrient management.

In another comparison of organic and conventional farming systems, Fagerberg et al. [56] showed that nutrient levels of the organic part of a split farm remained adequate for several years after conversion from conventional, but that later some nutrients become depleted and productivity fell. The likely reason was that these nutrients had accumulated during several decades of fertilizer application before half the farm was converted to organic farming.

Residual nutrients from previous fertilizer application are not the only source of N in organic farming. In parts of north America and western Europe, the deposition of mineral N from the atmosphere contributes a large part of crop requirement [20].

The sources are ammonia emitted from manure in intensive animal industries and nitrogen oxides emitted from vehicles. Both sources of N originate from industrial operations, so it is difficult to understand how any farming conducted in such regions can be designated as organic.

Some strands of organic farming propose application of plant extracts and microbial preparations to the soil to promote crop growth, and such products have spread in the decades since there was regulation of agricultural chemicals. Reports of the effectiveness of some of these products were reviewed by Edmeades [57], who concluded that they gave no significant yield benefit when used as recommended. A manufacturer of one such product objected to Dr. Edmeades' comments and took legal action against him in New Zealand courts. Dr. Edmeades won the case but effectively lost his scientific career and wasted a year enmeshed in the legal system [58]. It is not clear how reputable science should deal with claims that "alternative" products provide production and environmental benefits out of all proportion to their nutrient composition. Even if one such product is proved to be ineffective and removed from the market, it is likely that others will take its place. A solution may be to strengthen consumer protection laws so that manufacturers can be penalized for making unsubstantiated claims.

Environmental Benefits of Fertilizers

Fertilizers provide environment benefit by increasing soil organic matter in nutrient-deficient soils. For example, superphosphate provided P and S for vast areas of deficient soil in Australia, which provided that trigger for biological nitrogen fixation by pasture legumes [59]. Even more spectacular increases in soil organic matter came when micronutrient deficiencies were corrected with fertilizers in the Western Australian sandplain. These are the only positive environmental effects of fertilizer and other effects are neutral or negative.

Pollution of Surface Water and Groundwater

When there is nitrate in the soil and the water supply from precipitation and irrigation exceeds evaporation, the nitrate will be leached and eventually enter the groundwater. Levels of groundwater nitrate began to increase in the second half of the twentieth century, most notably in Europe and North America, and have stubbornly remained high. For example, Johansson and Gustafson [60] reported that after N-fertilizer application to an arable field in southern Sweden was discontinued, there was a delay of 20 years before the concentration of subsoil nitrate decreased.

The best-known environmental damage from nutrients is water pollution in lakes, rivers, and parts of the ocean. The pollution causes hypoxia, algal blooms, and death of pelagic fish in many water bodies, the largest of which are the Gulf of Mexico [61], the Baltic Sea [62], and the Yellow Sea [63]. The delay in affecting nutrient levels in such water bodies is probably longer than for groundwater, as shown by the constant levels of dissolved N in the Baltic Sea 10 years after N fertilizer usage almost stopped in the eastern Baltic countries after the breakup of the Soviet bloc [62].

Both N and P from fertilizer contribute to pollution of large water bodies but even if N from fertilizer were not present, blue-green algae would be able to obtain their N requirements from biological fixation. Other evidence for the importance of P in water pollution is that the size of the "dead zone" in the Gulf of Mexico is more closely related to the discharge of P than of N [64]. The source of nonpoint P pollution may not be confined to fertilizer, and streambanks disturbed by humans and grazing animals have the potential to release soluble P into water bodies. A promising method to reduce phosphate release into streams is to exclude grazing animals from riparian strips.

Systems to limit nutrient pollution of water have included fertilizer taxes and quantitative limits of nutrient loading from both fertilizer and manure. N fertilizer was taxed in Sweden for many years but there was little change in the rate of N application to crops. In the Netherlands, a system of nutrient accounting of inputs and outputs limits the nutrient balance of farms to reduce nutrient movement into groundwater and streams [65]. A promising method is to define sensitive zones, based on infiltration rate or proximity to streams, where fertilizers are banned, limited, or taxed [66].

Intensive research continues on the nitrogen cascade, a phrase that recognizes a chain of intended and unintended consequences when reactive N moves on land and in the air and water. An example of coordinated research in western Europe is given by Sutton et al. [67].

Greenhouse Gas and Ammonia Emissions

Nitrous oxide (N_2O) and N_2 are released from soil into the atmosphere by the processes of nitrification [68] and denitrification [69]. N_2O absorbs long-wave radiation and remains in the atmosphere for a long time so its greenhouse effect is much greater than that of CO_2. The International Panel on Climate Change estimates the emission of N_2O from agriculture as a percentage of the N fertilizer applied. This approach takes no account of nitrification and denitrification of non-fertilizer sources, mainly from mineralization of organic N. Since fertilizers provide about half of the N used for food production, the contribution of the organic N to N_2O emission is probably underestimated. The most promising strategy to reduce N_2O loss are to minimize waterlogging, since denitrification proceeds most rapidly in anaerobic conditions.

Gaseous loss of NH_3 is greatest when ammonium is on or near the surface of an alkaline soil, or in the floodwater of rice. Windy conditions at the soil surface also contribute to rapid loss. In the worst conditions, the loss of fertilizer N can be up to 10–15% per day [15], but the loss can be reduced or eliminated by injecting ammonia-based fertilizers below the soil surface, or, if topdressing is unavoidable, by applying fertilizer before forecast rain, so that the granules are dissolved and transported below the soil surface.

Soil Acidification

Ammonia-based fertilizers, including urea, acidify the soil when protons are released during nitrification. Soil pH falls more rapidly in light-textured and poorly buffered soils than in heavy-textured and highly buffered soils, but the process continues irrespective of soil type. The consequence of acidification is reduced plant growth, not because of the protons, but because of increasing concentrations of aluminum and manganese in the soil solution that accompany reduced pH. The most common cure is to apply lime, which represents a deferred and indirect cost of N fertilizers and an environmental cost since the reaction of lime on acid soil releases CO_2. A less common response is to apply nonacidifying fertilizers such as those containing nitrate, but these are more expensive than ammonia-based products.

Does N Fertilizer Deplete Soil Organic Matter (SOM)?

Until recently, there was a general belief that N fertilizer had little or no effect on SOM but this was challenged from analysis of soil data from the Morrow Plots, a long-term experiment at the University of Illinois in Urbana. Khan et al. [70] analyzed soil C and Mulvaney et al. [71] analyzed soil N from these plots and came to the startling conclusion that adding N fertilizer decreased SOM, apparently because the N fertilizer stimulated soil respiration. If true, this conclusion would prove that the modern N fertilizer industry is unsustainable.

Reid [72] and Powlson et al. [73] criticized these interpretations of the Morrow data. Their criticism was that the experiment had been confounded because the plot that received a large N dose from 1965 to 2005 had previously (1904–1966) received large annual doses of manure that had built up SOM to very high levels. After the manuring finished on that plot in 1967, the SOM decreased simply because it was above the equilibrium level for the environment, and not because of N fertilizer. This is a strong argument because there is ample evidence that soil maintains a high level of SOM only if there is a constant input of organic matter to offset soil respiration, and this material contains C, N, P, and S at ratios close to that of SOM. Kirkby et al. [74] surveyed data on SOM content and estimated that the average C:N:P:S ratio of humus was 1000:80:20:14. After manuring stops, the SOM decreases for many decades until it returns to the equilibrium for the environment [73]. The decrease in SOM in the Morrow plots can be explained more by the oxidation of manure than the effect of N fertilizer. The specific criticisms by Reid [72] and Powlson [73] about the confounded treatments have not been challenged after 3 years of debate on the subject [75] so it appears that the concern about N fertilizer depressing SOM was a false alarm.

Nutrient Transfer from Farmland to Natural Terrestrial Environments

It has long been known that nutrients move from farmland to natural environments on land, as well as to freshwater and oceans, as described above. Pathways for nutrient transfer are water, gas, dust, and wild

animals [76]. Movement by water is likely to remain in the stream system so is relatively unimportant for transfer to terrestrial environments. The only nutrient moved as gas is ammonia, and the main agricultural source of which is housed animals rather than emissions from fertilizer, although the original source of the nutrients is mostly fertilizer.

Nutrient movement in dust has not been widely discussed in the agricultural literature but is of more interest for earth science. Nutrients move in dust when wind lifts clay particles, which normally contain a large proportion of organic matter [77]. When the wind abates, the particles may land on another agricultural field where the nutrient transfer is relatively unimportant, and in natural environment where the nutrients may cause environmental damage.

The other source of nutrient transfer is through animals. Where farmland is adjacent to natural vegetation, it is common to see wild animals grazing on crops and pastures during the morning and evening and retreating into the cover of bush during the day. Examples are deer in Europe and North America, kangaroos in Australia, and birds almost everywhere. The obvious attraction to animals of the margin between farm and bushland is access highly nutritious feed on the farms and cover in the natural vegetation. Nutrient transfer occurs through urine and dung deposited in the bushland. A consequence of nutrient enrichment of natural vegetation from all sources is eutrophication of the landscape, analogous to eutrophication of water [78], with weedy annual plants competing with native perennials. Nutrient transfer over short distances could place limits on the development of mosaic farming systems where farming and natural vegetation are distributed through a landscape according to land capability.

Human Health

Heavy metals can enter the human food chain from fertilizer. Cadmium (Cd) is the most toxic and is a contaminant in many phosphatic fertilizers [79]. When Cd is applied to a field it accumulates first in crops, grazing animals, and then in the tissue of people who consume the products. The most promising way of reducing Cd levels in fertilizer is by manufacturing phosphatic fertilizer from sources of rock phosphate that contain low levels of Cd [80].

Another supposed health hazard from fertilizer was nitrate, but the concern turned out to be a false alarm. From the 1940s until the 1990s, nitrate was believed to cause blue-baby syndrome (methemoglobinemia) because some cases of this disease that were associated with groundwater containing high nitrate levels, and this was the main reason for an official limit of 50 mg/L of nitrate in drinking water. Evidence reviewed by Addiscott [20] showed that nitrate at this concentration was harmless and that the original basis for this limit was faulty because water containing high nitrate concentrations also contained bacterial contamination, which was the real culprit for methemoglobinemia.

There are however beneficial effects of fertilizers on human health through micronutrients that are essential for human health. The best example is Zn, which is widely deficient in the human diet. The World Health Organization concluded that human Zn deficiency was one of the most serious causes of poor health [81]. Low levels and low bioavailability of Zn in human food are the cause. Increased use of zinc fertilizer would be a boon to human health, particularly to people who rely on a vegetarian diet, since plant products contain less Zn than meat. In view of the difficulty in correcting Zn deficiency in crops, supplementation of human food with Zn may be more effective [45].

Resource Availability

World food supply relies on increasing amounts of nonrenewable resources to provide fertilizers. In the case of N, the resource involved is energy, and about 1% of the world's supply of fossil fuels is consumed in its production. Production of all the other nutrients depends on mining as well as relatively small amounts of energy used in manufacture and transport.

The energy cost of producing N fertilizer has decreased as technology improved. For example, the amount of natural gas needed to synthesize ammonia in the most efficient plants halved in the last 40 years of the twentieth century, and by 2000 was within about 25% of the highest possible efficiency [2]. With diminishing scope for improved efficiency and exhaustion of natural gas, it is likely that the real cost of N fertilizer will rise.

The nutrient at greatest risk of exhaustion is P. Cordell et al. [82] estimated that "peak phosphorus" had been reached and that reserves would be exhausted by 2030. This conclusion is based on the extrapolation of sigmoid curves of production, similar to those used to predict the exhaustion of crude oil reserves. Alternative approaches reviewed by Cornish [83] comes up with estimates of reserves lasting at least until 2100, based on current rates of extraction and the amounts and quality or known reserves compiled by the US Geological Survey. Whether the reserves will last for 30 or 100 years, there is increasing concern that P will be the most limiting nutrient in the long term. The most promising ways of prolonging supplies is to reduce overuse of P fertilizer and find ways to minimize phosphate fixation in soils. If exhaustion of P is as imminent as the worst predictions, it will be necessary to find ways of recycling at the important points of loss, which are in food preparation and in human urine. Recycling options are discussed by the Global Phosphorus Research Initiative [84].

Reserves of high-quality K fertilizers are likely to outlast those of P and probably N [83]. One concern about K supplies is that they are concentrated in the hands of relatively few producers who could be in a position to corner the market. Another concern is that the largest reserves of K fertilizer in Canada and Russia are remote from the regions of greatest K deficiency in Asia.

Future Directions

Existing science and technology shows that crops can respond reliably and profitably to fertilizers when used cautiously. This involves applying fertilizer so that the combined supply from fertilizer and the soil is sufficient for, but does not exceed, crop requirement. This principle has not penetrated far into farming systems, and fertilizer management varies from generally inadequate applications in sub-Saharan Africa to excess use of N in East Asia. Many farms in Europe and North America have passed the period of excess N use. However the efficiency of fertilizer use is still generally low and improvement will require incremental research on farms to refine methods to apply fertilizer efficiently and in ways that are compatible with farming systems. As farming systems change, as they inevitably will, fertilizer management will have to change also. The most promising methods are to arrange fertilizer rate, time of application, spacing and depth of placement to synchronize supply with crop demand, and to adjust these parameters in relation to zones in the field and region. The methods will have to be cost effective, so it will be important to continue to develop methods to manage low-cost fertilizers appropriately rather than resorting to high-cost additives, which may have to be supported by subsidies. Science should also critically evaluate and comment on "alternative" fertilizer products and systems that make outrageous claims and will lead to reduced food security if widely used.

More fundamental science should be directed to liberating the large amounts of unavailable soil nutrients, while recognizing that release of these bound nutrients will only delay the need for additional fertilizer. The largest environmental improvements related to nutrient overuse are likely to come from research to increase fertilizer efficiency on farms. The delay between conduct of farm-related research and its benefits on a regional scale can be many decades [85], so well-resourced field research needs to be maintained.

Acknowledgments

Tony Good provided insights into fertilizer technology and John Passioura and Mark Peoples made helpful suggestions about the article.

Bibliography

Primary Literature

1. Stewart WM, Dibb DW, Johnston AE, Smyth TJ (1995) The contribution of commercial fertilizer nutrients to food production. Agron J 97:1–6
2. Smil V (2001) Enriching the earth. Fritz Haber, Carl Bosch, and the transformation of world food production. MIT Press, Cambridge
3. www.fao.org/waicent/portal/statistics_en.asp
4. Bumb BL (1995) World nitrogen supply and demand: an overview. In: Bacon PE (ed) Nitrogen fertilization in the environment. Marcel Dekker, New York, pp 1–40
5. Dobermann A, Fairhurst T (2000) Rice: nutrient disorders and nutrient management. PPI and IRRI, Singapore/Los Baños
6. Bar-Tal A, Yermiyahu U, Beraud J, Keinan M, Rosenberg R, Zohar D, Rosen V, Fine P (2004) Nitrogen, phosphorus, and potassium uptake by wheat and their distribution in soil following successive annual compost applications. J Environ Qual 33:1855–1865

7. Bown SR (2007) A most damnable invention. Dynamite, nitrates and the making of the modern world. Penguin, London
8. Tisdale SL, Nelson WL, Beaton JD, Havlin JL (1993) Soil fertility and fertilizers. Macmillan, New York
9. World Bank (2008) Agriculture for development. World Bank, Washington, DC
10. Sanchez PA (2002) Soil fertility and hunger in Africa. Science 295:2019–2020
11. Angus JF, Fazekas de St. Groth C, Tasic RC (1990) Between-farm variability in yield response to inputs of fertilizers and herbicide applied to rainfed lowland rice. Agric Ecosyst Environ 39:219–234
12. Angus JF, Marquez DA, Tasic RC (2004) Diagnosing variable nutrient deficiencies in rainfed lowland rice using strip trials. In: Fourth international crop science congress, Brisbane. http://www.cropscience.org.au/icsc2004/poster/4/1/2/678_angusjf.htm
13. www.icis.com
14. Freney JR (1997) Strategies to reduce gaseous emissions of nitrogen from irrigated agriculture. Nutr Cycl Agroecosyst 48:155–160
15. Peoples MB, Freney JR, Mosier AR (1995) Minimizing gaseous losses of nitrogen. In: Bacon PE (ed) Nitrogen fertilization in the environment. Marcel Dekker, New York, pp 565–602
16. Martens DA, Bremner JM (1997) Inhibitory effects of fungicides on hydrolysis of urea and nitrification of urea nitrogen in soil. Pesticide Sci 49:344–352
17. Chen D, Freney JR, Rochester I, Constable GA, Mosier AR, Chalk PM (2007) Evaluation of a polyolefin coated urea (Meister) as a fertilizer for irrigated cotton. Nutr Cycl Agroecosyst 81:245–254
18. Angus JF (2001) Nitrogen supply and demand in Australian agriculture. Aust J Exp Agric 41:277–288
19. Cottrell R (1987) The sacred cow: the folly of Europe's food mountains. Grafton, London
20. Addiscott TM (2005) Nitrate, agriculture and the environment. CABI, Wallingford
21. Mengel K, Kirkby EA (2001) Principles of plant nutrition. Kluwer, Dordrecht
22. Cakmak I, Yilmaz A, Kalayci M, Ekiz H, Torun B, Erenoglu B, Braun HJ (1996) Zinc deficiency as a critical problem in wheat production in central Anatolia. Plant Soil 180:165–172
23. Alloway BJ (2004) Zinc in soils and plant nutrition. International Zinc Association, Brussels, http://www.zinc-crops.org/library_general_zni_publications.html
24. Russell CA, Dunn BW, Batten GD, Williams RL, Angus JF (2006) Soil tests to predict the response of rice to fertiliser nitrogen. Field Crops Res 9:286–301
25. Webster R, Oliver MA (2007) Geostatistics for environmental scientists. Wiley, Chichester
26. Myers RJK (1984) A simple model for estimating the nitrogen fertilizer requirement of a cereal crop. Fertilizer Res 5:95–108
27. Angus JF (1995) Modelling N fertilization requirements for crops and pastures. In: Bacon PE (ed) Nitrogen fertilization and the environment. Marcel Dekker, New York, pp 109–127
28. Sinclair TR, Park WI (1993) Inadequacy of the Liebig limiting-factor paradigm for explaining varying crop yields. Agron J 85:742–746
29. van Herwaarden AF, Farquhar GD, Angus JF, Richards RA, Howe GN (1998) 'Haying-off', the negative grain yield response of dryland wheat to nitrogen fertiliser. I. Biomass, grain yield and water use. Aust J Agric Res 49:1067–1081
30. Ellen J, Spiertz JHJ (1980) Effects of rate and timing of nitrogen dressings on grain yield formation of winter wheat (*T. aestivum* L.). Fertilizer Res 1:177–190
31. Angus JF, Fischer RA (1991) Grain and protein responses to nitrogen applied to wheat growing on a red earth. Aust J Agric Res 42:735–746
32. De Datta SK (1987) Advances in soil fertility research and nitrogen management for lowland rice. In: Efficiency of nitrogen fertilizers for rice. International Rice Research Institute, Los Baños, pp 27–41
33. Fujisaka S (1993) Were farmers wrong in rejecting a recommendation? The case of nitrogen at transplanting for irrigated rice. Agric Syst 43:271–286
34. Peng S, Cassman KG (1998) Upper thresholds of nitrogen uptake rates and associated N fertilizer efficiencies in irrigated rice. Agron J 90:178–185
35. Mi G, Tang L, Zhang F (2000) Is nitrogen uptake after anthesis in wheat regulated by sink size? Field Crops Res 68:183–190
36. Good AG, Johnson SJ, De Pauw M, Carroll RT, Savidov N, Vidmar J, Lu Z, Taylor G, Stroeher V (2007) Engineering nitrogen use efficiency with alanine aminotransferase. Can J Bot 85:252–262
37. Ju X, Liu X, Zhang F, Roelcke M (2004) Nitrogen fertilization, soil nitrate accumulation, and policy recommendations in several agricultural regions of China. Ambio 33:300–305
38. Mitsch WJ, Day JW Jr, Gillliam JW, Groffman PM, Hey DL, Wang N (2001) Reducing nitrogen loading to the Gulf of Mexico from the Mississippi River Basin: strategies to counter a persistent ecological problem. Bioscience 51:373–388
39. van der Ploeg JD (1994) Styles of farming: an introductory note on concepts and methodology. In: van der Ploeg JD, Long A (eds) Born from within: practices and perspectives of endogenous rural development. van Gorcum, Assen, pp 7–30
40. Fischer RA, Byerlee D, Edmeades GO (2009) Can technology deliver on the yield challenge to 2050? FAO, Rome, ftp://ftp.fao.org/docrep/fao/012/ak977e/ak977e00.pdf
41. van Raij B, Quaggio JA, da Silva NM (1986) Extraction of phosphorus, potassium, calcium, and magnesium from soils by an ion-exchange resin procedure. Commun Soil Sci Plant Anal 17:547–566
42. Holloway RE, Bertrand I, Frischke AJ, Brace DM, McLaughlin MJ, Shepperd W (2001) Improving fertilizer efficiency on calcareous and alkaline soils with fluid sources of P, N and Zn. Plant Soil 236:209–219
43. Cornish PS (1987) Effects of direct drilling on the phosphorus uptake and fertilizer requirements of wheat. Aust J Agric Res 38:775–790
44. Sillanpäa M (1990) Micronutrient assessment at the country level: an international study. FAO, Rome

45. Ryan MH, McInerney JK, Record IR, Angus JF (2008) Zinc bioavailability in wheat grain in relation to phosphorus fertilizer, crop sequence and mycorrhizal fungi. J Sci Food Agric 88:1208–1216
46. McCown RL (2002) Changing systems for reporting farmers' decisions: problems, paradigms and prospects. Agric Syst 74:179–220
47. McBratney A, Whelan B, Ancev T, Bouma J (2005) Future directions of precision agriculture. Precision Agric 6:7–23
48. Blackmer AM, White SE (1997) Using precision farming technologies to improve management of soil and fertiliser nitrogen. Aust J Agric Res 49:555–564
49. Angus JF, Norton RM, Pedler JF, Walker CN (2004) Cereal response to N fertiliser in relation to subsoil limitations. In: Proceedings of the fourth international crop science congress, Brisbane. http://www.cropscience.org.au/icsc2004/poster/2/5/1/1170_angusjf.htm
50. Wong MTF, Asseng S (2006) Determining the causes of spatial and temporal variability of wheat yields at sub-field scale using a new method of upscaling a crop model. Plant Soil 283:203–215
51. Sudduth KA, Kitchen NR, Bollero GA, Bullock DG, Wiebold WJ (2003) Comparison of electromagnetic induction and direct sensing of soil electrical conductivity. Agron J 95:472–482
52. Raun WR, Solie JB, Taylor RK, Arnall DB, Mack CJ, Edmonds DE (2008) Ramp calibration strip technology for determining midseason nitrogen rates in corn and wheat. Agron J 100:1088–1093
53. Reusch S, Link A, Lammel J (2002) Tractor-mounted multispectral scanner for remote field investigation. In: Roberts PC (ed) Sixth international conference on precision agriculture. ASA/CSSA/SSSA, Madison, pp 1385–1393
54. Li Y, Chen D, Walker CN, Angus JF (2010) Estimating the nitrogen status of crops using a digital camera. Field Crops Res 118:221–227
55. Kirchmann H, Bergström L (eds) (2008) Organic crop production – ambitions and limitations. Springer, Heidelberg
56. Fagerberg B, Salomon E, Jonsson S (1996) Comparisons between conventional and ecological farming systems at Örjebyn. Nutrient flows and balance. Swed J Agric Res 26:169–180
57. Edmeades DC (2002) The effects of liquid fertilisers derived from natural products on crop, pasture, and animal production: a review. Aust J Agric Res 53:965–976
58. Edmeades DC (2002) Science friction. The Maxicrop case and the aftermath. Fertiliser Information Services, Hamilton
59. Williams CH, Donald CM (1957) Changes in organic matter and pH in a podzolic soil as influenced by subterranean clover and superphosphate. Aust J Agric Res 8:179–189
60. Johansson G, Gustafson A (2004) Discharge and nutrient losses for the agrohydrological year 2002/03 and a long-term review. Swedish University of Agricultural Sciences, Division of Water Quality Management, Uppsala
61. Goolsby DA, Battaglin WA, Lawrence GB, Artz RS, Aulenbach BT, Hooper RP, Keeney DR, Stensland GJ (1999) Flux and sources of nutrients in the Mississippi-Atchafalaya River Basin. NOAA Coastal Ocean Program, Silver Spring, Maryland
62. Wulff FV, Rahm LA, Larsson P (2001) A systems analysis of the Baltic Sea. Springer, Berlin
63. Duan S, Zhang S, Huang H (2000) Transport of dissolved inorganic nitrogen from the major rivers to estuaries in China. Nutr Cycl Agroecosyst 57:13–22
64. Snyder CS (2008) Nutrients and hypoxia in the Gulf of Mexico – an update on progress, 2008. Better Crops 92:16–22
65. Ondersteijn CJM, Beldman ACG, Daatselaar CHG, Giesen GWJ, Huiurne RBM (2002) The Dutch mineral accounting system and the European nitrate directive: implications of N and P management and farm performance agriculture. Ecosyst Environ 92:283–296
66. Wright S, Mallia C (2003) The potential for eco-taxes as instruments of sustainability. J Transdiscipl Environ Stud 2:1–14, www.journal-tes.dk
67. Sutton MA, Howard CM, Erisman JW, Billen G, Bleeker A, Grennfelt P, van Grinsven H, Grizzetti B (2011) The European nitrogen assessment. Cambridge University Press
68. Norton JM (2008) Nitrification in agricultural soils. In: Scheppers JS, Raun WR (eds) Nitrogen in agricultural systems. ASA/CSSA/SSSA, Madison, pp 173–199
69. Coyne MS (2008) Biological denitrification. In: Scheppers JS, Raun WR (eds) Nitrogen in agricultural systems. ASA/CSSA/SSSA, Madison, pp 201–253
70. Khan SA, Mulvaney RL, Ellsworth TR, Boast CW (2007) The myth of nitrogen fertilization for soil carbon sequestration. J Environ Qual 36:1821–1832
71. Mulvaney RL, Khan SA, Ellsworth TR (2009) Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. J Environ Qual 38:2295–2314
72. Reid DK (2008) Comment on "The myth of nitrogen fertilization for soil carbon sequestration". J Environ Qual 37:739
73. Powlson DS, Jenkinson DS, Johnston AE, Poulton PR, Glendining MJ, Goulding KWT (2010) Comments on "Synthetic nitrogen fertilizers deplete soil nitrogen: A global dilemma for sustainable cereal production". J Environ Qual 39:749–752
74. Kirkby CA, Kirkegaard JA, Richardson AE, Wade LJ, Blanchard C, Batten G (2011) Stable soil organic matter: a comparison of C:N:P:S ratios in Australian and other world soils. Geoderma 163:197–208
75. Mulvaney RL, Khan SA, Ellsworth TR (2010) Reply to comments on "Synthetic nitrogen fertilizers deplete soil nitrogen: A global dilemma for sustainable cereal production". J Environ Qual 39:753–756
76. Likens GE, Bormann FH (1974) Linkages between terrestrial and aquatic ecosystems. Bioscience 24:447–456
77. Boon KF, Kiefert L, McTainsh GH (1998) Organic matter content of rural dusts in Australia. Atmos Environ 32: 2817–2823
78. Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671–677

79. McLaughlin MJ, Tiller KG, Naidu R, Stevens DP (1996) Review: the behaviour and environmental impact of contaminants in fertilizers. Aust J Soil Res 34:1–54
80. Lægreid M, Bøckman OC, Kaarstad O (1999) Agriculture, fertilizers and the environment. CABI, Cambridge
81. WHO (2002) World health report. World Health Organization, Geneva, www.who.int/whr/2002
82. Cordell D, Drangert J-O, White S (2009) The story of phosphorus: global food security and food for thought. Glob Environ Change 19:292–305
83. Cornish P (2010) A postscript to "peak P" - an agronomist's response to diminishing P reserves. Proc. 15th Australian Agronomy Conference. www.regional.org.au/au/pdf/asa/2010/7452_cornish.pdf
84. http://phosphorusfutures.net/
85. Alston JM, Andersen MA, James JS, Pardey PG (2010) Persistence pays: U.S. agricultural productivity growth and the benefits from public R&D spending. Springer, New York

Books and Reviews

Bacon PE (ed) (1995) Nitrogen fertilization in the environment. Marcel Dekker, New York
Hatfield JR (ed) (2005) The farmer's decision. Soil and Water Conservation Society, Ankeny
Marschner H (1995) Mineral nutrition of higher plants. Academic, London
Mosier AR, Syers JK, Freney JR (eds) (2004) Agriculture and the nitrogen cycle. Island Press, Washington

Genetic Engineering of Crops for Insect Resistance

John A. Gatehouse
School of Biological and Biomedical Sciences, Durham University, Durham, UK

Article Outline

Glossary

Coding sequence The part of a gene which determines the sequence of the protein product

Domain A region of a protein which forms a distinct 3-D structure, and will often form this structure even when separated from the rest of the protein

Genetic engineering Introduction of a specific DNA sequence into an organism by artificial means

Insect orders Lepidoptera=butterflies and moths; diptera=flies; coleoptera=beetles; hemiptera/homoptera=sucking insects such as aphids

Mutagenesis Alteration to a DNA sequence, often resulting in alteration to the sequence of a protein which the DNA specifies

Oligomerization Formation of polymers containing a relatively low number of repeating units

Proteolysis Introduction of breaks in the chain of amino acids making up a protein by a proteinase

Transgenic Organism into which a gene has been introduced by genetic engineering technology

Definition of the Subject

Genetic engineering of crops for insect resistance is the introduction of specific DNA sequences into crop plants to enhance their resistance to insect pests. The DNA sequences used usually encode proteins with insecticidal activity, so that in plants which contain introduced DNA, an insecticidal protein is present. However, other strategies to improve plant defenses against insects have been explored. Genetically engineered crops that are protected against major insect pests by production of insecticidal proteins from a soil bacterium, *Bacillus thuringiensis*, have become widely used in global agriculture since their introduction in 1996.

Introduction

Twenty years have elapsed since the first publications describing transgenic plants, which showed enhanced resistance to insect herbivores, as a result of the expression of a foreign gene encoding *Bacillus thuringiensis* (*Bt*) toxin [1–3]. In the intervening years, crops expressing these toxins have become widely used in global agriculture, and have led to reductions in pesticide usage and lower production costs [4] At the same time, the predictions made by lobby groups supporting "organic" crop production, that irreversible environmental damage would be caused by genetically engineered (GE) crops resistant to insect pests, have not been realized [5]. Despite all the controversy that GE crops have caused in many countries, it is difficult to dispute that the use of this technology to combat insect pests has had a positive impact on global agriculture.

This entry has two aims: first, to provide a summary of how and why *Bt* toxins have become the insect resistance genes of choice for commercial GE crop applications, and to anticipate some further developments of this technology; second, to consider some of the other approaches to engineering insect resistance in

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

plants, and to assess their potential for future development in the development of sustainable agriculture.

Insecticidal Proteins from *Bacillus thuringiensis*

The presence of insecticidal toxins in the soil bacterium *Bacillus thuringiensis* (*Bt*) has enabled both the bacteria themselves, and genes derived from them, to be exploited as plant protectants. The toxicity is almost invariably based on proteins produced during sporulation of the bacteria, which form crystalline deposits associated with the spores. The insecticidal *Bt* proteins are encoded by genes present on plasmids, and the presence of these plasmids is the main feature which distinguishes *Bt* from other spore-forming bacilli [6]. Preparations of *Bt* spores have been used since the 1920s as a conventional, spray insecticide (and, as a "natural" product, are approved for use in organic agriculture), but their efficacy in the field is limited by inactivation and low persistence.

The ecological niche occupied by *Bt* appears to be simple to define. The life cycle starts with a spore and associated crystalline protein body which may be present in the soil. On being eaten by an insect, the protein deposit associated with the spore is dissolved and digested, converting the crystalline protoxin to an active toxin. The insect is then killed, and the carcass provides nutrients for the growing bacteria, which multiply rapidly. When the insect carcass is exhausted, the bacteria sporulate; the spores are dispersed, and the cycle recommences. However, this cycle is clearly too simplistic, as the target insects for *Bt* toxins are only rarely soil dwellers, and the dose of spores required to kill an insect larva is too large for dispersed spores to have much effect. Although *Bt* is widely distributed, levels of the bacterium in soils are generally too low to have any effect on insects, and spraying plants with spores does not result in persistent protection as a result of the establishment of a high bacterial population. The species has been described as an opportunistic pathogen, which has evolved the sporulation mechanism as a "backup" system to ensure its survival under unfavorable conditions [7]. *Bt* is naturally present in the phylloplane, as well as in soil, and has been detected on cabbage foliage [8], and in vegetative form on clover [9] at low levels, without any insecticidal effect. However, the insecticidal characteristic must be of benefit to the bacterium, since most of the insecticidal proteins are encoded by plasmids, and the plasmids are maintained in the *Bt* population as a whole, despite the obvious metabolic costs of producing large quantities of spore-associated proteins. Not only are toxin-encoding plasmids maintained, but there is also a huge reservoir of diversity in the toxins themselves, and much effort has been put into screening bacterial isolates for strains of *Bt* with novel pesticidal activities [10].

Bt toxins are now classified on the basis of amino acid sequence similarity (an earlier classification system based on pesticidal activity has been superseded), in a systematic hierarchical system [11]. For the purposes of this contribution, only the major distinctions need be considered. There are four types of insecticidal proteins produced by *Bt*:

1. Proteins associated with *Bt* spores, usually as crystalline deposits; three domain structure; single toxins; designated by the symbol Cry
2. Proteins associated with *Bt* spores, usually as crystalline deposits; binary toxins and other similar proteins, including truncated versions of three-domain toxins; also designated by the symbol Cry
3. Proteins associated with *Bt* spores, usually as crystalline deposits; single domain structure; cytolytic; single toxins; designated by the symbol Cyt
4. Proteins expressed vegetatively by *Bt*; single chain and binary toxins; designated by the symbol Vip

Each type of toxin is subdivided (on the basis of sequence similarity) into families (number; same number $\geq$45% sequence identity) and then further subdivided using capital letters (same letter $\geq$78% sequence identity), small letters (same letter $\geq$95% sequence identity) and numbers successively. The resulting system yields designations for specific toxins such as Cry1Aa. A single *Bt* strain can produce spores which contain only a single toxin, or a complex mixture, such as the *Bt* subspecies *israelensis*, whose spores contain Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, and Cyt2Ba toxins [12].

All four types of proteins have been proposed for use as crop protection agents, although Cyt toxins have not as yet been used in commercial insect-resistant transgenic plants, and three-domain Cry toxins are by far the most commonly used type. Cry and Cyt toxins belong to the class of proteins referred to as bacterial

pore-forming toxins, and show structural similarity to the α-helical and β-barrel groups of toxins, respectively (where α-helical and β-barrel refer to the structures of the membrane-spanning parts of the toxin; reviewed by Parker and Feil [13]).These pore-forming toxins show common features of activity; they are produced as water-soluble proteins, and interact with specific receptors on cell surfaces, often after proteolytic activation by host proteinases. Binding to cell surfaces triggers a conformational change leading to oligomerization, which allows insertion into the cell membrane through promotion of a fluid, partially denatured structure. Insertion of the toxin into the membrane can either cause cell death directly, or result in effects on intracellular metabolism which lead to cell death.

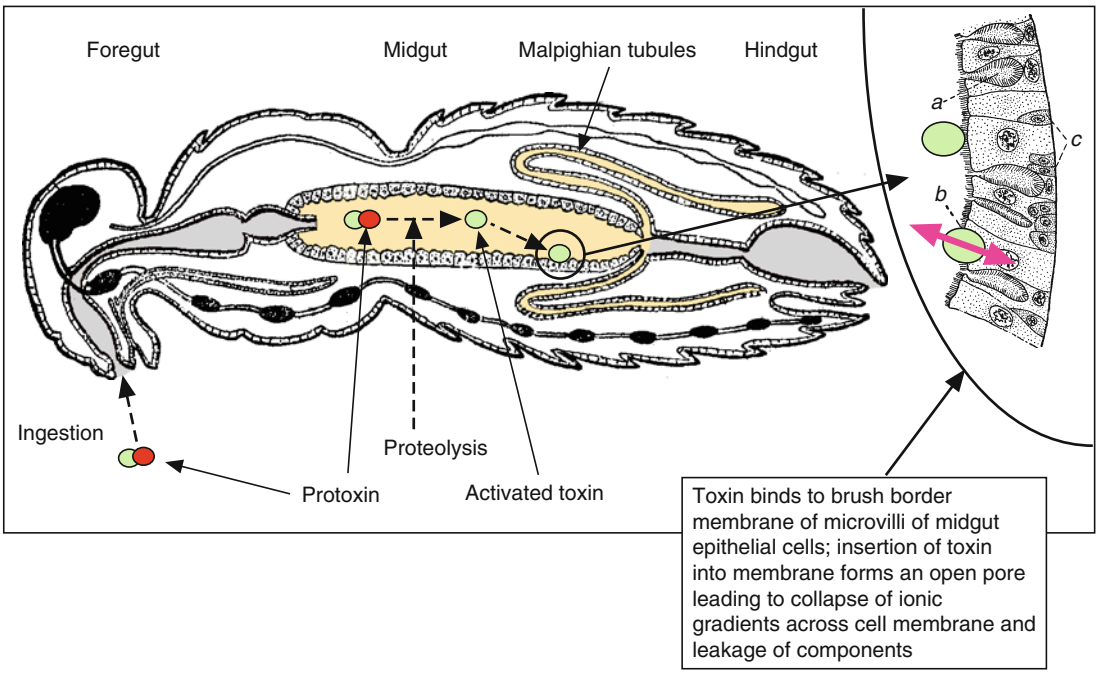
How Do *Bt* Toxins Work?

Three-Domain Cry Toxins

The mechanism of action of the "conventional" three-domain Cry toxins is now well understood, and can be divided into four stages:

1. Solubilization of the protoxin, and proteolytic activation by proteinases in the insect gut to produce active toxin
2. Interaction of the toxin with one or more receptors on cell surfaces in the insect gut epithelium
3. Oligomerization of the toxin
4. Insertion of the oligomerized toxin into cell membranes, leading to the formation of open pores, and cell death (see Fig. 1)

Following the pioneering work of Ellar's group [14] tertiary structures of six different three-domain Cry toxins are known – Cry1Aa [15], Cry2Aa [16], Cry3Aa [14], Cry3Bb [17], Cry4Aa [18], and Cry4Ba [19]; whereas most structures are for the active form, the structure of Cry2Aa includes the N-terminal pro-region. These toxins all show a high degree of structural similarity, and thus the formulation of a general model for their mode of action is justified. The three domains present in the active forms of these proteins are designated I, II, and III, and are normally contained in a single polypeptide of approximately 600 amino acid residues (in some cases proteolytic cleavages are



Genetic Engineering of Crops for Insect Resistance. Figure 1
Action of *Bt* toxins on the insect gut epithelium. Death of insect results from disintegration of gut epithelium (due to cell death) and proliferation of gut microflora

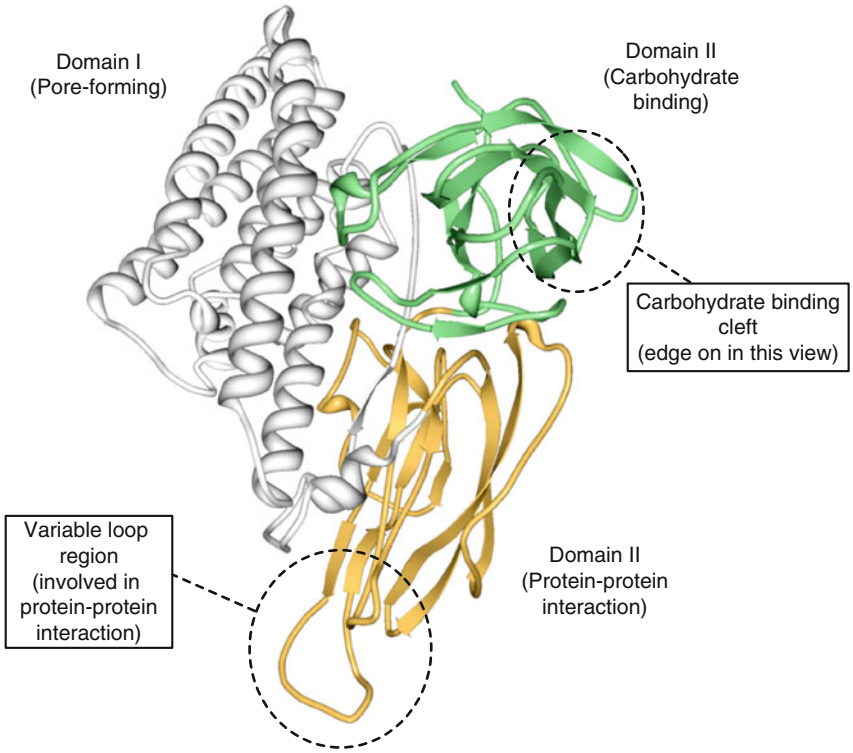
present within the active three-domain structure as a result of protoxin activation, resulting in multiple polypeptides making up the toxin, but the overall three-domain structure is conserved.). While conservation of structure and sequence is observed in the active forms of three-domain toxins, many toxins are synthesized with C-terminal extensions, which are variable in sequence between *Bt* strains, and in length between Cry families. The presence of C-terminal extensions leads to a large degree of heterogeneity in the size of the protoxins present in bacterial spores, with sizes ranging from approximately 600 amino acids (similar to the active toxin) to approximately 1,200 amino acids. These C-terminal extensions are not required for toxin function, and are removed during toxin activation, although their removal is not sufficient for toxicity to be shown. They are thought to play a role in the formation of crystalline inclusions in the bacterium during the spore-forming process.

The three domains of the active toxin are clearly distinguished in their structures.

1. Domain I, approx. 260 aa, contains seven α-helices, of which six are amphipathic and one hydrophobic. This structure is typical of pore-forming toxins, with the hydrophobic and amphipathic helices being responsible for membrane insertion and pore formation. The hydrophilic sides of the amphipathic helices form the surface lining the pore, so that polar species such as ions are able to cross the membrane.
2. Domain II, approx. 170 aa, forms a "β-prism" structure, with three β-sheets, and exposed loops on its surface.
3. Domain III, approx. 160 aa, has a compact structure with two anti-parallel β-sheets in a "jellyroll" formation, and is structurally similar to carbohydrate-binding domains such as the cellulose-binding domain in cellulases [20]. A general model for three-domain toxins is shown in Fig. 2.

The Proteolytic Activation Process Ingestion of the Cry protoxins by the insect leads to solubilization of the proteins, and exposure to digestive proteinases in the insect gut. Although removal of the C-terminal protoxin region occurs at this stage, the essential step in protoxin activation is the proteolytic cleavage and removal of an N-terminal peptide, which varies from approx. 25–60 amino acids in different Cry proteins. A non-activatable Cry1Ac mutant toxin could not form pores in insect membrane vesicles derived from gut epithelial cells [21], and it is thought that the N-terminal peptide "masks" a region of the toxin involved with interaction with receptors [16]. The activated toxin is fairly resistant to further proteolytic cleavage, which enables it to survive long enough in the gut to reach its site of action, the gut epithelial surface (Fig. 1).

This summary overlooks a number of factors which contribute to toxicity. First, the location of the proteolysis may be important, since many insects, such as diptera (flies), carry out digestion in the foregut, which is chitin-lined and does not contain epithelial surfaces, or even outside the insect altogether, by secreted saliva or regurgitated gut contents. Under these circumstances, the toxin will need to be more resistant to proteolysis, or more effective, since the time between activation and reaching the site of action will be longer. Secondly, gut conditions vary significantly between insects from different orders, or even within orders; in general, larvae of lepidoptera (moths and butterflies) have a highly alkaline midgut environment (pH 10–11 in many major crop pests), whereas larvae of coleoptera (beetles) have an acidic gut environment (pH approx. 5 for many species). These differences in conditions will affect both the activation and survival of the protein, although they may be less relevant to steps taking place at the gut surface, where there is a separation from the gut lumen by the peritrophic membrane (a macroscopic porous chitin-based structure) and by lipids sloughed off from the gut surface. Finally, the nature of the digestive enzymes present in the insect gut differs considerably between different orders; whereas most insects use serine proteinases with an alkaline pH optimum as their major endoproteinases, many coleopteran larvae use cathepsin-type cysteine proteinases with an acidic pH optimum (similar to lysosomal proteinases). On the other hand, protoxin activation does not appear to be very sequence specific. Many lepidopteran-specific Cry proteins can be activated in vitro by mild treatment of the protoxin with bovine trypsin, yielding products that appear to be similar to those formed in vivo. This suggests that it is the



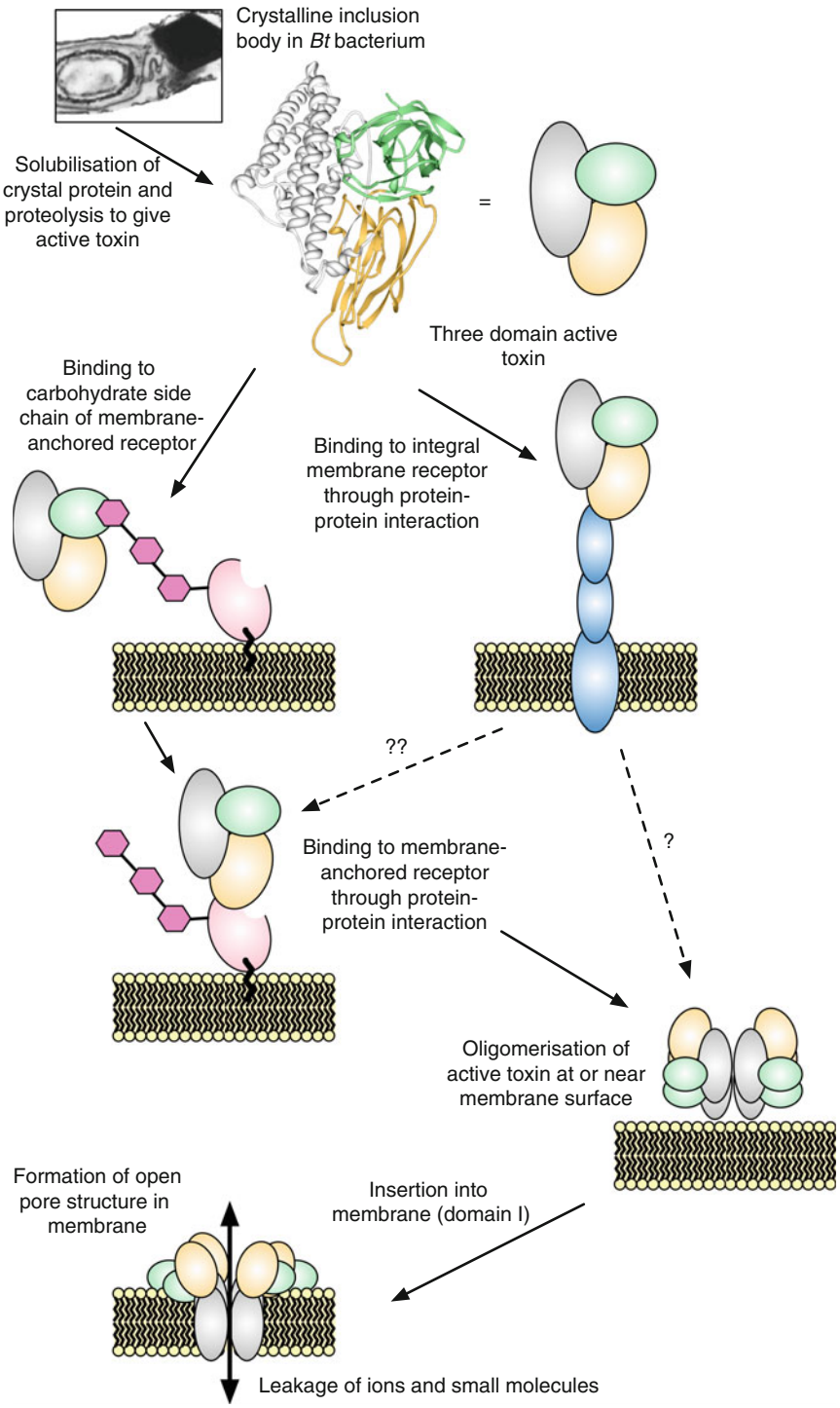
Genetic Engineering of Crops for Insect Resistance. Figure 2
Model structure for three-domain *Bt* toxins. Ribbon diagram showing backbone structure of *Bt* toxin Cry1Aa (PDB 1ciy; [15]); structure of active toxin shown. The three domains are color coded: domain I, silver; domain II, orange, domain III, green. Features as shown on diagram

three-dimensional structure of the protoxin that determines where proteolysis takes place, unless forcing conditions are used.

Interactions with Receptors Proteins to which Cry proteins bind in the insect gut are termed "receptors," although the specificity of interaction is determined by the Cry protein itself, and the ligands to which it binds do not show the properties of receptors as normally understood. Binding takes place on the microvillar membranes of the cells forming the midgut epithelium, and involves interactions with relatively abundant proteins, either attached to the cell membrane by glycosylphosphatidyl-inositol (GPI)-anchors, or integral to the membrane with large extracellular domains. The overall process is summarized in Fig. 3.

Methods for identifying receptors to which Cry proteins bind have largely been based on immunoblotting of proteins prepared from brush border membrane vesicles (BBMV). This method is not a good mimic of conditions in vivo, and may result in interactions with lower affinity, or which are dependent on protein conformations maintained by membranes, not being observed. Nevertheless, the major binding partners for Cry proteins which have been identified show binding when assayed as purified proteins, and as components of BBMVs, with binding constants in the range 1–100 nM.

The initial identification of membrane-anchored aminopeptidase N [23] and an integral membrane cadherin-like protein designated *Bt*-R1 [24] as Cry1A toxin receptors in lepidopteran insects has been supplemented more recently by identification of a 270 kDa glycoprotein [25] and alkaline phosphatase (membrane anchored; [26]) as additional potential receptors. Alkaline phosphatase appears to be the major receptor in mosquitoes [27]. A recent proteomic



Genetic Engineering of Crops for Insect Resistance. Figure 3

Mechanism of action of three-domain *Bt* toxins. The scheme shown is adapted from the "two-receptor" model [22]

analysis has identified further potential receptors, such as V-ATP synthase subunit 1 [28]. However, this analysis also showed binding to actin, which could not be present at the cell surface, showing that results from blotting experiments need to be interpreted critically.

Functional roles as "receptors" for aminopeptidase N and cadherin *Bt*-R1 in Cry protein toxicity are supported by numerous studies. Strains of lepidopteran insects resistant to Cry1 toxins have been identified which show mutations in the gene encoding *Bt*-R1, leading to the production of a truncated cadherin lacking the extracellular domains [29, 30]. The correlation with loss of function of cadherin with loss of susceptibility to Cry toxins suggests that binding to the extracellular domains of cadherin is a necessary step for toxicity. Binding of Cry1A toxin to the cadherin extracellular domains has been demonstrated in vitro, and the binding regions have been identified in some detail [31]. Both gain-of-function and loss-of-function assays have been used to provide further evidence for involvement of cadherin in toxicity; when transiently expressed in mammalian cells that were not normally susceptible to Cry toxin, Bt-R1 genes from silkworm conferred sensitivity to Cry1A toxins [32]; whereas suppression of cadherin expression by RNA interference in tobacco hornworm (*Manduca sexta*) decreased sensitivity to Cry1Ab toxin [33]. In the case of aminopeptidase N, similar correlations between resistance to *Bt* toxin and lack of expression of specific isoforms of the protein have been observed [34], but more direct evidence has come from downregulation of aminopeptidase by RNA interference using double-stranded RNA. This technique has been carried out in lepidopteran larvae, giving decreased sensitivity to Cry1C toxin [35], and in lepidopteran cell cultures, giving decreased sensitivity to Cry1Ac [36]. A gain of function experiment in which transgenic fruit flies (*Drosophila*) expressing lepidopteran aminopeptidase N became sensitive to the lepidopteran-specific toxin Cry1Ac [37] showed elegantly and convincingly that this receptor plays a key role in toxicity. Binding to aminopeptidase N involves interaction of Cry toxins with the carbohydrate side-chains of the protein [38, 39], with specificity toward GalNAc residues being shown (this sugar can inhibit binding; [40]). Binding to carbohydrate facilitates subsequent protein–protein interactions, which are thought to be necessary for toxicity [41]. Functional evidence for alkaline phosphatase acting as a Cry toxin receptor has again been provided by correlative observations, in that insect lines resistant to Cry1Ac toxins have lower alkaline phosphatase levels than susceptible lines [26]. Interactions with protein-bound carbohydrate also seem to be involved in the binding of Cry toxins to alkaline phosphatase.

The roles of the different domains of Cry proteins in the interaction with receptors are clearly distinguished. Despite the presence of the N-terminal propeptide which must be removed for activity, domain I plays little or no role in the interaction with receptors, whereas domain II is responsible for most protein–protein interactions (see Fig. 2), and domain III is responsible for binding to carbohydrates. This division of roles is consistent with the observation that a single toxin can interact with more than one type of "receptor"; for example, Cry1Ac interacts with both Bt-R1 and aminopeptidase N [22]. The protein–protein interactions mediated by domain II have been localized to variable loop regions on the surface of the domain, whereas the carbohydrate-binding region of domain III is a typical binding site cleft, which is spatially well-separated from the domain II loops.

Oligomerization Oligomerization is a common feature in bacterial pore-forming toxins, and Cry proteins appear to conform to the model, with the formation of oligomeric structures (probably tetramers) observed for toxins from the Cry1 and Cry3 families. Mutants of the Cry1Ab protein that have impaired oligomerization ability, but bind to the receptor, show much reduced toxicity or no toxicity toward lepidopteran larvae [42]. Similarly, monomeric Cry proteins have much lower intrinsic pore-forming abilities on synthetic membranes than oligomerized preparations [43]. Oligomerization is promoted by binding to a receptor; in the case of Cry1Ab protein binding to the cadherin Bt-R1 receptor, this process involves an additional proteolytic cleavage at the N-terminal end of the protein, in domain I [44]. The proteolytic cleavage, carried out by host enzymes, may aid the oligomerization process. The importance of oligomerization in promoting toxicity has been shown by two complementary studies. First, a peptide corresponding to the region of cadherin to which Cry1A binds has been shown to act as a synergist, increasing the toxicity of

Cry1A toward lepidopteran larvae [45], presumably as a result of the binding between the peptide and Cry1A promoting oligomerization of the toxin prior to interaction with the gut epithelium. Secondly, mutants of Cry1Ab toxin have been produced which contain deletions corresponding to the proteolysis in helix 1 of domain I which occurs on binding to cadherin. These mutated toxins form oligomers in the absence of cadherin binding, and are effective against insects that have cadherin expression suppressed, or which have a cadherin mutation which leads to resistance to unmodified toxin [33]. These results have led to a current view that cadherin is the primary receptor for Cry toxins, since it is necessary to promote oligomerization, with other molecules taking the role of "secondary receptors" [33].

Insertion into the Cell Membrane The oligomeric Cry protein must partially unfold in order for the pore-forming domains (domain I) to insert into the membrane. In the case of bacterial pore-forming toxins active against mammalian cells, this partial denaturation process is stimulated by acidic pH at the cell surface [13]. A similar mechanism could occur with Cry proteins active against lepidopteran insects, although the gut pH is very alkaline; the partial denaturation could still be triggered by a decrease in pH at the cell surface. The pH optimum for aminopeptidase N in lepidopteran larvae (8.0; [46]) is at least 2 pH units less than bulk gut content pH (>10), suggesting that a decrease in pH occurs near the cell surface. The involvement of lipid rafts, microdomains which are less fluid than the membrane as a whole, in pore formation has been suggested [47]. However, membrane-anchored proteins are selectively associated with these lipid rafts, and it is not clear whether lipid rafts are necessary for pore formation, or whether their involvement is a result of the presence of receptors. The trans-membrane cadherin-like *Bt*-R1receptor is not associated with lipid rafts.

A current model for pore formation by Cry1A toxins suggests that interaction with two receptors is necessary; an initial binding step with the cadherin-like *Bt*-R1 receptor leads to toxin oligomerization, followed by interaction of the oligomer with the aminopeptidase N receptor and insertion into the membrane [22, 48]. While this model is plausible, the details of the mechanism of toxicity must differ for different toxins, and a "two-receptor" model should not be assumed to be generally applicable. The gain of function experiments described above show that only one receptor is necessary for toxicity to be shown, and only a few lepidopteran-specific Cry toxins have been shown to interact with cadherin-like proteins [49]. If the major determinant of Cry protein toxicity is the assembly of oligomeric complexes at the surface of cells in the gut epithelium, then this requirement can be met in diverse ways, involving different "receptor" proteins to localize the toxin and promote oligomerization (although the interaction is always likely to involve the most abundant proteins at the cell surface). A "global" diversity of interactions is not inconsistent with specificity when interactions between specific toxins and hosts are considered.

Once the insertion of Cry toxin into the cell membrane leads to pore formation, the gut epithelial cell is unable to maintain its internal solute balance, as the open pore allows free exchange of ions and other small molecules between the gut lumen and the cytoplasm. The cytoplasm of gut cells has markedly different concentrations of ions (including H^+) than the gut lumen; this difference in concentrations is used to drive active transport processes, such as amino acid transport [50]. Free movement of ions thus causes massive disruption to cell physiology, leading to death. The leakage of cell contents also causes proliferation of gut microflora, so that dying insects show massive bacterial infection of collapsing gut tissue. Cry proteins may also produce toxic effects through interference with signaling pathways. Binding of Cry1Ab to the transmembrane *Bt*-R1 receptor has been shown to activate a G-protein-mediated intracellular signaling pathway, resulting in the formation of cAMP by adenylyl cyclase, and activation of protein kinase A [51]. This process led to cytological changes typical of *Bt* toxin activity.

Binary Cry and Vip Toxins

The binary Cry toxins are exemplified by toxins active against corn rootworm [52]. These toxins are only active as a combination of two proteins, designated as families Cry34 (14 kDa protein) and Cry35 (44 kDa protein). The two proteins are the product of a single

operon in the commonly used *Bt* strains. The binary toxin acts on the insect gut epithelium, and leads to swelling and vesicle production from epithelial cells, resulting in the disappearance of microvilli, and extensive disruption of the epithelium. However, it is not clear whether these symptoms are solely a result of open pore formation, or whether other modes of toxicity, such as ADP-ribosylation (see below) are occurring. No structural information on these proteins is available at present. There is evidence that the 44 kDa toxin protein Cry35 is evolutionarily related to an insecticidal toxin from *Bacillus sphaericus* [53]. The *B. sphaericus* toxins have received some attention due to their toxicity toward mosquitoes and other dipteran insects. They also bind to membrane-anchored receptors (α-glucosidase, in the case of the mosquito *Culex pipiens* [54]) and cause disruption of the gut epithelium [55]. However, their detailed mechanism of action is not known. Like Cry34/35, the *B. sphaericus* proteins are binary toxins, although in this case one component does show limited activity in the absence of the other. The designation of the corn rootworm binary *Bt* toxin by the symbol Cry obscures the fact that these toxins have little in common with the three-domain toxins, besides being found in crystalline deposits in *Bt*, and being insecticidal as a result of acting on the insect gut epithelium.

The *Bt* insecticidal Vip1/2 proteins (active against corn rootworm) are also binary toxins with similarity to the *B. sphaericus* toxins [56]. The mechanism of action of Vip1/2 toxins involves ADP-ribosylation by the active component, which disrupts actin polymerization in cellular microfilaments, similar to other bacterial ADP-ribosylating toxins such as botulinum toxin [57]. The inhibition of actin polymerization leads to massive disruption of cellular functions. The Vip1Ac binding component of the binary toxin interacts with membranes to form oligomeric channels, allowing the active component to gain access to the cell cytoplasm [58].

A further class of Vip proteins, Vip3, (active against lepidoptera) has been identified; these protein are single chain toxins which lyse insect gut cells by pore formation in membranes, and have no sequence similarity to Vip1/2 [59, 60]. Vip3 binds to brush border membrane vesicles prepared from target insect gut epithelial cells, but does not bind to the same receptors as Cry1 and Cry2 proteins [61]. Binding to 80 and 100 kDa membrane proteins is observed in ligand binding experiments [62], but these receptors have not been characterized. These proteins are promising candidates for further development; chimeric toxins containing regions from different Vip3 toxins have been produced and show extended ranges of toxicity toward lepidopteran pests [63].

Cyt Toxins

The cytolytic Cyt toxins, also found in crystalline inclusions in some *Bt* strains, are single polypeptides, of approx. 250 amino acids; the N-terminal region contains α-helices which wrap around a C-terminal β-sheet core in the three-dimensional structure [64]. Pore formation results from insertion of the β-sheet region into membranes [65]. Unlike the three-domain Cry toxins, this membrane insertion is not receptor-mediated [66]; the Cyt toxins insert directly into membranes, and are thus cytolytic to a wide range of cells. Like the three-domain Cry toxins, Cyt toxins are synthesized as inactive protoxins which are activated by proteolysis. Activation involves removal of propeptides from both the N- and C-termini of the protoxin; in the case of Cyt2Aa, 32 aa are removed from the N-terminus and 15 aa from the C-terminus to generate active toxin [67]. This process does not require specific proteinases.

The combination of Cry and Cyt toxins found in crystalline inclusions in some *Bt* strains, specifically in the strains of *Bt* subsp. *israelensis* active against mosquito larvae, is highly effective as a toxin due to synergistic interactions between its components. Not only are the three domain Cry protein components in these crystals more effective toxins in the presence of Cyt proteins, but the Cyt proteins also prevent resistance to Cry proteins from developing when insects are exposed to purified protein preparations under laboratory conditions [68]. This synergistic effect could result from the two types of toxin producing complementary disruption of the insect gut epithelial cell membranes, but evidence has been presented that Cry and Cyt toxins can interact directly. Specifically, Cry11Aa and Cyt1Aa bind strongly to each other, both in solution and in a membrane-bound state, and binding of Cry11Aa to mosquito gut epithelial cell

membranes was enhanced by pretreating the membranes with Cyt1Aa [69]. The interaction with Cyt1Aa takes place through the loop region in Cry11Aa involved in protein–protein interactions with its "normal" receptor (membrane GPI-anchored alkaline phosphatase). Insertion of Cyt1Aa into gut cell membranes, which is not dependent on receptor mediation, thus generates additional "receptors" for Cry11Aa, increasing its toxicity, and preventing resistance developing by mutation of the insect-encoded "receptor."

Expression of Genes Encoding *Bt* Insecticidal Proteins in Transgenic Plants

Expression of Three-Domain Cry Toxins from Transgenes in the Nuclear Genome

Almost all the insect-resistant transgenic crops currently in use express three-domain Cry proteins from *Bt* as their protective agent. The initial laboratory-based experiments expressed Cry1 toxins in plants to give protection against lepidopteran larvae, and this has remained the main focus of *Bt* gene utilization up to the present day. However, the three-domain Cry proteins pose a number of problems in terms of expression in plants. The technology involved in achieving sufficient levels of accumulation of these proteins to give adequate levels of protection was initially challenging, but developed rapidly, so that within 5 years of the initial reports of engineered resistance, the methodology for gene manipulation was essentially complete. The slower pace of transfer of this technology into major crop species observed subsequently has had much more to do with technical difficulties in plant transformation (particularly regenerating viable plants), than with any problems at the level of gene constructs. The minimum level of Cry protein expression in leaf tissue to give high levels of mortality of sensitive lepidopteran larvae under laboratory conditions is approximately 0.05% of total protein, but to give effective field protection against species which are less sensitive to *Bt* toxins, and to manage resistance to the toxin in pests (see later), levels of expression an order of magnitude higher (i.e., 0.5% of total protein) are desirable.

Engineering genes encoding three-domain Cry proteins for expression in transgenic plants has been extensively described (the review by Mazier et al. [70], gives a particularly comprehensive survey), but a short summary of the main considerations which had to be taken into account is relevant here. These were:

1. How much of the protein coding sequence should be expressed in plants?
2. Which promoters should be used to drive expression of the Cry protein coding sequence in plants?
3. How should the coding sequence be altered to avoid poor expression?

Protein Coding Sequence The C-terminal part of protoxins for three-domain Cry proteins is variable, and absent in some toxins. Its role in directing the formation of crystalline inclusions in *Bt* sporulation is not required when the proteins are expressed in plants (and might result in disruption of cells unless the protoxin was exported into intracellular spaces). All constructs which result in insecticidal activity have omitted this part of the molecule from the coding sequence expressed in plants. The initial research suggests that a complete protoxin accumulates in plant tissue at levels 10–50-fold less than a protoxin truncated so the C-terminal region is absent [3]. Since removal of the C-terminal region of the protoxin does not result in active toxin being produced, retention of the N-terminal activation peptide ensures that the initial protein product in transgenic plant tissue is not active, and proteolytic activation takes place as normal within the gut of insect herbivores. The coding sequence utilized thus corresponds to the three-domain structure shown in Fig. 2, plus the additional N-terminal propeptide.

Promoters The problems experienced in achieving levels of expression of Cry proteins high enough to confer effective protection meant that the initial use of promoter sequences which only gave low levels of expression, such as those from *Agrobacterium tumefaciens* Ti plasmids, was rapidly superseded by strongly expressed promoters, most of which were based on the Cauliflower Mosaic Virus 35S RNA promoter (CaMV 35S). Constitutive expression of the Cry protein in all plant tissues does not appear to cause significant problems either in a yield penalty, or

deleterious effects due to the accumulated protein. However, tissue-specific promoters have also been used, such as the ribulose-bisphosphate carboxylase small subunit promoter (e.g., [71]) or the phosphoenolpyruvate carboxylase promoter (e.g., [72]), both of which are specific for green tissue. The CaMV 35S promoter was initially considered to be specific for dicots, but further experience showed that it could also be functional in monocots, and, with suitable modification, could be used to direct Cry protein expression (e.g., [73]). However, many researchers have preferred to use promoters derived from constitutively expressed monocot genes in Cry protein expression constructs for use in cereal transformation (e.g., the maize ubiquitin-1 promoter; [74]). Root-expressed promoters have been used in constructs designed to protect cereals against corn rootworm [75].

Considerable research has also been undertaken on the use of promoters whose expression is only induced under specific conditions. The use of wound-induced promoters to direct Cry protein expression has the apparent advantage that production of Cry proteins in transgenic plants is, for the most part, only induced on attack by insect pests. Any potential deleterious effects on phenotype caused by production of the toxin in transgenic plants would therefore be minimized, and toxin residues in plant tissues would be reduced. A wound-inducible maize proteinase inhibitor gene promoter has been used to direct expression of Cry1B in transgenic rice, and has been shown to give effective protection against insect attack (against striped stem borer; [76]). However, the protection afforded by transgene constructs containing wound-inducible promoters is lower than when constitutive promoters are used, both in the laboratory and in the field [77].

While achievement of expression levels of *Bt* toxins sufficient to confer protection in transgenic plants is now considered routine, considerable technical problems may still need to be overcome when specific crop species are considered (e.g., soybean; [78]). These include the construction of the synthetic coding sequence for the toxin, choice of an appropriate promoter for the expression construct, developing protocols for efficient transformation and regeneration of the plant species, and production of homozygous progeny lines containing the transgene.

Engineering the Coding Sequence to Optimize Expression The initial experiments in which Cry toxins were produced in transgenic plants showed that only low levels of Cry protein were accumulated, generally of the order of 0.01% of total protein, or less. Levels of Cry proteins were at least one order of magnitude lower than when plant proteins were expressed using similar promoters in expression constructs, leading to the deduction that the Cry protein coding sequence contained features which decreased protein production as a result of posttranscriptional events. Cry protein coding sequences are generally A-T rich compared to plants (coding%GC in *Bacillus thuringiensis*, 36%; in *Arabidopsis thaliana*, 45%; in *Oryza sativa*, 55%; Codon Usage Database, http://www.kazusa.or.jp/codon/) and codon usages thus differ significantly. Cry protein genes were reengineered, modifying the nucleotide sequence without altering the encoded amino acid sequence, to change the codon usage to one more appropriate for plants, resulting in either partially or wholly synthetic genes (reviewed by Mazier et al. [70]). Codon optimization for both dicots and monocots has been carried out. Codon-optimized synthetic genes show accumulation levels of Cry proteins of up to 1% of total protein in leaf tissue, which is adequate for complete protection of plants against pest insects [79].

The basis for poor expression of Cry proteins in transgenic plants has received comparatively little attention. Evidence suggests that the major problem is not codon usage, but instability of RNA transcripts [80, 81]. Expression of unmodified Cry protein coding sequences leads to accumulation of short, polyadenylated transcripts resulting from incorrect recognition of polyadenylation addition signal sequences within the protein coding sequence [82]. Specific modification of A-T-rich regions within the coding sequence of Cry1Ac toxin putatively responsible for transcription termination and polyadenylation (both AATAAA signal addition sequences and ATTTA upstream motifs) has been shown to lead to increased protein expression in transgenic tobacco [83]. Changing codon usage to increase GC content has eliminated these A-T-rich regions in synthetic Cry protein genes, which therefore can produce high levels of stable mRNA.

Expression of Three-Domain Cry Toxins from Transgenes in the Chloroplast Genome

The bacterial origin of the chloroplast is reflected in differences in both the genome composition and organization, and the biochemistry of transcription and translation within the organelle, compared to the nuclear genome and transcription and translation in the nucleus and cytoplasm. The bacterial origin of the genes encoding Cry proteins suggests that expression in the plastid, from transgene constructs introduced into the plastid genome, might result in high levels of protein production. This prediction was confirmed in 1995 with a report showing that incorporation of a construct containing a complete coding sequence for the Cry1Ac protoxin protein and the plastid rRNA operon promoter into the genome of tobacco chloroplasts led to accumulation of Cry1Ac protoxin (approx. 130 kDa – i.e., with the C-terminal crystal-forming region intact) in tobacco leaves to levels of 3–5% of total protein [84]. The high level of Cry protein accumulation meant that transformed plants were effectively protected against attack by several major lepidopteran pests, even beet armyworm (*Spodoptera exigua*), a species relatively insensitive to *Bt* toxins.

Despite this highly promising initial report, expression of Cry proteins via plastid transformation has not been widely adopted, and is not used in the current commercial crops. Reasons for this are difficult to pinpoint; there are significant technical problems in achieving stable transformation of plastids, since all of the copies of the plastid genome in the cell (up to 10,000) must be transformed [85], and plastid transformation has been problematic in species other than tobacco [86]. Nevertheless, methods exist to overcome these problems [87]. Cry1, Cry2, and Cry9 proteins have been expressed in plastids of tobacco [88–91], and Cry1Ab has been expressed in soybean plastids [92], all giving high levels of protection against lepidopteran pests to the resulting plants. Overexpression of the Cry2Aa2 operon is particularly effective in giving broad-spectrum protection against a range of pests.

Commercial introduction of transgenic insect-resistant crops based on plastid transformation is almost certainly feasible, but may as yet be restricted by economic considerations, or concern over long-term stability of the transgene phenotype. The maternal inheritance of plastid-encoded characteristics shown by most plants, which means that pollen cannot disperse the transgene to non-transgenic plant stocks, is a further advantage to the method, which could be used to overcome objections to coexistence of transgenic and "organic" agricultural practices by environmental pressure groups.

Expression of Other Genes Encoding Insecticidal *Bt* Toxins

Gene constructs for expression of other *Bt* toxins follow the same principles as those outlined above for three-domain Cry toxins. For example, corn expressing the binary Cry34/35 toxin (for protection against corn rootworm) was transformed with a construct containing a constitutive promoter (maize ubiquitin-1) and synthetic coding sequences for the 44 and 14 kDa polypeptides [52], giving expression levels of up to 0.9% and 0.2% respectively of total soluble proteins in plant tissues. Details of the constructs used for expressing these, and other *Bt* toxins, are apparently not reported in the scientific literature.

Taking Transgenic Plants Expressing *Bt* Toxins into the Field

Dealing with Pest Resistance to *Bt* Toxins

The development of successful strategies for commercial deployment of "first generation" insect-resistant crops expressing a single three-domain Cry toxin has focused on a single major potential problem, the development of resistance to the insecticidal compound by the targeted pest species. Development of resistance to exogenously applied chemical pesticides has occurred in over 500 insect species [93], and field resistance to *Bt* sprays has been observed in the lepidopteran pest diamondback moth (*Plutella xylostella*). Resistance to *Bt* toxins can be produced in the laboratory within a small number of generations of many pests, showing that resistance alleles are present in pest populations at a nonnegligible level, although resistance to high doses of specific toxins is only shown in individuals homozygous for the resistance allele. This topic has been ably

reviewed in the context of the commercialization of *Bt* crops by [94]. The most common mechanism of resistance to Cry toxins in insects is mutation in a toxin receptor, leading to a failure to bind sufficient levels of toxin for lethal effects to be shown; however, the involvement of more than one "receptor" in current models for three-domain Cry toxin mechanisms of toxicity (see above) implies that multiple genetic loci for resistance in the pest are possible. Other mechanisms, such as altered proteolysis of toxins, have been proposed to account for the resistance to multiple toxins which can be produced in the laboratory.

The practical solution to prevent the development of resistance in pest populations, the "high-dose/refuge" strategy, has been extensively reviewed elsewhere [94]. In its simplest form, this strategy couples transgenic plants that are expressing sufficient levels of a specific toxin to kill all pest insects which are homozygous negative, or heterozygous, for a resistance allele, with a reservoir of untransformed plants which maintain a population of pests which have a normal frequency of resistance alleles. It assumes that the frequency of occurrence of resistance alleles is low ($<10^{-3}$). Surviving pests on the transgenic plants will be almost all homozygous positives for the resistance alleles, but will be few in number due to the low frequency of occurrence of these alleles. The nontransformed plants will produce a large number of pest insects, most of which are homozygous negative for resistance alleles. Provided that transgenic and untransformed plants are not spatially separated, mating between resistant insects selected on transgenic plants will be a rare event, and most progeny will be homozygous negative or heterozygous for resistance alleles, and thus susceptible to the insecticidal activity of the transgenic plants. In this way, both the pest population is suppressed, and any increase in the frequency of resistance alleles in the population is minimized by the continuous "diluting out" effect.

This approach has been almost wholly successful in controlling pest resistance to *Bt* toxins in agricultural use of transgenic crops over 10 years. That it has been so successful may be a result of factors other than those originally considered, since the assumption that *Bt* toxin resistance alleles occur at a very low frequency in natural populations has been called into question. Although some insect populations show resistance allele frequencies in the 10^{-3} to 10^{-2} range (e.g., tobacco budworm, *Heliothis virescens* in USA; [95]; *Sesamia nonagrioides* in Spain and Greece; [96]), estimates for pink bollworm (*Pectinophora gossypiella*) in Arizona, USA in 1997 were as high as 0.16 [97]. No evidence for selection for resistance was observed, since the frequency of resistance alleles did not increase over a 3-year monitoring period in which transgenic cotton expressing *Bt* toxins was extensively employed. A subsequent follow-up study [98] confirmed that frequencies of resistance alleles in this insect had not increased over an 8-year monitoring period, with values generally $<10^{-2}$, despite almost continuous exposure to Cry1Ac via transgenic cotton. The possibility that resistance alleles in the insect carry a significant fitness penalty is one additional factor that could account for these observations.

The success of the refuge strategy is dependent on farmers sacrificing part of their crop (untransformed plants) to maintain a pest population. This has been successfully enforced in the industrialized agriculture of developed countries, but may be more difficult to ensure when insect-resistant transgenic crops become available to rural farmers. Although greater agricultural diversity may play the same role as the refuge strategy in maintaining a pest population and decreasing selection pressure, emergence of resistance in pests to *Bt* crops has been delayed, not eliminated, and further strategies to manage it will be necessary.

Pests That Are Not Susceptible to *Bt* Toxins

As described above, most of the *Bt* toxins that have been investigated, and introduced into transgenic crops, are active against lepidopteran or coleopteran insect pests. This is partly a result of the practical requirements of agriculture, since these orders include most of the major pests. However, there are significant insect herbivores which remain outside the range of activity of *Bt* toxins that have been expressed in transgenic plants.

Dipteran pests, such as fruit flies and root flies, are serious pests in many crops, and *Bt* toxins active against diptera have been thoroughly investigated. A major problem with introducing protection against these pests into plants is that *Bt* strains active against dipteran insects usually contain a mixture of toxins, often

including both Cry and Cyt proteins (see above). These toxins act synergistically, and individual components are only of low toxicity. Introduction of genes encoding the mixture of toxins found in a typical dipteran-active *Bt* strain into a transgenic plant has yet to be attempted, although it is not beyond the capacity of existing technology.

The major order of insect herbivores outside the range of *Bt* toxins is Hemiptera, which includes aphids, plant- and leafhoppers, whitefly, and other sap-suckers which feed directly on the contents of phloem and/or xylem vessels, predominantly sucrose and free amino acids. These insects are important pests and virus vectors. No *Bt* toxins with activity against them have been found. The reason for this is not clear; receptors similar to those in other insect orders are present in these insects [99], but generally they contain very low levels of digestive proteolytic activity, as a result of ingesting nitrogen in the form of amino acids rather than protein. This lack of digestive proteolytic activity may interfere with activation of *Bt* toxins, and prevent enough activated toxin to have effects on the insect being present in the gut.

Why Haven't Plants Evolved Their Own *Bt*?

Despite the problems encountered in managing resistance of pests to *Bt* toxins, transgenic plants expressing these insecticidal proteins have proved their value in the field. However, the necessity for resistance management suggests that this solution to defense of plants against insect herbivores may not be viable on an evolutionary timescale. Endogenous expression of *Bt* toxins is not a "natural" method of defense against herbivores, since plants do not produce similar insecticidal proteins themselves. This failure on the part of plants to exploit a viable strategy for protection seems puzzling, and the obvious explanation, that plants lack the capacity to produce *Bt* toxin-like proteins, is not correct. Since introduction of suitably modified *Bt* genes gives adequate levels of protein expression for protection, there is no reason why plants could not have evolved a similar capacity. As discussed in the following section, plants have evolved a diverse array of defensive mechanisms, but make little use of proteins which are highly toxic to insect herbivores. Possibly, this is due to the relative ease with which insects can develop resistance to protein toxins which exert a very strong selection pressure on the population; although alternative hypotheses, such as the balance between investing plant resources into defense versus growth not favoring this strategy, or practical difficulties for a sessile organism in delivering toxins, should also be considered. Unfortunately, the experiments which would enable this issue to be investigated, namely, an evaluation of the "fitness" of *Bt*-expressing plants in a natural ecosystem in competition with varieties relying on endogenous defenses, and the persistence of *Bt* genes in a natural population, are unlikely to be carried out in the near future, due to obvious regulatory issues.

Whatever the reason for plants "in the wild" not using defensive proteins similar to *Bt* toxins, there is no reason to suppose that transgenic plants with engineered insect resistance will not continue to be useful in the artificial growing conditions of agriculture. Manipulation of crop plants by conventional breeding has successfully introduced characteristics such as large seed size, which were not present, and would not be viable, in their wild progenitors. Characteristics introduced into cultivated plants by plant genetic engineering do result from a process that is fundamentally different from selection, but both conventional breeding and genetic engineering are aiming for the same end results, agriculturally desirable phenotypes. Their products should be evaluated by similar criteria.

Developments to "First-Generation" Crops Expressing *Bt* Toxins

Plants Expressing Multiple Toxins ("Pyramiding")

The specificity of a single Cry toxin toward specific target pests can be a problem in the field where a secondary, minor pest species can replace the primary pest and cause serious damage to crops. An obvious method to counter this problem is to add or introduce a second *Bt cry* gene into the crop to extend the range of pests against which protection is afforded. The availability of a wide range of gene constructs encoding Cry toxins has made this a realistic possibility, with crossing singly transformed lines, or repeated transformation, or transformation with a construct containing two genes as alternative methods for introducing the

genes into one line. Monsanto's Bollgard transgenic cotton was improved by introducing a second *Bt* gene as early as 1999. Laboratory trials showed that cotton plants expressing both Cry1Ac and Cry2Ab proteins were more toxic to bollworms (*Helicoverpa zea*) and two species of armyworms (*S. frugiperda* and *S. exigua*) than cotton expressing Cry1Ac alone, even though doses in this trial were sublethal [100]. Subsequent evaluations in greenhouse and field trials [101] confirmed the superior insect resistance of plants expressing both toxins.

A further potential advantage of transgenic plants expressing two Cry proteins with differing specificities, that target different receptors in the insect, is in preventing the appearance of resistance in the pest, since multiple mutations are required to produce the loss of sensitivity to the toxins. This hypothesis was confirmed directly in work reported by [102], in which transgenic broccoli plants expressing either Cry1Ac, or Cry1C, or both proteins were produced. Plants were exposed to a population of diamondback moth (*P. xylostella*) which carried Bt resistance genes at a relatively low frequency in an extended greenhouse experiment, and results showed that selection over 24 generations led to a significant delay in the appearance of resistance in insects exposed to the pyramided two-gene plants. The success of these experiments has led to suggestions that the refuge approach to resistance management may be redundant for crops expressing multiple toxins [103]. However, some care is needed in the selection of genes in relation to potential pests, as resistance to multiple toxins has been observed in several cases. For example, a strain of the lepidopteran cotton pest *H. virescens* which has simultaneous resistance to Cry1Ac and Cry2Aa has been identified, in which the genetic bases of resistance to each toxin are different [104].

Many subsequent programs which have aimed to produce insect-resistant crops expressing *Bt* toxins have adopted the two-gene approach to broaden and improve protection against diverse pests, and to prevent resistance developing in insects (e.g., [105]). Although engineering to produce combinations of different three-domain Cry toxins is the most common approach, other potential resistance genes have been included also, such as those encoding Vip proteins [106], or even proteinase inhibitors (e.g., cowpea trypsin inhibitor; [107]). The "pyramiding" or "stacking" of resistance transgenes has been enthusiastically adopted by commercial organizations, and the recent announcement of a transgenic maize variety containing eight different transgenes by Monsanto and Dow Agrosciences [108] exemplifies this trend. This variety contains insect-resistance genes derived from both companies' research programs, active against corn rootworm and lepidopteran pests (Herculex RW = Cry34Ab1 + Cry35Ab1, Herculex I = Cry1F; YieldGard VT Rootworm/RR2 = modified Cry3Bb1, YieldGard VT PRO = Cry1A.105 + Cry2Ab2), as well as two herbicide tolerance genes (giving resistance against glyphosate and glufosinate-ammonium), and is intended to be a "one-stop" solution to pest and weed problems.

Domain Exchange in Three-Domain Cry Toxins

The separate roles played by the different domains in the process of interaction of three-domain *Bt* toxins with their receptors, and their structural independence, suggested to investigators that hybrid toxins, in which domains from different naturally occurring toxins were grafted together, would be likely to be active, and could show novel specificities in their activity toward insects. This process can be made to occur in vivo in *Bacillus thuringiensis*, using a site-specific recombination vector [109], or can be carried out in vitro using conventional molecular biology techniques, followed by expression in a microbial host. Transfer of domain III between different Cry1 proteins led to identification of this domain as conferring primary specificity to different lepidopteran species, and the generation of hybrids with broader specificity than naturally occurring toxins [110]. Subsequent work generated a Cry1Ab-Cry1C hybrid, which was highly toxic to *S. exigua*, an insect resistant to Cry1A toxins [111], and identified Cry1Ca domain III as sufficient to confer toxicity toward *Spodoptera* in a variety of hybrids [112]. In contrast to the results obtained when exchanging domain III, exchange of domain I between different Cry1 toxins did not yield biologically active proteins [113].

A measure of the potential for improvement in "natural" *Bt* toxins is shown by experiments reported by [114], in which a hybrid Cry protein, constructed by fusing domains I and III from Cry1Ba with domain II

of Cry1Ia, was expressed in transgenic potato. Plants expressing the hybrid toxin at levels up to 0.3% of total soluble protein were produced, and not only showed resistance to the lepidopteran pest potato tuber moth (*Phthorimaea operculella*), but also had a high level of resistance to Colorado potato beetle. The "parental" Cry proteins have high toxicity towards lepidopterans, but only very limited toxicity towards coleopterans such as the potato beetle. The hybrid has effectively created a novel toxicity, which is suggested to be based on interaction with a novel receptor.

Mutagenesis of Three-Domain Cry Toxins

Modification of *Bt* toxins by site-directed mutagenesis to increase toxicity towards target pests has been employed as an alternative to the "domain swap" approach. Most mutagenesis experiments on *Bt* toxins have been carried out to explore structure-function relationships in these proteins (see above; reviewed by Dean et al. [115]), but the accumulated knowledge of which parts of the protein determine specificity of interactions with receptors in the insect have been exploited to produce variants with increased activity toward target pests.

The key role of domain II in three-domain Cry proteins in mediating interactions with insect receptors was shown by a mutagenesis experiment in which altering amino acid residues in the loop regions in this domain of Cry1Ab increased its toxicity toward larvae of gypsy moth (*Lymantria dispar*) by up to 40-fold, with a corresponding increase in binding affinity to brush border membrane vesicles [116]. These results were based on expression of the recombinant protein in microbial hosts. A similar strategy was used to increase the toxicity of Cry3A protein toward target coleopteran pests [117], and of Cry4Ba toxin [118, 119] and Cry19Aa toxin [120] toward mosquito larvae. The level to which rational design of toxins is possible is shown by the engineering of toxicity toward mosquito larvae into the lepidopteran-specific toxin Cry1Aa [121]. Alternatively, a directed evolution system based on phage display technology for producing toxins with improved binding to a receptor, and thus increased toxicity, has been described [122]. Mutagenesis of domain I has also been attempted, with claims that alteration of alpha helix 7 in Cry1Ac to resemble the corresponding helix in diphtheria toxin led to increased toxicity toward cotton bollworm (*Helicoverpa armigera*) larvae [123].

The impressive achievements of toxin engineering at the level of recombinant proteins, have led to the technology being used for gene constructs designed for expression in transgenic plants, although toxins with unmodified amino acid sequences continue to be widely used (largely as they give adequate protection). One example where toxin engineering has been successfully carried out is the current commercial transgenic corn variety with resistance to corn rootworm, MON863, which expresses a modified version of the *Bt* Cry3Bb1 toxin [75]. Unmodified Cry3Bb1 is active against a number of coleopteran species, including Colorado potato beetle and corn rootworm [124], but toxicity toward western corn rootworm (*Diabrotica virgifera virgifera*) was not sufficient to give adequate protection at levels of expression achievable in corn. Modifications to the amino acid sequence increased the toxicity of the protein toward corn rootworm approximately eightfold. The nature of the modifications has not been described in the scientific literature, and is only available through reference to a series of patents (see [75]).

Fusions

As a logical extension to the transformation of plants with separate gene constructs encoding two Cry proteins, some workers have chosen to produce a single construct containing a single translationally fused coding sequence encoding both proteins. This approach has been successfully demonstrated by producing a Cry1Ab-Cry1B translational fusion protein in transgenic maize [125], although there is no apparent advantage over simpler methods for introducing two genes. The Cry1Ab-Cry1B fusion protein has also been expressed in transgenic rice [126], which was fully resistant to yellow stem borer (*Scirpophaga incertulas*).

A more interesting possibility is the introduction of extra functionality into Cry toxins by addition of sequences from other proteins which could lead to binding interactions with more potential receptors in the insect gut, extending the range of toxicity and hindering development of resistance. In work reported by Mehlo et al. [127], the galactose-binding lectin

domain (B-chain) from the ribosome-inactivating protein ricin was fused C-terminally to domain III of Cry1Ac, producing a Cry1Ac-ricin B-chain fusion protein. The fusion protein thus has the ability to bind to galactose residues in side chains of glycoproteins or glycolipids in the insect gut epithelium, as well as *N*-acetyl galactosamine residues which are bound by domain III. The fusion protein was expressed in transgenic maize and rice plants, and was shown to afford a high level of protection to larvae of stemborers (*Chilo suppressalis*) and leaf armyworm (*Spodoptera littoralis*), whereas plants expressing the unmodified Cry1Ac were susceptible to both insects. The transgenic maize plants were also resistant to a homopteran plant pest, the leafhopper *Cicadulina mbila*, although it is possible that this was an effect of the lectin domain in the fusion (see later section Lectins), since *Bt* toxins are not effective against homopteran insects.

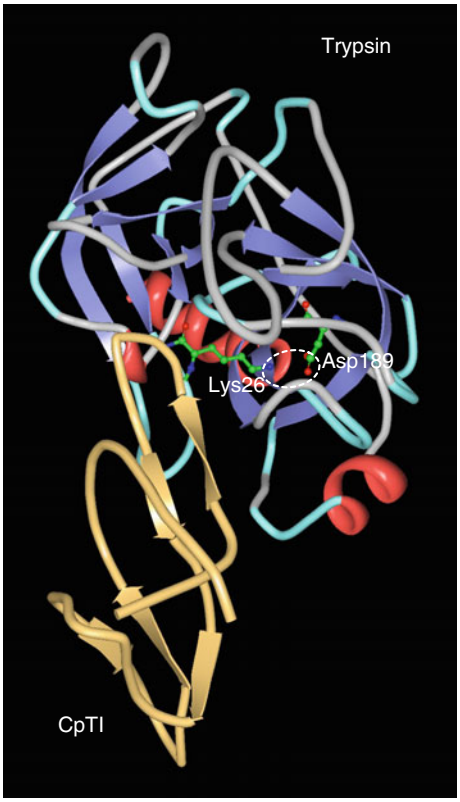
The engineering of extended binding properties into three-domain Cry proteins to increase the range of toxicity toward insect pests is clearly possible, but needs to be approached with some caution. There is a risk that the extended range of activity will include mammalian toxicity, which would negate one of the major advantages of these insecticidal proteins.

Exploitation of Endogenous Plant Defensive Mechanisms Against Insect Herbivores

Plants have a range of endogenous mechanisms to defend themselves against insect herbivores, and use both static defense mechanisms based on the accumulation of pre-synthesized insecticidal compounds, and active defense mechanisms in which gene expression is induced as a result of insect damage (response to wounding, and responses to insect secretions), leading to the synthesis of insecticidal compounds [128]. Conventional breeding has sought to exploit endogenous insecticidal genes within a plant species, but the use of transgenic technology allows defensive compounds and mechanisms to be transferred between species, or allows the control of existing defensive systems to be altered to improve their effectiveness. The molecular biology involved in transfer of genes between plant species is technically straightforward, and does not involve the kind of reengineering necessary to make bacterial genes suitable for use in plants. This approach to increasing insect resistance in transgenic plants has almost as long a history as engineering for *Bt* Cry toxin expression, but to date has not resulted in a commercial product, or widescale adoption in agriculture. Some of the reasons for the lack of practical outcomes for this strategy will be discussed below.

Proteinase Inhibitors

Protein proteinase inhibitors (PIs) are ubiquitous in plant species. They are major components of both "static" and "active" defense in that they are accumulated in specific tissues ("static" defense), and are the major end-product in the induced response to wounding ("active" defense). They are generally small proteins, ranging in size from 4 to 25 kDa, with many different sequence families having been identified. They form tightly bound complexes with their target proteinases, which usually involve a "loop" on the inhibitor fitting into the enzyme active site (Fig. 4), blocking the site, and inactivating the enzyme. The observation that most of these inhibitors were active against digestive serine proteinases from higher animals, and not endogenous plant proteinases (where serine proteinases are comparatively rare, and not involved in protein digestion) suggested that they were defensive compounds, and bioassays in which purified PIs were fed in artificial diet confirmed that an antimetabolic effect was exerted on insect herbivores which relied on protein digestion for nitrogen supply, shown as a slower growth rate, retarded development, and increased mortality (reviewed by Garcia-Olmedo et al. and Ryan [130, 131]). Besides a direct effect on digestion of ingested proteins, PIs cause a loss of nitrogen to the insect by preventing the reabsorption of nitrogen used to produce digestive proteinases, which are normally (self)-degraded in the gut rather than excreted. The role of these proteins in induced defense against insects was shown by blocking the normal wounding response in transgenic tobacco plants by suppression of expression of the prosystemin gene, which produces the peptide hormone systemin, using antisense RNA. The transformed plants were unable to synthesize wound-induced PIs and were significantly more susceptible to herbivory by lepidopteran larvae [132]. The importance of the wounding response to plant defense in natural ecosystems has been



Genetic Engineering of Crops for Insect Resistance. Figure 4

Structure of a complex between a typical plant protein proteinase inhibitor (*PI*) and a target proteinase (from PDB 2g81; [129]). Structure shown in backbone representation is the complex between beta-trypsin (*top*, secondary structure color-coded in red and blue) and a Bowman-Birk PI from cowpea (*Vigna unguiculata*; *bottom*, gold). This inhibitor ("CpTI") has been expressed in transgenic plants to give partial resistance to lepidopteran larvae. The side chains responsible for the specificity-defining ion-pair interaction (dotted ellipse) are shown in ball-and-stick representation; they are Asp189 (S1′) in the substrate binding pocket of the enzyme, and Lys26 (S1) on the active site loop of the inhibitor. Other interactions take place across the contact surface between inhibitor and enzyme to form a tightly bound complex

extensively studied by Baldwin's group (reviewed in [133]); this outstanding body of work has established a synthesis of responses in the plant under attack, responses in neighboring plants, and responses of natural enemies of insect herbivores, with communication via volatile signals produced by the plant under attack.

A seed-expressed Bowman-Birk-type serine proteinase inhibitor from cowpea, which contained two inhibitory sites active against bovine trypsin (CpTI) was the first plant PI to be produced in another species [134], using a gene construct containing a CaMV 35S promoter. The resulting transgenic tobacco plants expressed CpTI at up to 1.0% of total soluble protein, and decreased growth and survival of tobacco budworm (*H. virescens*) by up to 50%, with similar effects on other lepidopteran larvae. Subsequent experiments carried out with wound-induced PIs showed that these also had similar effects when constitutively expressed in transgenic plants; for example, the tomato inhibitor II gene, when expressed in tobacco, was also shown to confer insect resistance [135], as did potato PI-II [136]. Both CpTi and PI-II were subsequently expressed in rice, where partial protection against stem borers was observed [137, 138]. The constitutive expression of foreign PIs could be mimicked in transgenic tomato plants by constitutive expression of the prosystemin gene (see above) leading to constitutive expression of wound-induced tomato PIs [139]. Tobacco plants modified in this manner show partial resistance to insect herbivores similar to that produced by expressing foreign PIs [140].

The problem with this strategy for producing insect-resistant plants soon became obvious; in contrast to the expression of Cry proteins, which, when optimized, routinely gave transgenic plants virtually complete protection against susceptible pests (mortality100%, damage minimal) expression of PIs only produced partial resistance. Investigation of the digestive biochemistry showed that exposure to PIs in the diets of lepidopteran and coleopteran herbivores resulted in the appearance of proteinase activities which were insensitive to the inhibitor(s) present [141, 142], or were able to degrade the ingested PIs [143]. These insects contain large families of genes encoding dietary proteinases, whose expression could be up- or downregulated by dietary inhibitors [144]. In effect, these insect herbivores were preadapted to be partially resistant to dietary PIs, as a result of similar or identical compounds being present routinely in their diet. Although expression of resistance to PIs in herbivorous

insects has a fitness penalty, shown by reduced growth on diets to which inhibitors are added, or on plants which are expressing foreign PIs, or over-expressing endogenous PIs (see above), this is not sufficient to cause mortality at a level which affords more than partial protection. In some cases, low levels of expression of a foreign PI in transgenic plants can actually result in improved insect performance, as when tobacco and *Arabidopsis* plants expressing mustard trypsin inhibitor 2 were exposed to larvae of cotton worm (*S. littoralis*; [145]).

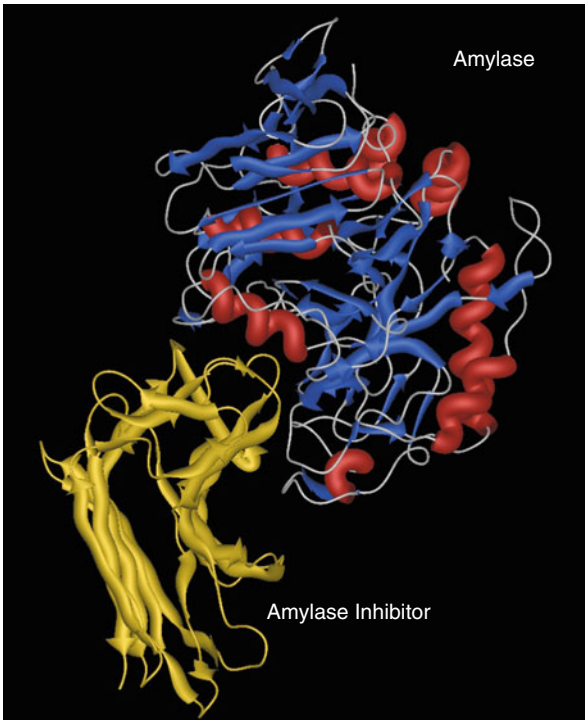
A number of investigators have attempted to select PIs for expression in transgenic plants which are optimally active against the dietary proteinases present in specific insect pests. Attempts to develop inhibitors active against specific lepidopteran digestive serine proteinases induced by dietary PIs have not been successful. On the other hand, not all pest insects rely on serine proteinases for digestion. Many herbivorous coleopteran larvae utilize cysteine proteinases, rather than serine proteinases, as their major digestive endoproteinases, and these proteinases can be inhibited by cystatins, a family of proteins present in all kingdoms of organisms. Enzyme assays in vitro were used to characterize digestive proteinases of a coleopteran pest, *Chrysomela tremulae,* as cysteine proteinases, and to show that a cystatin from rice, oryzacystatin, was an effective inhibitor. Transgenic poplar seedlings expressing oryzacystatin were produced, and leaves from these plants were shown to be toxic to larvae of the pest [146]. This promising result does not seem to have been followed up. Expression of oryzacystatin in transgenic potato only gave partial protection against larvae of Colorado potato beetle [147], suggesting preadaptation in this pest, which is known to employ a diverse range of digestive proteinases. In an attempt to use proteinase inhibitors which insects would not be preadapted to, synthetic multidomain cysteine proteinase inhibitors based on domains found in animal and plant sources (kininogen, stefin, cystatin C, potato cystatin, and equistatin) were assembled and expressed in transgenic potato; the plants were deterrent to thrips, and gave partial resistance in greenhouse trials, but complete protection was not observed [148, 149]. Attempts to express the sea anemone cysteine/aspartic proteinase inhibitor equistatin itself in transgenic potato did not give significant levels of resistance to Colorado potato beetle, due to degradation of the inhibitor in the plant [150]. Multiple proteinase inhibitors (potato PI-II and PCI) active against two families of proteinases, serine proteinases and carboxypeptidases, have been expressed in transgenic tomato plants [151], but still only afforded partial protection against lepidopteran larvae due to adaptive mechanisms present in the insects.

In conclusion, the expression of suitable PIs in transgenic plants can give protection against lepidopteran and coleopteran pests, but has not been able to produce results comparable with those achieved by use of *Bt* toxins.

Amylase Inhibitors

The widespread occurrence of protein inhibitors of mammalian amylases in plants has become accepted as another defensive mechanism against herbivores (reviewed by Franco et al. [152]). Like proteinase inhibitors, these are generally small proteins, resistant to proteolysis, ranging in size from approx. 8–30 kDa. Although they are also active against insect amylases, it is not clear to what extent these proteins contribute to insect resistance in most cases, since the relatively low nitrogen content of plant tissues compared to insects means that most herbivorous insects are nitrogen limited, not carbon limited, and starch digestion is unlikely to be a limiting factor in growth. However, in the case of coleopteran herbivores whose larvae attack seeds specifically, such as seed weevils (bruchids), there is good evidence for α–amylase inhibitors from legume seeds being highly insecticidal [153], and in being causative factors in the resistance of specific varieties of legumes to bruchids [154]. These proteins belong to a different sequence family than the more common types of α–amylase inhibitors found in cereals, and are similar to legume lectins in sequence [155].

Like proteinase inhibitors, amylase inhibitors form tightly bound complexes with their target amylase (Fig. 5), although the same interaction of a loop on the inhibitor with the active site of the enzyme is not possible, since the enzyme substrate is a polysaccharide, not a polypeptide. The mechanism of toxicity clearly involves inhibition of starch digestion, since bruchid larvae exposed to the α–amylase inhibitor from French



Genetic Engineering of Crops for Insect Resistance. Figure 5

Structure of a complex between a plant protein α-amylase inhibitor and an insect amylase enzyme (from PDB 1viw; [156]). Structures shown in backbone representation; α-amylase from larvae of the coleopteran storage pest *Tenebrio molitor* (yellow mealworm beetle) is shown *top right*, in red and blue (secondary structure color coding); the α-amylase inhibitor from *Phaseolus vulgaris* (French bean) is shown in gold *bottom left*. This inhibitor has been expressed in several transgenic legume species to give resistance to coleopteran pests. The inhibitor shows the typical "all β-sheet" structure of the legume lectin family of proteins. Interaction between the binding loop of the protein and the starch-binding site of the enzyme occurs across the contact surface, sterically blocking access by polysaccharides to the active site

bean (*Phaseolus vulgaris*) show induction of amylase enzymes [157], although other mechanisms of toxicity may also be present, since these proteins can cause 100% mortality in susceptible insect species at levels of <1.0% of total protein. Alternatively, these highly specialized herbivores may lack the adaptive mechanisms to plant defensive proteins shown by species that feed on a wide range of plant foodstuffs [128]. High levels of toxicity toward insects have not been observed in general with amylase inhibitors. For example, α–amylase inhibitors are not strongly toxic to lepidopteran larvae, where the alkaline environment of the gut may interfere with the formation of inhibitor-enzyme complexes. The α–amylase inhibitor from French bean is inactivated by high pH.

The isolation of a lectin-like α–amylase inhibitor gene from *P. vulgaris* [155] stimulated research in this area, and in a ground-breaking series of experiments, this gene was assembled into a construct with a strong seed-specific promoter (from the *P. vulgaris* seed lectin gene), and expressed in seeds of transgenic garden pea. The resulting seeds contained up to 3% of the foreign protein, and were highly resistant to larvae of cowpea and Azuki bean weevils [158], which do not normally attack garden peas in the field, but are stored product pests, and to larvae of the pea weevil *Bruchus pisorum* [159], which is a field pest of garden pea. In all cases larval development from eggs laid on seeds was halted at a very early stage, and damage to the crop was minimal. Subsequent experiments showed that transgenic azuki beans could also be protected against bruchid storage pests [160], and that transgenic garden pea was protected against pea weevil under field conditions [161]. The success of this strategy led to hopes that the *Phaseolus* α–amylase inhibitor gene could be incorporated into a range of crops, particularly other grain legumes such as lentils, mungbean, groundnuts, and chickpeas to give protection against a variety of bruchids. Technical problems with transformation of some of these crop species have delayed this goal being achieved, but transgenic chickpeas expressing high levels of the *Phaseolus* α–amylase inhibitor have been successfully produced [162].

Despite the success of this strategy, full agricultural deployment of transgenic crops expressing the *Phaseolus* α–amylase inhibitor gene has not taken place. Commercial reasons have played a major part in preventing widescale adoption, but safety concerns have also arisen. The protein product of the *Phaseolus* α–amylase inhibitor gene expressed in pea shows minor structural differences to the native product (i.e., expressed in *P. vulgaris*) as a result of differences in posttranslational processing (differences in the extent of glycosylation, and in minor components

resulting from proteolysis). Whereas consumption of the native form of the *Phaseolus* α–amylase inhibitor by mice did not result in immunological responses, consumption of transgenic peas expressing this protein led to the presence of circulating antibodies directed against it, and systemic immunological responses including inflammatory responses (i.e., allergic responses) to inhaled or injected protein [163]. In contrast to some earlier work claiming that consumption of transgenic plant material was harmful, this study has been published in a fully peer-reviewed journal and the quality of the research has not been disputed. Further research will be necessary to identify, and remove, the cause of this increased antigenicity. An additional potential drawback was revealed by feeding trials of transgenic peas expressing *Phaseolus* α–amylase inhibitor with pigs and chickens. These trials did not show immunological effects on animal health, but did show that starch utilization by the animals was significantly decreased due to the presence of the inhibitor in the transgenic peas when compared to non-transgenic peas, consistent with the effect of the protein on higher animal amylases [164, 165]. This factor would limit the utility of transgenic peas as animal feed.

Lectins

Lectins, or carbohydrate-binding proteins, occur throughout the plant kingdom, and in many species are accumulated in plant tissues as defensive proteins, being particularly abundant in seeds and other storage tissues, where they can account for up to 1% or more of total protein (reviewed by Peumans and van Damme and van Damme et al. [166, 167]). They are multimeric proteins containing polypeptides which range from 10 to 35 kDa in size. The insecticidal activity of lectins was first observed in assays with larvae of coleopteran species (e.g., LE QA done [168, 169]), where retardation of development, and in some cases, mortality, was observed when lectins were incorporated into diets at 1–5% of total protein. Lectins have only relatively low antimetabolic effects on lepidopteran larvae when fed in diet [170], possibly as a result of high gut pH inactivating the carbohydrate-binding activity. The mechanism of toxicity of these proteins remains obscure, but is dependent on carbohydrate binding.

Although transgenic tobacco and potato plants expressing lectins from garden pea [171] and snowdrop [172] have been produced by standard transformation techniques, and have been shown to confer partial resistance to lepidopteran larvae (>50% reductions in plant damage, with increased larval mortality and decreased growth), the availability of better insecticidal genes specific for these pests has directed this approach toward different targets. Homopteran plant pests, which are not affected by known *Bt* toxins, were shown to be susceptible to lectin toxicity when the proteins were delivered via artificial diet [173]. Susceptibility varied between species, and between lectins, but LC_{50} values as low as 6 μM have been estimated (for snowdrop lectin fed to rice brown planthopper (*Nilaparvata lugens*); [174]). Expression of the mannose-specific snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) in transgenic rice plants was carried out, using both a phloem-specific (rice sucrose synthase) and a constitutive (maize ubiquitin-1) promoter [175]. The resulting plants were partially resistant to rice brown planthopper, with reductions of up to 50% in survival of immature insects to adulthood, and reduced development and fertility of survivors. Results were confirmed by independent transformations of indica rice varieties [176]. GNA-expressing rice was also resistant to other homopteran plant pests, such as green leafhopper (*Nephotettix virescens*; [177]) and whitebacked planthopper (*Sogatella furcifera*; [178]). Plants expressing both GNA and Cry1Ac were protected against both brown planthopper and striped stem borers (*C. suppressalis*), but no synergistic effects between the two insecticidal proteins was observed [179]. Further progress on this research has been limited, due to concerns about possible adverse consequences to higher animals of ingesting snowdrop lectin. While earlier data must be regarded as unreliable, a recent study found that no adverse effects of consumption of transgenic rice expressing GNA by rats, although significant differences in some parameters to a control group were observed [180]. GNA expression has also been engineered into potato [181] and maize [182], to give partial resistance to peach–potato aphid (*Myzus persicae*) and corn leaf aphid (*Rhopalosiphum maidis*), respectively. However, these insects are insensitive to lectin toxicity, and only marginal effects on fecundity were observed.

Introduction of foreign lectin genes into plants has become established as a potential method for engineering insect resistance, although with the lectins tested at best only partial protection against homopteran pests is conferred, and some species are relatively insensitive to the effects of lectins. As is the case with PIs, it is likely that plant pests are preadapted to the presence of lectins as defensive compounds, and are able to tolerate the toxic effects to varying degrees, although responses induced in insects by ingested lectins have not been characterized. Attempts have been made to select lectins which are the most effective toxins against target insect pests; a mannose-specific lectin expressed specifically in garlic leaves (ASA-L) was observed to show a high level of toxicity toward homopteran pests [183]. A gene encoding this lectin has been engineered into a variety of transgenic plant species, including tobacco [184] and Indian mustard [185], in both cases producing partial resistance to aphid species, with reduced survival and fecundity. Expression of this lectin in transgenic rice using constitutive [186] or phloem-specific promoters [187] gave protection against homopteran pests comparable to, or slightly better than, earlier transformations using gene constructs encoding GNA. The transgenic rice plants expressing ASA-L were shown to decrease transmission of Rice Tungro Virus by its insect vector (green leafhopper), presumably by causing decreased feeding by the pest [188].

Despite these encouraging results, widescale adoption of transgenic crops expressing lectins will probably not occur unless a major commercial company is able to gain exclusive marketing rights, and invests in pushing the transgenic varieties through the regulatory process. This is unlikely to happen, as the technology is not readily protectable by patenting.

Oxidative Enzymes

Induction of polyphenol oxidase (PPO) synthesis is one of the end-results of the plant wounding response [189], and it would seem reasonable to suppose that increased levels of this enzyme would lead to enhanced resistance to insect attack. PPO activity leads to tissue browning, which has been correlated with enhanced insect resistance. The oxidative cross-linking of tannins to proteins catalyzed by PPO decreases protein digestibility, and limits nitrogen availability [190]. However, there is little or no evidence that PPO levels are correlated with insect resistance (e.g., [191]). High-level, constitutive over expression of a poplar PPO gene in transgenic poplar seedlings led to levels of PPO up to 50x higher than normal in plant tissues [192], but these plants had only marginal effects on larvae of the lepidopteran insect pest forest tent caterpillar (*Malacosoma disstria*). No feeding deterrence was observed, and there was no effect on larval growth or survival except under conditions where larval survival was poor on controls. PPO activity was detected in insect gut and frass, so the negative results were not due to enzyme inactivation. The conclusion that herbivorous insects are preadapted to be able to deal with PPO activity, as a result of exposure to the wounding response on an evolutionary timescale (in a similar manner to preadaptation to PIs – see above) is difficult to avoid.

Peroxidase activity is also induced when plants are stressed, or attacked by pathogens, as part of a lignification response, and several attempts have been made to over-express peroxidases in transgenic plants to enhance insect resistance, despite a lack of clear-cut evidence that peroxidase activity in plant tissues is toxic to insect herbivores. Initial results using tobacco as the host plant, with over-expression of tobacco anionic peroxidase, showed only marginal effects [193], although limited broad-range protection against a variety of pests was observed in the field [194]. The limited protection afforded by this technique argues against further development.

Other Plant Proteins

Ribosome inactivating proteins (RIPs) and chitinases have also been viewed as defensive proteins in plants, although it is not clear that they are part of defense against insect herbivores. Both types of proteins have been expressed in transgenic plants, with variable results in conferring insect resistance. Expression of a maize RIP in transgenic tobacco resulted in very low levels of protection against corn earworm (*H. zea*), which were barely statistically significant [195]. Plant chitinases in general show low toxicity toward insects, but a poplar chitinase, designated WIN6, was selected on the basis that its expression was induced by insect attack. Expression of WIN6 in trangenic tomato plants

led to partial protection against larvae of Colorado potato beetle, with retardation of larval development observed [196]. Expression of the chitin-degrading enzyme *N*-acetylhexosaminidase from *Arabidopsis* in various transgenic plant tissues also gave some protective effects against lepidopteran larvae [197], but it is difficult to see what advantages over other strategies this approach could give. Orally ingested insect chitinases are strongly toxic to lepidopteran larvae (e.g., [198]). However, expression in transgenic plants gave only partial protection against insect herbivores [199], or, in one case, increased susceptibility to attack [200]. Expression of chitinase A from baculovirus AcMNPV in transgenic tobacco gave similar results, with only small effects on lepidopteran larvae and aphids [201].

Engineering Secondary Metabolism for Plant Defense

Compounds synthesized as the end-products of secondary metabolism play major roles in both constitutive and induced defense against insect herbivores in many plant species (reviewed by Wittstock and Gershenon [202]). The idea that these compounds could be used as insecticides has been a part of agriculture for thousands of years, and has been exploited successfully by synthetic chemistry in the production of classes of insecticides such as pyrethroids, based on terpenoid esters produced by flowers of pyrethrums (Chrysanthemums). Although the concept of synthesizing a foreign, insecticidal secondary metabolite in a transgenic plant developed concurrently with plant transformation technology, the biosynthesis of most secondary compounds was poorly understood, and the necessity of cloning and introducing a series of genes expressing biosynthetic enzymes to produce a secondary metabolite was considered beyond the techniques available at the time. Anticipation of problems in ensuring controlled co-expression of a series of biosynthetic genes has proved to be over-pessimistic, and plants containing multiple expressing transgenes have been produced without difficulty.

The explosion of knowledge brought about by large-scale cDNA sequencing programs and the *Arabidopsis* genome program has resulted in a much better understanding of secondary metabolism, with many biosynthetic pathways now reasonably well understood, and clones encoding biosynthetic enzymes available. The first successful demonstration that a foreign secondary compound could confer insect resistance in a transgenic plant [203] exploited a biosynthetic pathway for cyanogenic glycosides. The cereal *Sorghum bicolor* produces a cyanogenic glycoside, dhurrin, by a biosynthetic pathway starting from the amino acid tyrosine, a product of primary metabolism. Two oxidation reactions catalyzed by cytochrome P450 oxidases generate *p*-hydroxymandelonitrile, which is then glycosylated by a UDP-glycosyltransferase to form dhurrin. The three sorghum enzymes responsible were cloned and assembled into expression constructs using constitutive (CaMV 35S) promoters [204], and *Arabidopsis* plants were successively transformed with a construct containing both P450 oxidase sequences, and the glycosyl transferase sequence. All the enzymes were localized correctly (to endoplasmic reticulum membranes) and functioned properly. Surprisingly, little disruption to endogenous metabolism was observed in the transgenic plants expressing medium levels of dhurrin, and accumulation of pathway intermediates was not observed. The implication is that the plastic nature of plant metabolism can accommodate and regulate activity in new biosynthetic pathways that are introduced. The resulting plants included individuals producing levels of dhurrin similar to sorghum plants in leaf tissue (up to 4 mg/g fresh weight) and produced hydrogen cyanide on tissue damage (due to the hydrolysis of dhurrin by an endogenous *Arabidopsis* enzyme). The dhurrin-expressing plants showed enhanced resistance to attack by the flea beetle *Phyllotreta nemorum*, a specialist feeder on crucifers; adult beetles avoided feeding on dhurrin-expressing leaves when offered a choice, and larvae under no-choice conditions either failed to initiate feeding, or on initiating feeding showed a significant level of mortality. These initial results clearly imply that production of high levels of dhurrin in transgenic *Arabidopsis* caused phenotypic abnormalities, but subsequent refining of the technology allowed accumulation of dhurrin at up to 4% dry weight in *Arabidopsis* tissues without deleterious effects on plant growth [205]; expression levels of the UPD-glycosyl transferase must be high enough to prevent accumulation of the *p*-hydroxymandelonitrile intermediate.

Although these results represent science of the highest quality, this method is of marginal usefulness for crop protection as it stands, due to the dhurrin end product being toxic to higher organisms, due to the production of hydrogen cyanide when it is hydrolyzed. Worse, many insect herbivores, particularly those which have a polyphagous feeding habit, can detoxify cyanide [206]. However, the feasibility of engineering secondary metabolism in crop plants has now been established. Expression of the cassava cyanogenic glycosides, linamarin and lotaustralin (derived from valine and isoleucine respectively), has also been achieved in *Arabidopsis* [207], and grape vine root cultures have been engineered to produce dhurrin [208], although in this case no protection against root aphids was observed. Other types of secondary metabolites have also been exploited; production of the alkaloid caffeine from its precursor xanthosine in tobacco was achieved by the introduction of three genes encoding *N*-methyl transferases [209]. The resulting plants contained up to 5 μg/g fresh weight caffeine in leaves, and showed a strong feeding deterrent effect toward a generalist lepidopteran herbivore, *Spodoptera litura*. An alternative approach to modifying secondary metabolism was taken by [210], who introduced a gene encoding β-glucosidase from *Aspergillus niger* into tobacco plants, and demonstrated that transgenic plants expressing the enzyme had insecticidal activity toward whiteflies (*Bemisia* spp.) and dipterans (flies), putatively due to hydrolysis of unidentified glycosides in the plant (although the greater density of secretory trichomes observed in transgenic plants may also have been significant). Further developments in this area can be expected.

Besides engineering, secondary metabolism to produce defensive compounds normally present in other plant species, the biosynthetic capacity of plants can be used to produce a variety of volatile secondary compounds used for communication. Better understanding of the terpenoid biosynthesis pathways has led to the production of a number of transgenic plants with altered volatile composition (reviewed by Aharoni et al. [211]). Suppression of expression of a cytochrome P450 oxidase gene expressed in trichomes by RNAi led to transgenic tobacco plants which deterred aphid colonization [212], due to the final step in production of the diterpenoid cembratriene-diol being blocked, resulting in accumulation of the precursor, cembratriene-ol. These compounds are both volatile and components of trichome secretions. Transgenic *Arabidopsis* plants constitutively over-expressing a dual linalool/nerolidol synthase in plastids produced significant amounts of linalool, both as a free alcohol (volatile) and as glycosylated derivatives, and were repellent to aphids (*M. persicae*) when tested in a choice experiment [213]. Modifications to isoprenoid synthesis in *Arabidopsis* have also been shown to attract predatory mites, which could protect plants by destroying pests [214]. This strategy of attracting natural enemies to pests has also been exemplified by transforming *Arabidopsis* with the maize terpene synthase gene TPS10, which is responsible for producing sesquiterpene volatiles emitted by maize. The resulting plants emitted the volatiles normally produced in maize and attracted parasitoid wasps which attack maize pests [215]. A different approach to utilizing terpene production in transgenic plants exploits the activity of the sesquiterpene (E)-β-farnesene as an alarm pheromone in aphids, which causes cessation of feeding and avoidance, as well as acting as an attractant for aphid predators and parasitoids [216]. *Arabidopsis* was transformed with an (E)-β-farnesene synthase gene from mint, under control of a constitutive promoter (CaMV 35S); resulting plants produced (E)-β-farnesene as a volatile. The transgenic plants showed significant levels of aphid deterrence in choice experiments, and were attractive to the aphid parasitoid *Diaeretiella rapae*. Experiments which engineer the volatiles emitted by plants are an exciting area of research at present, which has established the role that volatiles emitted by plants play in the interactions between plants, herbivores, and natural enemies at the tritrophic level. This technology has yet to show that it is a practical method for crop protection in the field, but practical applications look likely to follow.

Some Novel Approaches

Many other approaches to engineering insect resistance in transgenic plants have been proposed, and progressed to varying degrees. The following section gives an overview of some of the most promising of these approaches, which have been taken forward to the stage of demonstrating feasibility by producing insect-resistant plants.

Of necessity, many other interesting ideas have had to be omitted, such as transformation of plants with transcription factors which alter gene expression [217, 218], or the use of transgenic plants expressing potentially toxic proteins from insects [219] or insect peptide hormones [220]. Despite the lack of commercial deployment of any of the insect-resistant transgenic plant other than those expressing proteins derived from *Bt*, this field of research is active and new approaches will continue to be put forward and evaluated.

Photorhabdus luminescens Insecticidal Proteins

Photorhabdus luminescens is an enterobacterial symbiont of entomophagous (insecticidal) nematodes of *Heterorhabditis* species, used for small-scale biological control of insect pests. The bacteria are present in the nematode gut, and when nematodes enter an insect host, bacterial cells are released into the insect circulatory system. The bacterial cells release toxins which cause cell death, leading to a lethal septicemia, providing a substrate for both bacteria and nematodes to grow on [221, 222]. The toxins are present as high-molecular-weight (M_r approx. 10^6) complexes, which are toxic when injected or fed to insects from four major orders of agricultural pests. The complex has been separated into four components, encoded by genetic loci *tca*, *tcb*, *tcc*, and *tcd*; the products of *tca* and *tcd* are toxic individually when fed to lepidopteran larvae. The mechanism of action of the toxins remains unresolved. Subsequent investigation has shown that *Photorhabdus* contains a large number of potentially insecticidal components, some of which are only toxic by injection, whereas others are orally toxic (reviewed by ffrench-Constant [223]); a variety of mechanisms of toxicity, including promotion of apoptosis, seems to be exploited by the bacterium. This presence of a reservoir of redundant insecticidal activities, reminiscent of the situation in *Bacillus thuringiensis*, led to *Photorhabdus* being put forward as a successor to *Bt* as a source of insecticidal genes for expression in transgenic plants.

In order to be able to exploit insecticidal genes, investigators have sought to isolate single toxic proteins from *Photorhabdus*. Two proteins, designated toxin A and toxin B, were isolated from culture supernatant and shown to be orally toxic [224]. They exist as high-molecular-weight complexes (approx. 860 kDa) in solution, and each consist of two polypeptides, 201 and 63 kDa molecular weight. The mature polypeptides are produced from single precursor protoxin polypeptides of 283 kDa by proteolysis by endogenous bacterial proteinases. The 283 kDa protoxin A is the product of a gene designated *tcdA in Photorhabdus,* which has been cloned and assembled into expression constructs for use in transgenic plants. Expression levels of mRNA and protein were improved by adding 5′ and 3′ UTR sequences from a tobacco osmotin gene, but the coding sequence was not reengineered. Expression in transgenic *Arabidopsis* gave plants that contained intact protoxin, with a range of expression levels [225]; expression of toxin A at levels above 0.07% of total soluble protein in leaves gave almost complete protection against larvae of the lepidopteran tobacco hornworm (*M. sexta*). The toxin is not species specific, and leaf extracts were also toxic to the coleopteran corn rootworm (*Diabrotica undecimpunctata*). Commercial development of this technique is highly likely.

Entomophagous nematodes of *Steinernema* species also contain mutualistic bacteria, of *Xenorhabdus* species, which produce insecticidal toxins. These proteins could also be exploited to produce insect resistance in transgenic plants, but have not yet received as much attention as *Photorhabdus* toxins [223].

Cholesterol Oxidase

The identification of a protein from *Streptomyces* that was highly insecticidal to larvae of the coleopteran pest cotton boll weevil (*Anthonomus grandis*) resulted from a screening program assaying culture filtrates of different bacterial species [226]. The protein, which was toxic at levels comparable to a *Bt* three-domain Cry protein, was identified as a cholesterol oxidase. It was able to lyse the midgut epithelium in the insect. The mechanism of action involves the activity of the enzyme, since no activity is seen in lepidopteran larvae where the gut pH is high, and the enzyme has low activity, but may also involve effects on membrane-bound alkaline phosphatase [227]. Oxidation of membrane sterols such as cholesterol in the insect gut epithelium can destabilize membranes, leading to cell lysis as observed. However, expression of this protein in transgenic plants could prove problematic, since

it is equally capable of oxidizing sterols in plant cell membranes. The encoding gene for the cholesterol oxidase was isolated, and assembled into expression constructs containing either the complete coding sequence, the mature protein coding sequence, or the coding sequence fused to a chloroplast targeting peptide from the *Arabidopsis ribulose* bisphosphate carboxylase (RuBisCO) small subunit gene [228]. No codon optimization was carried out. Transgenic tobacco plants were produced by transformation of the nuclear genome, and all constructs were shown to result in synthesis and accumulation of active enzyme. The constructs which omitted the chloroplast targeting peptide caused protein to accumulate in the cytoplasm, and these plants were developmentally abnormal, possibly as a result of interference with plant sterol hormone signaling pathways. Plants in which the enzyme was localized in chloroplasts were phenotypically normal. Leaf tissue from all transgenic plants was toxic to boll weevil larvae when fed as a component of an artificial diet.

This work does not seem to have been progressed beyond the stage of a demonstration of concept, and no further references to it are present in the scientific literature. This gene would seem a good candidate for introduction into the chloroplast genome to engineer insect resistance, although potential effects on chloroplast membrane systems would remain a drawback.

Avidin as an Insecticidal Protein

Exploitation of the biotin-binding properties of the avian egg white protein avidin (and its bacterial functional homologue, streptavidin) in a variety of biochemical techniques has obscured its role as a defensive protein, which is toxic to bacteria. The antibacterial activity is based on its essentially irreversible binding of biotin, leading to this essential enzyme cofactor being unavailable. The insecticidal activity of avidin was recognized in 1993, when assays carried out in artificial diet showed toxicity to coleopteran and lepidopteran larvae at levels as low as 10 ppm in diet (estimated as of the order of 0.01% of total protein), although the level necessary to show toxicity was up to 100x higher for other pest species. The toxic effect was eliminated by addition of biotin to diets, suggesting that the mechanism of avidin insecticidal activity is also through biotin sequestration. Both growth reduction and mortality were observed, and the suggestion was made that gene constructs expressing avidin could provide protection against insect pests in transgenic plants [229]. Subsequent assays confirmed that susceptibility to avidin as an insecticide varies widely between different insect species, and that biotin carried over in the egg between generations had a significant effect on subsequent avidin toxicity [230].

Initial reports of expression of avidin in transgenic maize were focused on producing the protein as a high-value product [231]. An expression construct containing a codon-optimized avidin coding sequence with an N-terminally fused signal peptide from barley α-amylase, driven by the maize ubiquitin-1 promoter, resulted in expression levels of avidin of >2.0% of total protein in seed. Seed from these plants was subsequently bioassayed for resistance to larvae of three different coleopteran storage pests, including red flour beetle (*Tribolium castaneum*), with 100% mortality at avidin levels above 100 ppm of seed (approx. 0.1% of total protein). However, not all pests were as susceptible; larvae of the larger grain borer, *Prostephanus truncatus*, were effectively insensitive to avidin, whether added to artificial diet or expressed in transgenic plant material. The engineered maize was nontoxic to mice over 21 days [232]. Subsequent reports confirmed the insecticidal effects of avidin expressed in transgenic plants: these include protection of tobacco against noctuid lepidopterans [233], using vacuolar targeting sequences from potato proteinase inhibitors to direct avidin accumulation in the vacuole at levels up to 1.5% of total leaf protein [234]; protection of apple against lepidopteran pests [235]; and protection of rice against coleopteran stored grain pests, using a similar approach to that used for maize [236]. Targeting of the foreign protein to vacuolar or similar compartments is important; expression of streptavidin in tomato using plant and bacterial signal peptides and strong promoters led to developmental abnormalities in the plants, which could be corrected by topical application of biotin, suggesting that sequestration of cellular biotin is equally detrimental for plants as well as insects [237].

Despite many promising results, this technology appears to have failed to gain any acceptance for agricultural crops, as illustrated by a recent study in which seed meal from transgenic avidin-expressing maize was tested as an insecticide for topical application to stored maize [238]. Studies have shown that avidin can increase the protection afforded by *Bt* expression in transgenic plants against insect pests which have limited susceptibility to the toxin (e.g., potato expressing Cry3A; [239]), but it is clear that little further development in this area is taking place.

RNA Interference Using Double-Stranded RNA

Downregulation of gene expression by double-stranded RNA (dsRNA) corresponding to part or all of a specific gene transcript has been used as a research technique in insect genetics since 1998. The method has been based on delivery of synthetic dsRNA produced in vitro by injection into insect cells or tissues, which is clearly not practical for applications in crop protection. However, recent results have shown that dsRNA can be introduced into insects as a component of artificial diet, and is effective in downregulating genes normally expressed in gut tissue. This technique has been used to downregulate the production of a gut carboxylesterase in larvae of the lepidopteran *Epiphyas postvittana* (light brown apple moth; [240]), leading to suppression of mRNA in the insect. More significantly, two recent papers show that dsRNA can be delivered to insect pests by expression in plant material, and that this can lead to an insecticidal effect when pests are exposed to plants. Transgenic tobacco and *Arabidopsis* plant material expressing dsRNA directed against a cotton bollworm detoxification enzyme (cytochrome P450 gene CYP6AE14) for gossypol suppressed expression of the gene, and caused the insect to become more sensitive to gossypol in the diet, leading to reduced performance compared to controls [241]. A similar technique was used to suppress expression of a V-type ATPase in larvae of the coleopteran *Diabrotica virgifera virgifera* (Western corn rootworm); transgenic corn plants producing dsRNA directed against this gene showed protection against feeding damage by the insect [242]. The feasibility of using dsRNA in crop protection strategies has thus been demonstrated. This approach holds great promise for future development, as it allows a wide range of potential targets for suppression of gene expression in the insect to be exploited.

Insect-Resistant Genetically Engineered Crops and Sustainability

The success of Bt-expressing crops in the field has been a direct result of taking "sustainability" into account in their introduction, particularly with respect to managing the emergence of pest resistance to the toxins through the refuge strategy, as described earlier. Even organizations hostile to Genetic Engineering technology, such as organic growers in the USA, have reported that *Bt* cotton and corn have reduced insecticide usage significantly (by up to 0.2 kg/ha/year), showing that these crops are compatible with the goals of "sustainable" agriculture [243].

The "sustainability" of transgenic insect-resistant crops has also been examined in terms of potential effects on the wider ecosystem in which the plants are grown. Numerous studies have been carried out to effects on predators and parasites at the third trophic level, and on nontarget insects and other invertebrates. Some initial reports which did report negative effects were based on dubious assumptions, or used experimental designs which had little relevance to field conditions (e.g., the supposed threat to monarch butterflies posed by transgenic *Bt* corn; reviewed by Gatehouse et al. [244]). Nevertheless, it must be the case that if a pest population is decreased as a result of endogenous resistance in crops, then there will be a "knock on" effect to the wider ecosystem, and particularly to predators and parasites of the pest species, when the resistant crop is compared to a nonresistant one that is not treated with pesticide. However, this is not a realistic comparison, since in agricultural practice a crop that does not have endogenous resistance is treated with exogenous insecticides. The use of the refuge strategy allows significant pest populations to be present, and thus can support both beneficial insects which attack the pest, and a wider ecosystem, which would be destroyed by exogenous insecticide application.

Looking to the future, wider use of insect-resistant transgenic crops could contribute positively to "sustainability" in agriculture in general, by further decreasing

insecticide usage and thereby decreasing energy inputs. However, the "sustainability" of the insect-resistant crops themselves is going to come under increasing pressure, as less controlled deployment of insect-resistant plants evades the present compulsory use of the refuge strategy, and use of crop varieties with multiple *Bt* toxins renders the refuge strategy apparently less necessary to prevent pest resistance to *Bt* toxins developing. Field resistance to *Bt* crops has been observed recently (reviewed by Tabashnik et al. [245]), but is manageable using existing practices, or modifications of them. The sustainability of relying on one mechanism of crop protection can be questioned, especially as plants in general have evolved mixed defense strategies [246]. In the longer term, a wider range of strategies for producing insect-resistant plants is going to be necessary, not only to deal with the potential for nonspecific resistance to *Bt* toxins, but to extend the range of crop pests that can be targeted, and further reduce the application of pesticides.

Future Directions

After 20 years, insect-resistant transgenic crops have been a greater success in some ways than the early experiments suggested, but have failed to meet all the hopes that were initially raised. The success is self-evident when the widescale adoption of the technology in certain crops such as cotton and maize is considered, and documented evidence of reductions in damage to human health and the environment as a result of decreases in the use of exogenously applied pesticides. The failure does not lie in any technical shortcomings in the science, although improvements and new strategies are always possible; it lies in a failure to disseminate the technology as widely as should have been the case, so that it remains largely in the hands of commercial organizations, and is limited to the major crops. Is it an unrealistic hope to anticipate that after another 20 years, amateur gardeners in developed countries will be able to choose to buy seed to grow genetically engineered cabbages, which will be resistant to cabbage white butterfly larvae, in their allotments and gardens? Or that rural farmers in developing countries will have free access to engineered rice varieties, suitable for their growth conditions, that are resistant to pests such as stemborers? Both these aims have been scientifically achievable for at least the last 10 years, and it is surely about time that a more rational approach, which cuts through both the largely futile debate about the rights and wrongs of plant genetic engineering, and the protectionism of agrochemical companies, was taken to address the looming problem of producing enough crops to meet humanity's needs.

Bibliography

Primary Literature

1. Barton KA, Whitely HR, Yang N-S (1987) *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to Lepidopteran insects. Plant Physiol 85: 1103–1109
2. Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SH, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. Bio/Technology 5:807–813
3. Vaeck M, Reynaerts A, Hofte H, Jansens S, De Beuckeleer M, Dean C, Zabeau M, Van Montagu M, Leemans J (1987) Transgenic plants protected from insect attack. Nature (London) 328:33–37
4. Toenniessen GH, O'Toole JC, DeVries J (2003) Advances in plant biotechnology and its adoption in developing countries. Curr Opin Plant Biol 6:191–198
5. Shelton AM, Zhao J-Z, Roush RT (2002) Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants. Annu Rev Entomol 47:845–881
6. Aronson AI, Shai Y (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. FEMS Microbiol Lett 195:1–8
7. de Maagd RA, Bravo A, Crickmore N (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet 17:193–199
8. Damgaard PH, Hansen BM, Pedersen JC, Eilenberg J (1997) Natural occurrence of *Bacillus thuringiensis* on cabbage foliage and in insects associated with cabbage crops. J Appl Microbiol 82:253–258
9. Bizzarri MF, Bishop AH (2007) Recovery of *Bacillus thuringiensis* in vegetative form from the phylloplane of clover (*Trifolium hybridum*) during a growing season. J Inverteb Pathol 94: 38–47
10. Bernhard K, Jarrett P, Meadows M, Butt J, Ellis DJ, Roberts GM, Pauli S, Rodgers P, Burges HD (1997) Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests. J Inverteb Pathol 70:59–68
11. Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62:807–813
12. Berry C, O'Neil S, Ben-Dov E, Jones AF, Murphy L, Quail MA, Holden MTG, Harris D, Zaritsky A, Parkhill J (2002) Complete

sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israeliensis*. Appl Environ Microbiol 68:5082–5095
13. Parker MW, Feil SC (2005) Pore-forming protein toxins: from structure to function. Prog Biophys Mol Biol 88:91–142
14. Li J, Carrol J, Ellar DJ (1991) Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature 353:815–821
15. Grochulski P, Masson L, Borisova S, Pusztai-Carey M, Schwartz JL, Brousseau R, Cygler M (1995) *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J Mol Biol 254:447–464
16. Morse RJ, Yamamoto T, Stroud RM (2001) Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure 9:409–417
17. Galitsky N, Cody V, Wojtczak A, Ghosh D, Luft JR, Pangborn W, English L (2001) Structure of the insecticidal bacterial δ-endotoxin CryBb1 of *Bacillus thuringiensis*. Acta Crystallogr D 57:1101–1109
18. Boonserm P, Mo M, Angsuthanasombat C, Lescar J (2006) Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-Angstrom resolution. J Bacteriol 188:3391–3401
19. Boonserm P, Davis P, Ellar DJ, Li J (2005) Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J Mol Biol 348:363–382
20. de Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu Rev Genet 37:409–433
21. Bravo A, Sánchez J, Kouskoura T, Crickmore N (2002) N-terminal activation is an essential early step in the mechanism of action of the *B. thuringiensis* Cry1Ac insecticidal toxin. J Biol Chem 277:23985–23987
22. Bravo A, Gill SS, Soberón M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49:423–435
23. Knight P, Crickmore N, Ellar DJ (1994) The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol Microbiol 11:429–436
24. Vadlamudi RK, Weber E, Ji I, Ji TH, Bulla LA Jr (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J Biol Chem 270:5490–5494
25. Valaitis AP, Jenkins JL, Lee MK, Dean DH, Garner KJ (2001) Isolation and partial characterization of Gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. Arch Insect Biochem Physiol 46:186–200
26. Jurat-Fuentes JL, Adang MJ (2004) Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur J Biochem 271: 3127–3135
27. Fernández LE, Aimanova KG, Gill SS, Bravo A, Soberón M (2006) A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochem J 394:77–84
28. Krishnamoorthy M, Jurat-Fuentes JL, McNall RJ, Andacht T, Adang MJ (2007) Identification of novel CryIAc binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. Insect Biochem Mol Biol 37:189–201
29. Gahan LJ, Gould F, Heckel DG (2001) Identification of a gene associated with *Bt* resistance in *Heliothis virescens*. Science 293:857–860
30. Yang YJ, Chen HY, Wu SW, Yang YH, Xu XJ, Wu YD (2006) Identification and molecular detection of a deletion mutation responsible for a truncated cadherin of *Helicoverpa armigera*. Insect Biochem Mol Biol 36:735–740
31. Xie R, Zhuang M, Ross LS, Gómez I, Oltean DI, Bravo A, Soberón M, Gill SS (2005) Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J Biol Chem 280:8416–8425
32. Tsuda Y, Nakatani F, Hashimoto K, Ikawa S, Matsuura C, Fukada T, Sugimoto K, Himeno M (2003) Cytotoxic activity of *Bacillus thuringiensis* Cry proteins on mammalian cells transfected with cadherin-like Cry receptor gene of *Bombyx mori* (silkworm). Biochem J 369:697–703
33. Soberón M, Pardo-López L, López I, Gómez I, Tabashnik BE, Bravo A (2007) Engineering modified *Bt* toxins to counter insect resistance. Science 318:1640–1642
34. Herrero S, Gechev T, Bakker PL, Moar WJ, de Maagd RA (2005) *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four aminopeptidase N genes. BMC Genomics 6:96
35. Rajagopal R, Sivakumar S, Agrawal N, Malhotra P, Bhatnagar RK (2002) Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J Biol Chem 277: 46849–46851
36. Sivakumar S, Rajagopal R, Venkatesh GR, Srivastava A, Bhatnagar RK (2007) Knockdown of aminopeptidase-N from *Helicoverpa armigera* larvae and in transfected Sf21 cells by RNA interference reveals its functional interaction with *Bacillus thuringiensis* insecticidal protein Cry1Ac. J Biol Chem 282:7312–7319
37. Gill M, Ellar D (2002) Transgenic *Drosophila* reveals a functional *in vivo* receptor for the *Bacillus thuringiensis* toxin Cry1Ac1. Insect Mol Biol 11:619–625
38. Burton SL, Ellar DJ, Li J, Derbyshire DJ (1999) *N*-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J Mol Biol 287:1011–1022
39. Knight PJK, Carroll J, Ellar DJ (2004) Analysis of glycan structures on the 120 kDa aminopeptidase N of *Manduca sexta* and their interactions with *Bacillus thuringiensis* CrylAc toxin. Insect Biochem Mol Biol 34:101–112
40. de Maagd RA, Bakker PL, Masson L, Adang MJ, Sangadala S, Stiekema W, Bosch D (1999) Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding

to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol Microbiol 31:463–471
41. Jenkins JL, Lee MK, Valaitis AP, Curtiss A, Dean DH (2000) Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. J Biol Chem 275: 14423–14431
42. Jimenez-Juarez N, Munoz-Garay C, Gomez I, Saab-Rincon G, Damian-Almazo JY, Gill SS, Soberon M, Bravo A (2007) *Bacillus thuringiensis* Cry1Ab mutants affecting oligomer formation are non-toxic to *Manduca sexta* larvae. J Biol Chem 282:21222–21229
43. Rausell C, García-Robles I, Sánchez J, Muñoz-Garay C, Martínez-Ramírez AC, Real MD, Bravo A (2004) Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* [Say]. Biochem Biophys Acta 1660:99–105
44. Gómez I, Sánchez J, Miranda R, Bravo A, Soberón M (2002) Cadherin-like receptor binding facilitates proteolytic cleavage of helix a-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. FEBS Lett 513:242–246
45. Chen J, Hua G, Jurat-Fuentes JL, Abdullah MA, Adang MJ (2007) Synergism of *Bacillus thuringiensis* toxins by a fragment of a toxin-binding cadherin. Proc Natl Acad Sci USA 104: 13901–13906
46. Parenti P, Morandi P, McGivan JD, Consonnic P, Leonardi G, Giordana B (1997) Properties of the aminopeptidase N from the silkworm midgut (*Bombyx mori*). Insect Biochem Mol Biol 27:397–403
47. Zhuang M, Oltean DI, Gómez I, Pullikuth AK, Soberón M, Bravo A, Gill SS (2002) *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. J Biol Chem 277:13863–13872
48. Bravo A, Gómez I, Conde J, Muñoz-Garay C, Sánchez J, Zhuang M, Gill SS, Soberón M (2004) Oligomerization triggers differential binding of a pore-forming toxin to a different receptor leading to efficient interaction with membrane microdomains. Biochem Biophys Acta 1667:38–46
49. Pigott CR, Ellar DJ (2007) Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol Mol Biol Rev 71:255–281
50. Sacchi VF, Wolfsberger MG (1996) Amino acid absorption. In: Lehane MJ, Billingsley PF (eds) Biology of the insect midgut. Chapman and Hall, London, pp 265–292
51. Zhang X, Candas M, Griko NB, Taussig R, Bulla LA Jr (2006) A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. Proc Natl Acad Sci USA 103:9897–9902
52. Moellenbeck DJ, Peters ML, Bing JW, Rouse JR, Higgins LS, Sims L, Nevshemal T, Marshall L, Ellis RT, Bystrak PG, Lang BA, Stewart JL, Kouba K, Sondag V, Gustafson V, Nour K, Xu DP, Swenson J, Zhang J, Czapla T, Schwab G, Jayne S, Stockhoff BA, Narva K, Schnepf HE, Stelman SJ, Poutre C, Koziel M, Duck N (2001) Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. Nat Biotechnol 19:668–672
53. Ellis RT, Stockhoff BA, Stamp L, Schnepf HE, Schwab GE, Knuth M, Russell J, Cardineau GA, Narva KE (2002) Novel *Bacillus thuringiensis* binary insecticidal crystal proteins active on western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Appl Environ Microbiol 68:1137–1145
54. Darboux I, Nielsen-LeRoux C, Charles JF, Pauron D (2001) The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: molecular cloning and expression. Insect Biochem Mol Biol 31:981–990
55. Charles JF, NielsenLeRoux C, Delecluse A (1996) *Bacillus sphaericus* toxins: molecular biology and mode of action. Annu Rev Entomol 41:451–472
56. Warren GW (1997) Vegetative insecticidal proteins: novel proteins for control of corn pests. In: Carozzi NB, Koziel MG (eds) Advances in insect control: the role of transgenic plants. Taylor & Francis, London, UK, pp 109–121
57. Barth H, Aktories K, Popoff MR, Stiles BG (2004) Binary bacterial toxins: Biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. Microbiol Mol Biol Rev 68:373–402
58. Leuber M, Orlik F, Schiffler B, Sickmann A, Benz R (2006) Vegetative insecticidal protein (Vip1Ac) of *Bacillus thuringiensis* HD201: Evidence for oligomer and channel formation. Biochemistry 45:283–288
59. Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG (1996) Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc Natl Acad Sci USA 93:5389–5394
60. Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ (1997) The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl Environ Microbiol 63:532–536
61. Lee MK, Miles P, Chen JS (2006) Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem Biophys Res Commun 339:1043–1047
62. Lee MK, Walters FS, Hart H, Palekar N, Chen JS (2003) Mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab delta-endotoxin. Appl Environ Microbiol 69:4648–4657
63. Fang J, Xu X, Wang P, Zhao J-Z, Shelton AM, Cheng J, Feng M-G, Shen Z (2007) Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl Environ Microbiol 73:956–961
64. Li J, Pandelakis AK, Ellar DJ (1996) Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J Mol Biol 257:129–152
65. Du J, Knowles BH, Li J, Ellar DJ (1999) Biochemical characterization of *Bacillus thuringiensis* cytolytic toxins in association with a phospholipid bilayer. Biochem J 338:185–193
66. Promdonkoy B, Ellar DJ (2003) Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from *Bacillus thuringiensis*. Biochem J 374:255–259

67. Koni PA, Ellar DJ (1994) Biochemical characterization of *Bacillus thuringiensis* cytolytic δ-endotoxins. Microbiology 140:1869–1880
68. Wirth MC, Georghiou GP, Federeci BA (1997) CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. Proc Natl Acad Sci USA 94:10536–10540
69. Pérez C, Fernández LE, Sun J, Folch JL, Gill SS, Soberón M, Bravo A (2005) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proc Natl Acad Sci USA 102:18303–18308
70. Mazier M, Pannetier C, Tourneur J, Jouanin L, Giband M (1997) The expression of *Bacillus thuringiensis* toxin genes in plant cells. Biotechnol Annu Rev 3:313–347
71. Zheng SJ, Henken B, de Maagd RA, Purwito A, Krens FA, Kik C (2005) Two different *Bacillus thuringiensis* toxin genes confer resistance to beet armyworm (*Spodoptera exigua* Hubner) in transgenic *Bt*-shallots (*Allium cepa* L.). Transgenic Res 14: 261–272
72. Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified δ–endotoxin gene of *Bacillus thuringiensis*. Bio/Technology 11:194–200
73. Wunn J, Kloti A, Burkhardt PK, Biswas GCG, Launis K, Iglesias VA, Potrykus I (1996) Transgenic Indica rice breeding line IR58 expressing a synthetic cryIA(b) gene from *Bacillus thuringiensis* provides effective insect pest control. Bio/Technology 14:171–176
74. Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997) Transgenic elite indica rice plants expressing CryIAc delta-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). Proc Natl Acad Sci USA 94:2111–2116
75. Vaughn T, Cavato T, Brar G, Coombe T, DeGooyer T, Ford S, Groth M, Howe A, Johnson S, Kolacz K, Pilcher C, Purcell J, Romano C, English L, Pershing J (2005) A method of controlling corn rootworm feeding using a *Bacillus thuringiensis* protein expressed in transgenic maize. Crop Sci 45:931–938
76. Breitler JC, Cordero MJ, Royer M, Meynard D, San Segundo B, Guiderdoni E (2001) The-689/+197 region of the maize protease inhibitor gene directs high level, wound-inducible expression of the *cry1B* gene which protects transgenic rice plants from stemborer attack. Mol Breed 7:259–274
77. Breitler JC, Vassal JM, Catala MD, Meynard D, Marfa V, Mele E, Royer M, Murillo I, San Segundo B, Guiderdoni E, Messeguer J (2004) Bt rice harbouring *Cry* genes controlled by a constitutive or wound-inducible promoter: protection and transgene expression under Mediterranean field conditions. Plant Biotechnol J 2:417–430
78. Miklos JA, Alibhai MF, Bledig SA, Connor-Ward DC, Gao A-G, Holmes BA, Kolacz KH, Kabuye VT, MacRae TC, Paradise MS, Toedebusch AS, Harrison LA (2007) Characterization of soybean exhibiting high expression of a synthetic *Bacillus thuringiensis cry1A* transgene that confers a high degree of resistance to lepidopteran pests. Crop Sci 47:148–157
79. Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect controlling protein genes. Proc Natl Acad Sci USA 88:3324–3328
80. De Rocher EJ, Vargo-Gogola TC, Diehn SH, Green PJ (1998) Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol 117:1445–1461
81. Murray EE, Rocheleau T, Eberle M, Stock C, Sekar V, Adang M (1991) Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. Plant Mol Biol 16: 1035–1050
82. Diehn PJ, Chiu SH, De Rocher WL, Green EJ (1998) Premature polyadenylation at multiple sites within a *Bacillus thuringiensis* toxin gene-coding region. Plant Physiol 117:1433–1443
83. Misztal LH, Mostowska A, Skibinska M, Bajsa J, Musial WG, Jarmolowski A (2004) Expression of modified Cry1Ac gene of *Bacillus thuringiensis* in transgenic tobacco plants. Mol Biotechnol 26:17–26
84. McBride KE, Svab Z, Schael DJ, Hogan PS, Stalker KM, Maliga P (1995) Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Bio/Technology 13:362–365
85. Bock R (2001) Transgenic plastids in basic research and plant biotechnology. J Mol Biol 312:425–438
86. Maliga P (2003) Progress towards commercialization of plastid transformation technology. Trends Biotechnol 21:20–28
87. Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci 7:84–91
88. Chakrabarti SK, Lutz KA, Lertwiriyawong B, Svab Z, Maliga P (2006) Expression of the *cry9Aa2 B.t.* gene in tobacco chloroplasts confers resistance to potato tuber moth. Transgenic Res 15:481–488
89. De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the *Bt* cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19:71–74
90. Kota M, Daniell H, Varma S, Garczynski SF, Gould F, Moar WJ (1999) Overexpression of the *Bacillus thuringiensis* (*Bt*) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and *Bt*-resistant insects. Proc Natl Acad Sci USA 96:1840–1845
91. Reddy VS, Leelavathi S, Selvapandiyan A, Raman R, Giovanni F, Shukla V, Bhatnagar RK (2002) Analysis of chloroplast transformed tobacco plants with *cry1Ia5* under rice *psbA* transcriptional elements reveal high level expression of *Bt* toxin without imposing yield penalty and stable inheritance of transplastome. Mol Breed 9:259–269
92. Dufourmantel N, Tissot G, Goutorbe F, Garcon F, Muhr C, Jansens S, Pelissier B, Peltier G, Dubald M (2005) Generation and analysis of soybean plastid transformants expressing *Bacillus thuringiensis* Cry1Ab protoxin. Plant Mol Biol 58:659–668

93. Chrispeels MJ, Sadava DE (1994) Plants, genes and agriculture. Jones and Bartlett, London
94. Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726
95. Gould F, Anderson A, Jones A, Sumerford D, Heckel DG, Lopez J, Micinski S, Leonard R, Laster M (1997) Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. Proc Natl Acad Sci USA 94:3519–3523
96. Andreadis SS, Álvarez-Alfagene A, Sánchez-Ramos I, Stodola TJ, Andow DA, Milonas PG, Savopoulou-Soultani M, Castánera P (2007) Frequency of resistance to *Bacillus thuringiensis* toxin Cry1Ab in Greek and Spanish population of *Sesamia nonagrioides* (Lepidoptera: Noctuidae). J Econ Entomol 100:195–201
97. Tabashnik BE, Patin AL, Dennehy TJ, Liu Y-B, Carrière Y, Sims M, Antilla L (2000) Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc Natl Acad Sci USA 97:12980–12984
98. Tabashnik BE, Dennehy TJ, Carrière Y (2005) Delayed resistance to transgenic cotton in pink bollworm. Proc Natl Acad Sci USA 102:15389–15393
99. Cristofoletti PT, de Sousa FAM, Rahbe Y, Terra WR (2006) Characterization of a membrane-bound aminopeptidase purified from *Acyrthosiphon pisum* midgut cells. FEBS J 273:5574–5588
100. Stewart SD, Adamczyk JJ, Knighten KS, Davis FM (2001) Impact of *Bt* cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of noctuid (Lepidoptera) larvae. J Econ Entomol 94:752–760
101. Chitkowski RL, Turnipseed SG, Sullivan MJ, Bridges WC (2003) Field and laboratory evaluations of transgenic cottons expressing one or two *Bacillus thuringiensis* var. *kurstaki* Berliner proteins for management of noctuid (Lepidoptera) pests. J Econ Entomol 96:755–762
102. Zhao JZ, Cao J, Li YX, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21:1493–1497
103. Christou P, Capell T, Kohli A, Gatehouse JA, Gatehouse AMR (2006) Recent developments and future prospects in insect pest control in transgenic crops. Trends Plant Sci 11:302–308
104. Gahan LJ, Ma YT, Coble MLM, Gould F, Moar WJ, Heckel DG (2005) Genetic basis of resistance to Cry1Ac and Cry2Aa in *Heliothis virescens* (Lepidoptera: Noctuidae). J Econ Entomol 98:1357–1368
105. Bashir K, Husnain T, Fatima T, Riaz N, Makhdoom R, Riazuddin S (2005) Novel indica basmati line (B-370) expressing two unrelated genes of *Bacillus thuringiensis* is highly resistant to two lepidopteran insects in the field. Crop Prot 24:870–879
106. Dively GP (2005) Impact of transgenic VIP3A x Cry1Ab lepidopteran-resistant field corn on the nontarget arthropod community. Environ Entomol 34:1267–1291
107. Han LZ, Wu KM, Peng YF, Wang F, Guo YY (2006) Evaluation of transgenic rice expressing Cry1Ac and CpTI against *Chilo suppressalis* and intrapopulation variation in susceptibility to Cry1Ac. Environ Entomol 35:1453–1459
108. Grainnet (2007) Monsanto and Dow Agrosciences launch "SmartStax," industry's first-ever eight-gene stacked combination in corn. http://www.grainnet.com/articles/Monsanto_and_Dow_Agrosciences_Launch_SmartStax_Industry_s_First_Ever_Eight_Gene_Stacked_Combination_in_Corn_-48374.html
109. Sanchis V, Agaisse H, Chaufaux J, Lereclus D (1996) Construction of new insecticidal *Bacillus thuringiensis* recombinant strains by using the sporulation non-dependent expression system of *cry*IIIA and a site specific recombination vector. J Biotechnol 48:81–96
110. Bosch D, Schipper B, van der Kleij H, de Maagd R, Stiekema W (1994) Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. Bio/Technology 12:915–919
111. de Maagd RA, Kwa MSG, van der Klei H, Yamamoto T, Schipper B, Vlak JM, Stiekema WJ, Bosch D (1996) Domain III substitution in *Bacillus thuringiensis* delta-endotoxin Cry1A(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl Environ Microbiol 62:1537–1543
112. de Maagd RA, Weemen-Hendriks M, Stiekema W, Bosch D (2000) *Bacillus thuringiensis* delta-endotoxin Cry1C domain III can function as a specificity determinant for *Spodoptera exigua* in different, but not all, Cry1-Cry1C hybrids. Appl Environ Microbiol 66:1559–1563
113. Rang C, Vachon V, Coux F, Carret C, Moar WJ, Brousseau R, Schwartz JL, Laprade R, Frutos R (2001) Exchange of domain I from *Bacillus thuringiensis* Cry1 toxins influences protoxin stability and crystal formation. Curr Microbiol 43:1–6
114. Naimov S, Dukiandjiev S, de Maagd RA (2003) A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato. Plant Biotechnol J 1:51–57
115. Dean DH, Rajamohan F, Lee MK, Wu SJ, Chen XJ, Alcantara E, Hussain SR (1996) Probing the mechanism of action of *Bacillus thuringiensis* insecticidal proteins by site-directed mutagenesis: a minireview. Gene 179:111–117
116. Rajamohan F, Alzate O, Cotrill JA, Curtiss A, Dean DH (1996) Protein engineering of *Bacillus thuringiensis* delta-endotoxin: mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. Proc Natl Acad Sci USA 93:14338–14343
117. Wu SJ, Koller CN, Miller DL, Bauer LS, Dean DH (2000) Enhanced toxicity of *Bacillus thuringiensis* Cry3A delta-endotoxin in coleopterans by mutagenesis in a receptor binding loop. FEBS Lett 473:227–232
118. Abdullah MAF, Alzate O, Mohammad M, McNall RJ, Adang MJ, Dean DH (2003) Introduction of Culex toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. Appl Environ Microbiol 69:5343–5353

119. Tuntitippawan T, Boonserm P, Katzenmeier G, Angsuthanasombat C (2005) Targeted mutagenesis of loop residues in the receptor-binding domain of the *Bacillus thuringiensis* Cry4Ba toxin affects larvicidal activity. FEMS Microbiol Lett 242:325–332
120. Abdullah MAF, Dean DH (2004) Enhancement of Cry19Aa mosquitocidal activity against *Aedes aegypti* by mutations in the putative loop regions of domain II. Appl Environ Microbiol 70:3769–3771
121. Liu XS, Dean DH (2006) Redesigning *Bacillus thuringiensis* Cry1Aa toxin into a mosquito toxin. Protein Eng Des Sel 19:107–111
122. Ishikawa H, Hoshino Y, Motoki Y, Kawahara T, Kitajima M, Kitami M, Watanabe A, Bravo A, Soberon M, Honda A, Yaoi K, Sato R (2007) A system for the directed evolution of the insecticidal protein from *Bacillus thuringiensis*. Mol Biotechnol 36:90–101
123. Chandra A, Ghosh P, Mandaokar AD, Bera AK, Sharma RP, Das S, Kumar PA (1999) Amino acid substitution in alpha-helix 7 of Cry1Ac delta-endotoxin of *Bacillus thuringiensis* leads to enhanced toxicity to *Helicoverpa armigera* Hubner. FEBS Lett 458:175–179
124. Rupar MJ, Donovan WP, Groat RG, Slaney AC, Mattison JW, Johnson TB, Charles JF, Dumanior VC, DeBarjac H (1991) Two novel strains of *Bacillus thuringiensis* toxic to coleopterans. Appl Environ Microbiol 57:3337–3344
125. Bohorova N, Frutos R, Royer M, Estanol P, Pacheco M, Rascon Q, McLean S, Hoisington D (2001) Novel synthetic *Bacillus thuringiensis* cry1B gene and the cry1B-cry1Ab translational fusion confer resistance to southwestern corn borer, sugarcane borer and fall armyworm in transgenic tropical maize. Theor Appl Genet 103:817–826
126. Ho NH, Baisakh N, Oliva N, Datta K, Frutos R, Datta SK (2006) Translational fusion hybrid Bt genes confer resistance against yellow stem borer in transgenic elite vietnamese rice (*Oryza sativa* L.) cultivars. Crop Sci 46:781–789
127. Mehlo L, Gahakwa D, Nghia PT, Loc NT, Capell T, Gatehouse JA, Gatehouse AMR, Christou P (2005) An alternative strategy for sustainable pest resistance in genetically enhanced crops. Proc Natl Acad Sci USA 102:7812–7816
128. Gatehouse JA (2002) Plant resistance towards insect herbivores: a dynamic interaction. New Phytol 156:145–169
129. Barbosa JARG, Silva LP, Teles RCL, Esteves GF, Azevedo RB, Ventura MM, Freitas SM (2007) Crystal Structure of the Bowman-Birk inhibitor from *Vigna unguiculata* seeds in complex with beta-trypsin at 1.55 Å resolution and its structural properties in association with proteinases. Biophys J 92: 1638–1650
130. Garcia-Olmedo F, Salmedo G, Sanchez-Monge R, Gomez L, Royo J, Carbonero P (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. Oxf Surv Plant Mol Cell Biol 4: 275–334
131. Ryan CA (1990) Protease inhibitors in plants – genes for improving defenses against insects and pathogens. Annu Rev Phytopathol 28:425–449
132. Orozco-Cardenas M, McGurl B, Ryan CA (1993) Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. Proc Natl Acad Sci USA 90:8273–8276
133. Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annu Rev Plant Biol 53:299–328
134. Hilder VA, Gatehouse AMR, Sheerman SE, Barker RF, Boulter D (1987) A novel mechanism of insect resistance engineered into tobacco. Nature 330:160–163
135. Johnson R, Narvaez J, An G, Ryan C (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. Proc Natl Acad Sci USA 86:9871–9875
136. McManus MT, White DWR, McGregor PG (1994) Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. Transgenic Res 3:50–58
137. Duan X, Li X, Xue Q, Abo-El-Saad M, Xu D, Wu R (1996) Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. Nat Biotechnol 14:494–496
138. Xu DP, Xue QZ, McElroy D, Mawal Y, Hilder VA, Wu R (1996) Constitutive expression of a cowpea trypsin-inhibitor gene, CpTI, in transgenic rice plants confers resistance to 2 major rice insect pests. Mol Breed 2:167–173
139. McGurl B, Orozco-Cardenas M, Pearce G, Ryan CA (1994) Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. Proc Natl Acad Sci USA 91:9799–9802
140. Ren F, Lu Y-T (2006) Overexpression of tobacco hydroxyproline-rich glycopeptide systemin precursor A gene in transgenic tobacco enhances resistance against *Helicoverpa armigera* larvae. Plant Sci 171:286–292
141. Bolter CJ, Jongsma MA (1995) Colorado potato beetles (*Leptinotarsa decemlineata*) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. J Insect Physiol 41:1071–1078
142. Jongsma MA, Bakker PL, Peters J, Bosch D, Stiekma WJ (1995) Adaptations of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proc Natl Acad Sci USA 92:8041–8045
143. Harsulkar AM, Giri AP, Patankar AG, Gupta VS, Sainani MN, Ranjekar PK, Deshpande VV (1999) Successive use of non-host plant proteinase inhibitors required for effective inhibition of *Helicoverpa armigera* gut proteinases and larval growth. Plant Physiol 121:497–506
144. Bown DP, Wilkinson HS, Gatehouse JA (1997) Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochem Mol Biol 27:625–638
145. De Leo F, Bonade-Bottino MA, Ceci LR, Gallerani R, Jouanin L (1998) Opposite effects on *Spodoptera littoralis* larvae of high

expression level of a trypsin proteinase inhibitor in transgenic plants. Plant Physiol 118:997–1004
146. Leplé JC, Bonade-Bottino M, Augustin S, Pilate G, Letan VD, Delplanque A, Cornu D, Jouanin L (1995) Toxicity to *Chrysomela tremulae* (Coleoptera, Chrysomelidae) of transgenic poplars expressing a cysteine proteinase inhibitor. Mol Breed 1:319–328
147. Lecardonnel A, Chauvin L, Jouanin L, Beaujean A, Prevost G, Sangwan-Norreel B (1999) Effects of rice cystatin I expression in transgenic potato on Colorado potato beetle larvae. Plant Sci 140:71–79
148. Outchkourov NS, de Kogel WJ, Schuurman-de Bruin A, Abrahamson M, Jongsma MA (2004) Specific cysteine protease inhibitors act as deterrents of western flower thrips, *Frankliniella occidentalis* (Pergande), in transgenic potato. Plant Biotechnol J 2:439–448
149. Outchkourov NS, de Kogel WJ, Wiegers GL, Abrahamson M, Jongsma MA (2004) Engineered multidomain cysteine protease inhibitors yield resistance against western flower thrips (*Franklinielia occidentalis*) in greenhouse trials. Plant Biotechnol J 2:449–458
150. Outchkourov NS, Rogelj B, Strukelj B, Jongsma MA (2003) Expression of sea anemone equistatin in potato: effects of plant proteases on heterologous protein production. Plant Physiol 133:379–390
151. Abdeen A, Virgos A, Olivella E, Villanueva J, Aviles X, Gabarra R, Prat S (2005) Multiple insect resistence in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. Plant Mol Biol 57:189–202
152. Franco OL, Rigden DJ, Melo FR, Grossi-de-Sa MF (2002) Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases - structure, function and potential for crop protection. Eur J Biochem 269:397–412
153. Suzuki K, Ishimoto M, Kikuchi F, Kitamura K (1993) Growth-inhibitory effect of an alpha-amylase inhibitor from the wild common bean resistant to the mexican bean weevil (*Zabrotes subfasciatus*). Jpn J Breed 43:257–265
154. Ishimoto M, Kitamura K (1991) Effect of absence of seed alpha-amylase inhibitor on the growth inhibitory activity to azuki bean weevil (*Callosobruchus chinensis*) in common bean (*Phaseolus vulgaris* L.). Jpn J Breed 41:231–240
155. Moreno J, Chrispeels MJ (1989) A lectin gene encodes the $\alpha-$amylase inhibitor of common bean. Proc Natl Acad Sci USA 86:7885–7889
156. Nahoum V, Farisei F, Le-Berre-Anton V, Egloff MP, Rouge P, Poerio E, Payan F (1999) A plant-seed inhibitor of two classes of alpha-amylases: X-ray analysis of *Tenebrio molitor* larvae alpha-amylase in complex with the bean *Phaseolus vulgaris* inhibitor. Acta Crystallogr Sect D 55:360–362
157. Silva CP, Terra WR, de Sa MFG, Samuels RI, Isejima EM, Bifano TD, Almeida JS (2001) Induction of digestive alpha-amylases in larvae of *Zabrotes subfasciatus* (Coleoptera: Bruchidae) in response to ingestion of common bean alpha-amylase inhibitor 1. J Insect Physiol 47:1283–1290
158. Shade RE, Schroeder HE, Pueyo JJ, Tabe LM, Murdock LL, Higgins TJV, Chrispeels MJ (1994) Transgenic pea seeds expressing the alpha-amylase inhibitor of the common bean are resistant to bruchid beetles. Bio/Technology 12: 793–796
159. Schroeder HE, Gollasch S, Moore A, Tabe LM, Craig S, Hardie DC, Chrispeels MJ, Spencer D, Higgins TJV (1995) Bean alpha-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L). Plant Physiol 107:1233–1239
160. Ishimoto M, Sato T, Chrispeels MJ, Kitamura K (1996) Bruchid resistance of transgenic azuki bean expressing seed alpha-amylase inhibitor of common bean. Entomol Exp Appl 79: 309–315
161. Morton RL, Schroeder HE, Bateman KS, Chrispeels MJ, Armstrong E, Higgins TJV (2000) Bean alpha-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. Proc Natl Acad Sci USA 97:3820–3825
162. Sarmah BK, Moore A, Tate W, Molvig L, Morton RL, Rees DP, Chiaiese P, Chrispeels MJ, Tabe LM, Higgins TJV (2004) Transgenic chickpea seeds expressing high levels of a bean alpha-amylase inhibitor. Mol Breed 14:73–82
163. Prescott VE, Campbell PM, Moore A, Mattes J, Rothenberg ME, Foster PS, Higgins TJV, Hogan SP (2005) Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. J Agric Food Chem 53: 9023–9030
164. Collins CL, Eason PJ, Dunshea FR, Higgins TJV, King RH (2006) Starch but not protein digestibility is altered in pigs fed transgenic peas containing alpha-amylase inhibitor. J Sci Food Agric 86:1894–1899
165. Li XH, Higgins TJV, Bryden WL (2006) Biological response of broiler chickens fed peas (*Pisum sativum* L.) expressing the bean (*Phaseolus vulgaris* L.) alpha-amylase inhibitor transgene. J Sci Food Agric 86:1900–1907
166. Peumans WJ, van Damme EJM (1995) Lectins as plant defence proteins. Plant Physiol 109:347–352
167. van Damme EJM, Peumans WJ, Barre A, Rougé P (1998) Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Sci 17:575–692
168. Czalpa TH, Lang BA (1990) Effect of plant lectins on the larval development of European corn borer (Lepidoptera: Pyralidae) and southern corn rootworm (Coleoptera: Chrysomelidae). J Econ Entomol 83:2480–2485
169. Murdock LL, Huesing JE, Nielsen SS, Pratt RC, Shade RE (1990) Biological effects of plant lectins on the cowpea weevil. Phytochemistry 29:85–89
170. Fitches E, Gatehouse AMR, Gatehouse JA (1997) Effects of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on the development of tomato moth (*Lacanobia oleracea*) larvae in laboratory and glasshouse trials. J Insect Physiol 43:727–739

171. Boulter D, Edwards GA, Gatehouse AMR, Gatehouse JA, Hilder VA (1990) Additive protective effects of incorporating two different higher plant derived insect resistance genes in transgenic tobacco plants. Crop Prot 9:351–354
172. Gatehouse AMR, Davison GM, Newell CA, Merryweather A, Hamilton WDO, Burgess EPJ, Gilbert RJC, Gatehouse JA (1997) Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: growth room trials. Mol Breed 3:49–63
173. Powell KS, Gatehouse AMR, Hilder VA, Gatehouse JA (1993) Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinciteps*. Entomol Exp Appl 66:119–126
174. Powell KS, Gatehouse AMR, Hilder VA, van Damme EJM, Peumans WJ, Boonjawat J, Horsham K, Gatehouse JA (1995) Different antimetabolic effects of related lectins towards nymphal stages of *Nilaparvata lugens*. Entomol Exp Appl 75:61–65
175. Rao KV, Rathore KS, Hodges TK, Fu X, Stoger E, Sudhakar D, Williams S, Christou P, Bharathi M, Bown DP, Powell KS, Spence J, Gatehouse AMR, Gatehouse JA (1998) Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown planthopper. Plant J 15:469–477
176. Nagadhara D, Ramesh S, Pasalu IC, Kondala Rao Y, Krishnaiah NV, Sarma NP, Bown DP, Gatehouse JA, Reddy VD, Rao KV (2003) Transgenic indica rice resistant to sap-sucking insects. Plant Biotechnol 1:231–240
177. Foissac X, Loc NT, Christou P, Gatehouse AMR, Gatehouse JA (2000) Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). J Insect Physiol 46:573–583
178. Nagadhara D, Ramesh S, Pasalu IC, Rao YK, Sarma NP, Reddy VD, Rao KV (2004) Transgenic rice plants expressing the snowdrop lectin gene (gna) exhibit high-level resistance to the whitebacked planthopper (*Sogatella furcifera*). Theor Appl Genet 109:1399–1405
179. Loc NT, Tinjuangjun P, Gatehouse AMR, Christou P, Gatehouse JA (2002) Linear transgene constructs lacking vector backbone sequences generate transgenic rice plants which accumulate higher levels of proteins conferring insect resistance. Mol Breed 9:231–244
180. Poulsen M, Kroghsbo S, Schroder M, Wilcks A, Jacobsen H, Miller A, Frenzel T, Danier J, Rychlik M, Shu QY, Emami K, Sudhakar D, Gatehouse A, Engel KH, Knudsen I (2007) A 90-day safety study in Wistar rats fed genetically modified rice expressing snowdrop lectin Galanthus nivalis (GNA). Food Chem Toxicol 45:350–363
181. Gatehouse AMR, Down RE, Powell KS, Sauvion N, Rahbe Y, Newell CA, Merryweather A, Hamilton WDO, Gatehouse JA (1996) Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae*. Entomol Exp Appl 79:295–307
182. Wang ZY, Zhang KW, Sun XF, Tang KX, Zhang JR (2005) Enhancement of resistance to aphids by introducing the snowdrop lectin gene *gna* into maize plants. J Biosci 30: 627–638
183. Bandyopadhyay S, Roy A, Das S (2001) Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity. Plant Sci 161:1025–1033
184. Dutta I, Saha P, Majumder P, Sarkar A, Chakraborti D, Banerjee S, Das S (2005) The efficacy of a novel insecticidal protein, *Allium sativum* leaf lectin (ASAL), against homopteran insects monitored in transgenic tobacco. Plant Biotechnol J 3:601–611
185. Dutta I, Majumder P, Saha P, Ray K, Das S (2005) Constitutive and phloem specific expression of *Allium sativum* leaf agglutinin (ASAL) to engineer aphid (*Lipaphis erysimi*) resistance in transgenic Indian mustard (*Brassica juncea*). Plant Sci 169: 996–1007
186. Saha P, Majumder P, Dutta I, Ray T, Roy SC, Das S (2006) Transgenic rice expressing *Allium sativum* leaf lectin with enhanced resistance against sap-sucking insect pests. Planta 223:1329–1343
187. Saha P, Chakraborti D, Sarkar A, Dutta I, Basu D, Das S (2007) Characterization of vascular-specific *RSs1* and *rolC* promoters for their utilization in engineering plants to develop resistance against hemipteran insect pests. Planta 226:429–442
188. Saha P, Dasgupta I, Das S (2006) A novel approach for developing resistance in rice against phloem limited viruses by antagonizing the phloem feeding hemipteran vectors. Plant Mol Biol 62:735–752
189. Ryan CA (2000) The system in signaling pathway: differential activation of plant defensive genes. Biochim Biophys Acta 1477:112–121
190. Felton GW, Donato KK, Broadway RM, Duffey SS (1992) Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore, *Spodoptera exigua*. J Insect Physiol 38:277–285
191. Melo GA, Shimizu MM, Mazzafera P (2006) Polyphenoloxidase activity in coffee leaves and its role in resistance against the coffee leaf miner and coffee leaf rust. Phytochemistry 67: 277–285
192. Wang JH, Constabel CP (2004) Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*). Planta 220:87–96
193. Dowd PF, Lagrimini LM (1997) Examination of different tobacco (*Nicotiana* spp.) types under- and overproducing tobacco anionic peroxidase for their leaf resistance to *Helicoverpa zea*. J Chem Ecol 23:2357–2370
194. Dowd PF, Lagrimini LM (2006) Examination of the biological effects of high anionic peroxidase production in tobacco plants grown under field conditions. I. Insect pest damage. Transgenic Res 15:197–204
195. Dowd PF, Zuo WN, Gillikin JW, Johnson ET, Boston RS (2003) Enhanced resistance to *Helicoverpa zea* in tobacco

expressing an activated form of maize ribosome-inactivating protein. J Agric Food Chem 51:3568–3574
196. Lawrence SD, Novak NG (2006) Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. Biotechnol Lett 28:593–599
197. Dowd PF, Johnson ET, Pinkerton TS (2007) Oral toxicity of beta-*N*-acetyl hexosaminidase to insects. J Agric Food Chem 55:3421–3428
198. Fitches E, Wilkinson H, Bell H, Bown DP, Gatehouse JA, Edwards JP (2004) Cloning, expression and functional characterisation of chitinase from larvae of tomato moth (*Lacanobia oleracea*): a demonstration of the insecticidal activity of insect chitinase. Insect Biochem Mol Biol 34:1037–1050
199. Ding XF, Gopalakrishnan B, Johnson LB, White FF, Wang XR, Morgan TD, Kramer KJ, Muthukrishnan S (1998) Insect resistance of transgenic tobacco expressing an insect chitinase gene. Transgenic Res 7:77–84
200. Saguez J, Hainez R, Cherqui A, Van Wuytswinkel O, Jeanpierre H, Lebon G, Noiraud N, Beaujean A, Jouanin L, Laberche JC, Vincent C, Giordanengo P (2005) Unexpected effects of chitinases on the peach-potato aphid (*Myzus persicae* Sulzer) when delivered via transgenic potato plants (*Solanum tuberosum* Linne) and *in vitro*. Transgenic Res 14:57–67
201. Corrado G, Arciello S, Fanti P, Fiandra L, Garonna A, Digilio MC, Lorito M, Giordana B, Pennacchio F, Rao R (2007) The Chitinase A from the baculovirus AcMNPV enhances resistance to both fungi and herbivorous pests in tobacco. *Transgenic Res.* 17:557–571. (published online: DOI: 10.1007/s11248-007-9129-4)
202. Wittstock U, Gershenzon J (2002) Constitutive plant toxins and their role in defense against herbivores and pathogens. Curr Opin Plant Biol 5:300–307
203. Tattersall DB, Bak S, Jones PR, Olsen CE, Nielsen JK, Hansen ML, Hoj PB, Moller BL (2001) Resistance to an herbivore through engineered cyanogenic glucoside synthesis. Science 293:1826–1828
204. Bak S, Olsen CE, Halkier BA, Møller BL (2000) Transgenic tobacco and *Arabidopsis* plants expressing the two multifunctional sorghum cytochrome P450 enzymes, CYP79A1 and CYP71E1, are cyanogenic and accumulate metabolites derived from intermediates in dhurrin biosynthesis. Plant Physiol 123:1437–1448
205. Kristensen C, Morant M, Olsen CE, Ekstrom CT, Galbraith DW, Moller BL, Bak S (2005) Metabolic engineering of dhurrin in transgenic *Arabidopsis* plants with marginal inadvertent effects on the metabolome and transcriptome. Proc Natl Acad Sci USA 102:1779–1784
206. Ogunlabi OO, Agboola FK (2007) A soluble beta-cyanoalanine synthase from the gut of the variegated grasshopper *Zonocerus variegatus* (L.). Insect Biochem Mol Biol 37:72–79
207. Mikkelsen MD, Halkier BA (2003) Metabolic engineering of valine- and isoleucine-derived glucosinolates in *Arabidopsis* expressing CYP79D2 from cassava. Plant Physiol 131:773–779
208. Franks TK, Powell KS, Choimes S, Marsh E, Iocco P, Sinclair BJ, Ford CM, van Heeswijck R (2006) Consequences of transferring three sorghum genes for secondary metabolite (cyanogenic glucoside) biosynthesis to grapevine hairy roots. Transgenic Res 15:181–195
209. Kim YS, Uefuji H, Ogita S, Sano H (2006) Transgenic tobacco plants producing caffeine: a potential new strategy for insect pest control. Transgenic Res 15:667–672
210. Wei S, Semel Y, Bravdo BA, Czosnek H, Shoseyov O (2007) Expression and subcellular compartmentation of *Aspergillus niger* beta-glucosidase in transgenic tobacco result in an increased insecticidal activity on whiteflies (*Bemisia tabaci*). Plant Sci 172:1175–1181
211. Aharoni A, Jongsma MA, Bouwmeester HJ (2005) Volatile science? Metabolic engineering of terpenoids in plants. Trends Plant Sci 10:594–602
212. Wang E, Wang R, DeParasis J, Loughrin JH, Gan S, Wagner GJ (2001) Suppression of a P450 hydroxylase gene in plant trichome glands enhances natural-product-based aphid resistance. Nat Biotechnol 19:371–374
213. Aharoni A, Giri AP, Deuerlein S, Griepink F, de Kogel WJ, Verstappen FWA, Verhoeven HA, Jongsma MA, Schwab W, Bouwmeester HJ (2003) Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. Plant Cell 15:2866–2884
214. Kappers IF, Aharoni A, van Herpen TWJM, Luckerhoff LLP, Dicke M, Bouwmeester HJ (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. Science 309:2070–2072
215. Schnee C, Kollner TG, Held M, Turlings TCJ, Gershenzon J, Degenhardt J (2006) The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. Proc Natl Acad Sci USA 103:1129–1134
216. Beale MH, Birkett MA, Bruce TJA, Chamberlain K, Field LM, Huttly AK, Martin JL, Parker R, Phillips AL, Pickett JA, Prosser IM, Shewry PR, Smart LE, Wadhams LJ, Woodcock CM, Zhang YH (2006) Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior. Proc Natl Acad Sci USA 103:10509–10513
217. Johnson ET, Dowd PF (2004) Differentially enhanced insect resistance, at a cost, in *Arabidopsis thaliana* constitutively expressing a transcription factor of defensive metabolites. J Agric Food Chem 52:5135–5138
218. Johnson ET, Berhow MA, Dowd PF (2007) Expression of a maize Myb transcription factor driven by a putative silk-specific promoter significantly enhances resistance to *Helicoverpa zea* in transgenic maize. J Agric Food Chem 55: 2998–3003
219. Maiti IB, Dey N, Pattanaik S, Dahlman DL, Rana RL, Webb BA (2003) Antibiosis-type insect resistance in transgenic plants expressing a teratocyte secretory protein (TSP14) gene from a hymenopteran endoparasite (*Microplitis croceipes*). Plant Biotechnol J 1:209–219
220. Tortiglione C, Fogliano V, Ferracane R, Fanti P, Pennacchio F, Monti LM, Rao R (2003) An insect peptide engineered into the

tomato prosystemin gene is released in transgenic tobacco plants and exerts biological activity. Plant Mol Biol 53:891–902
221. Bowen DJ, Ensign JC (1998) Purification and characterization of a high-molecular-weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. Appl Environ Microbiol 64:3029–3035
222. Bowen D, Rocheleau TA, Blackburn M, Andreev O, Golubeva E, Bhartia R, ffrench-Constant RH (1998) Insecticidal toxins from the bacterium *Photorhabdus luminescens*. Science 280: 2129–2132
223. ffrench-Constant RH, Dowling A, Waterfield NR (2007) Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. Toxicon 49:436–451
224. Guo LN, Fatig RO, Orr GL, Schafer BW, Strickland JA, Sukhapinda K, Woodsworth AT, Petell JK (1999) *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins - purification and characterization of toxin A and toxin B. J Biol Chem 274:9836–9842
225. Liu D, Burton S, Glancy T, Li ZS, Hampton R, Meade T, Merlo DJ (2003) Insect resistance conferred by 283-kDa *Photorhabdus luminescens* protein TcdA in *Arabidopsis thaliana*. Nat Biotechnol 21:1222–1228
226. Purcell JP, Greenplate JT, Jennings MG, Ryerse JS, Pershing JC, Sims SR, Prinsen MJ, Corbin DR, Tran M, Sammons RD, Stonard RJ (1993) Cholesterol oxidase - a potent insecticidal protein active against boll weevil larvae. Biochem Biophys Res Commun 196:1406–1413
227. Shen Z, Corbin DR, Greenplate JT, Grebenok RJ, Galbraith DW, Purcell JP (1997) Studies on the mode of action of cholesterol oxidase on insect midgut membranes. Arch Insect Biochem Physiol 34:429–442
228. Corbin DR, Grebenok RJ, Ohnmeiss TE, Greenplate JT, Purcell JP (2001) Expression and chloroplast targeting of cholesterol oxidase in transgenic tobacco plants. Plant Physiol 126:1116–1128
229. Morgan TD, Oppert B, Czapla TH, Kramer KJ (1993) Avidin and streptavidin as insecticidal and growth-inhibiting dietary proteins. Entomol Exp Appl 69:97–108
230. Markwick NP, Christeller JT, Docherty LC, Lilley CM (2001) Insecticidal activity of avidin and streptavidin against four species of pest Lepidoptera. Entomol Exp Appl 98:59–66
231. Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh RJ, Hernan R, Kappel WK, Ritland D, Li CP, Howard JA (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306
232. Kramer KJ, Morgan TD, Throne JE, Dowell FE, Bailey M, Howard JA (2000) Transgenic avidin maize is resistant to storage insect pests. Nat Biotechnol 18:670–674
233. Burgess EPJ, Malone LA, Christeller JT, Lester MT, Murray C, Philip BA, Phung MM, Tregidga EL (2002) Avidin expressed in transgenic tobacco leaves confers resistance to two noctuid pests, *Helicoverpa armigera* and *Spodoptera litura*. Transgenic Res 11:185–198
234. Murray C, Sutherland PW, Phung MM, Lester MT, Marshall RK, Christeller JT (2002) Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences. Transgenic Res 11:199–214
235. Markwick NP, Docherty LC, Phung MM, Lester MT, Murray C, Yao JL, Mitra DS, Cohen D, Beuning LL, Kutty-Amma S, Christeller JT (2003) Transgenic tobacco and apple plants expressing biotin-binding proteins are resistant to two cosmopolitan insect pests, potato tuber moth and lightbrown apple moth, respectively. Transgenic Res 12:671–681
236. Yoza K, Imamura T, Kramer KJ, Morgan TD, Nakamura S, Akiyama K, Kawasaki S, Takaiwa F, Ohtsubo K (2005) Avidin expressed in transgenic rice confers resistance to the stored-product insect pests *Tribolium confusum* and *Sitotroga cerealella*. Biosci Biotechnol Biochem 69:966–971
237. Ginzberg I, Perl A, Genser M, Wininger S, Nemas C, Kapulnik Y (2004) Expression of streptavidin in tomato resulted in abnormal plant development that could be restored by biotin application. J Plant Physiol 161:611–620
238. Flinn PW, Kramer KJ, Throne JE, Morgan TD (2006) Protection of stored maize from insect pests using a two-component biological control method consisting of a hymenopteran parasitoid, *Theocolax elegans*, and transgenic avidin maize powder. J Stored Prod Res 42:218–225
239. Cooper SG, Douches DS, Grafius EJ (2006) Insecticidal activity of avidin combined with genetically engineered and traditional host plant resistance against Colorado potato beetle (Coleoptera: Chrysomelidae) larvae. J Econ Entomol 99: 527–536
240. Turner CT, Davy MW, MacDiarmid RM, Plummer KM, Birch NP, Newcomb RD (2006) RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Mol Biol 15:383–391
241. Mao Y-B, Cai W-J, Wang J-W, Hong G-J, Tao X-Y, Wang L-J, Huang Y-P, Chen X-Y (2007) Silencing a cotton bollworm P450 monoxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol 25:1307–1313
242. Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, Vaughn T, Roberts J (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25:1322–1326
243. Benbrook C (2009) Impacts of genetically engineered crops on pesticide use in the United States: The first thirteen years. *The Organic Centre; Critical Issue Report*. http://www.organic-center.org/reportfiles/13Years20091126_FullReport.pdf
244. Gatehouse AMR, Ferry N, Raemaekers RJM (2002) The case of the monarch butterfly: a verdict is returned. Trends Genet 18:249–251
245. Tabashnik BE, Van Rensburg JBJ, Carriere Y (2009) Field-evolved insect resistance to *Bt* crops: Definition, theory, and data. J Econ Entomol 102:2011–2025

246. Nunez-Farfan J, Fornoni J, Luis Valverde P (2007) The evolution of resistance and tolerance to herbivores. Annu Rev Ecol Evol Syst 38:541–566

Books and reviews

Carozzi N, Koziel M, eds. (1997) Advances in Insect Control; The Role of Transgenic Plants. Taylor and Francis, London

Romeis J, Shelton AM, Kennedy G eds. (2008) Integration of Insect-Resistant Genetically Modified Crops within IPM Programs (Progress in Biological Control). Springer

Lemaux PG (2008) Genetically engineered plants and foods: a scientist's analysis of the issues (part I). Annu Rev Plant Biol 59:771–812

Lemaux PG (2009) Genetically engineered plants and foods: a scientist's analysis of the issues (part II). Annu Rev Plant Biol 60:511–559

Park JR, McFarlane I, Phipps RH, Ceddia G (2011) The role of transgenic crops in sustainable development. Plant Biotech J 9:2–21

Genotype by Environment Interaction and Adaptation

Ignacio Romagosa[1], Gisela Borràs-Gelonch[1], Gustavo Slafer[1], Fred van Eeuwijk[2]
[1]Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain
[2]Biometris, Wageningen University and Research Centre, Wageningen, The Netherlands

Article Outline

Glossary

GE *Genotype by environment interaction* is differential genotypic expression across environments that may cause that a genotype selected among the best in one location to perform poorly in another. GE weakens association between phenotype and genotype, reducing genetic progress in breeding programs. In statistical terms, GE describes a situation in which the simultaneous effect of two classification variables (genotype and environment) on a continuous dependent third one, such as yield, does not follow an additive model.

MET A *multi-environment trial* is a series of trials sampling the target environmental range in which a particular set of genotypes is evaluated.

QTL A *quantitative trait locus* is a region in the genome associated with a particular quantitative phenotypic trait, such as crop yield, resource-use-efficiency, phenology, or height. QTL analysis is a statistical method that links phenotypic data (specific trait measurements on a series of individuals) and genotypic data (usually in the form of molecular markers taken on the same individual) in order to explain the genetic basis of complex traits. QTL number and the variation they explain on the phenotypic trait give clues about the genetic control of that trait, for example, if plant height is controlled by many genes of small effect, or by a few genes of large effect.

QTLxE QTL by environment interaction is differential QTL effect across environments that may cause that a favorable QTL in one environment may become irrelevant, or even unfavorable, in another.

Specific and wide adaptation A genotype is considered stable if it yields well relative to the productive potential of the environments in which is grown. If such concept of stability is shown for a wide agroecological array of environments, a genotype is considered to have general, wide, or broad adaptation. If stability is confined to a limited range, a genotype is said to have specific or narrow adaptation.

Definition

One of the first decisions farmers have to take is the selection of the variety to be grown in their fields based on expectation of economic returns, generally, in the form of the highest attainable yield. This is a critical choice that strongly determines the sustainability of the agricultural system. However, this is by no means trivial as it is very hard to identify the "best" variety across a diverse set of environments subjected to complex biotic and abiotic factors and interactions generally causing significant changes in varietal rank. Therefore, a major objective in plant breeding programs is to determine the potential adaptation of advanced breeding lines across a range of agroecological conditions. William S. Gosset (who signed as "Student [1]" in a landmark publication introducing the t distribution) wrote at the onset of modern breeding that the ultimate purpose of field experimentation was to determine what varieties pay farmers best. He thought that the design of experiments should aim, not only at determining the average yield, but also at identifying varieties whose yield, being within those of high average value, were relatively less responsive to variation in soil and climate.

Breeding programs normally aim to release cultivars to be successfully grown over a rather large cropping area, varying in soil quality attributes and in

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

average climate, and across several growing seasons, with interannual variations in climatic conditions. The target environment is defined as the set of soil × climatic conditions in which the released cultivars will be grown and to which the cultivars must be adapted. Therefore, a key step in applied plant breeding is the identification of advanced genotypes broadly or narrowly adapted across a wide range of target environments. Breeders focus in the first segregating generations on direct phenotypic selection of highly heritable traits, such as plant architecture and phenology to concentrate in later stages on complex quantitative traits like yield and end-use quality. Marker-assisted selection aims at complementing this phenotypic selection with direct marker screening for, mostly, oligogenic-controlled traits. The traditional approach to estimate the genotypic value in the context of breeding, varietal registration, and recommendation is deployment of extensive field evaluation schemes in a series of sites in which the assessed genotypes could be potentially grown. These collections of trials are generally denominated multi-environment trials (METs) in which a set of genotypes is evaluated in a series of trials that sample the target environmental range. Data from METs are typically summarized in the form of genotype by environment tables of means. Simple inspection of such tables of means will often reveal the presence of genotype by environment interaction (GE) or differences in performance of genotypes that are trial dependent. They also allow for the identification of those genotypes that are partially or generally adapted to the environmental range, showing specific or narrow versus general or wide adaptation, respectively.

The traditional outcome of METs is the identification of "which" cultivar and "where" has performed well. These studies are empirical, based on simple statistical characterizations of genotypic responses across environments and do not provide any physiological insight into the basis of the genotypic response to environmental changes. However, as one wants to move forward toward a *predictive* breeding scenario, the challenge beyond "which" and "where" is "why" narrow or wide adaptation happens, in terms of a thorough understanding of both the environment, the physiological behavior of the different cultivars and, eventually, of the genes responsible for adaptation. Identifying the "why" is not only a matter of satisfying curiosity: It would potentially allow more precise breeding through the direct manipulation of the genes responsible for the different adaptation patterns.

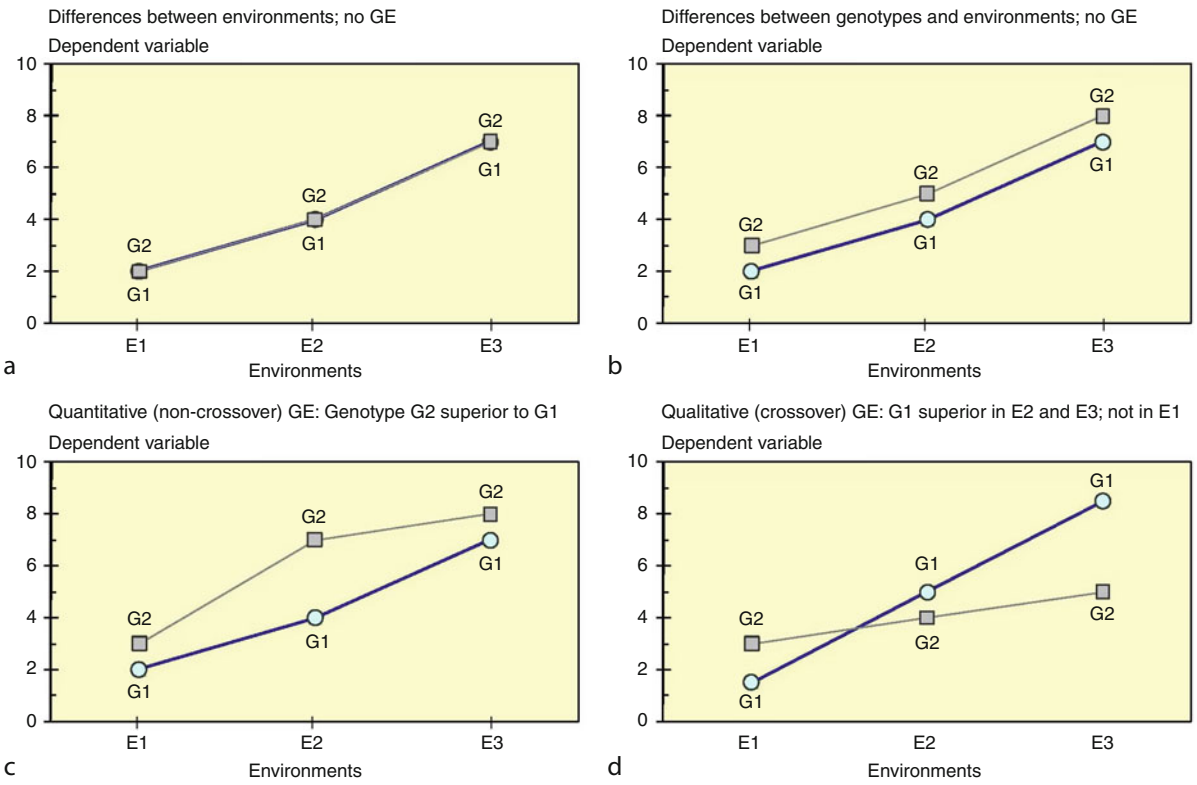
Introduction

Statistical analyses that detect and describe GE have been comprehensively reviewed [2–14]. Means across environments in METs are only adequate estimates of varietal performance in the absence of GE. When GE is significant, average values across environments may hide subsets of environments where genotypes differ markedly in relative performance.

As for any other statistical two-factor model, there are different types of interactions which originate from departure from additivity. In Fig. 1, the average for each of two genotypes, G1 and G2, for the dependent variable of interest, for example yield, is shown for three environments. Figure 1a represents the situation in which differences were detected only between environments. Figure 1b shows an additive model in which differences for both main effects, genotypes and environments, were observed but no GE. Figure 1c shows a quantitative or non-crossover interaction; in this scenario, genotypes with superior means can be recommended for all environments. In plant breeding, the most important GE is of the crossover or qualitative type (Fig. 1d), which implies changes in the rankings of genotypes across environments). In this case, variety G2 may be recommended for environment E1 but not for E2 and E3.

When there are genotypic differences among the varieties tested and the target environments include different soils and variable climate, MET analyses more often than not detect crossover GE (only MET with limited genotypic and/or narrow environmental diversity might reveal negligible or nonsignificant interaction). Thus, identification of superior genotypes is complicated by qualitative GE and largely depends on extensive field testing conducted over years at different locations. Therefore, there is a strong need to deploy powerful statistical models for MET data taking into account GE and their breeding implications.

Crossover interactions represent a double-edged sword [10]. Whereas they make breeding, testing, selection, and varietal recommendation more difficult, if the



Genotype by Environment Interaction and Adaptation. Figure 1
Performance of two hypothetical genotypes in three environments showing: (**a**) Only environmental differences; (**b**) No GE; (**c**) Quantitative, non-crossover, GE; (**d**) Qualitative, crossover, GE

underlying ecophysiological grounds of GE are known, identification of genotypes better adapted to certain specific niche conditions, allowing for increased genetic gains, is possible. If the traits conferring adaptation to these specific environments and/or the genes that control them are revealed, direct implementation in breeding may be feasible either by choosing parents for a new cross possessing the adaptive attributes or by directly selecting for the presence of such attributes in the progenies (through direct measurement of the attributes or through genotypic selection, see below).

METs are often carried out over a number of sites and years that are considered to be representative of the target environments. Standard analyses of variance partition the GE term into genotype by locations (GL), genotype by years (GY), and genotype by locations by years (GLY) interactions. The relative size of these terms allow for a statistical assessment of the spatial and temporal components of adaptation. If GL dominates over the other components, then specific adaptation is exploitable by identifying subsets of homogeneous locations for variety release and recommendation. Where GY and GLY terms dominate, as most often happens, no simplification involving spatial subdivision of growing regions is possible. In this context, specific recommendations may be only possible after counting with robust models trustworthily predicting the main climatic conditions of the growing season in advance to sowing.

Recent efforts have searched for the genetic factors underlying GE and, thus, to describe adaptation patterns. Quantitative trait loci (QTLs) responsible for individual complex traits (see, e.g., [15]), such as yield and adaptation have been reported in several populations for most crop species. QTL related to adaptation show different effects in different environments. The magnitude of individual QTL effects (expressed as the amount of GE variation explained

by a particular QTL) varied among populations and across environments. Therefore, implementation of marker-assisted selection strategies for these QTLs in applied breeding programs remains a challenge. Modern GE studies have introduced external environmental, physiological, and/or genetic information to develop statistical models whose parameters relate better to physiological knowledge [16, 17], and therefore offer better possibilities for implementation of QTL selection methodologies in breeding programs.

Breeding Implications

Historically most of the genetic progress in the last decades at the global level, particularly in cereals, has been attained through increases of yield potential and disease resistance. Genetic gains in yield under non-limited growing conditions, i.e., improving yield potential, have often brought about parallel gains in yield under a wide range of more realistic, largely stressful, growing conditions [18–20]; because physiological traits behind improved yield potential may often be constitutive and provide yield advantage over a range of conditions [21]. Thus, improving simultaneously for yield potential (which is directly linked to both attainable and on-farm yields; [22]) and for disease resistance has conferred not only clear progress under high-yielding conditions but also wide adaptation.

Thus, it is critical to further improve yield potential [23]. Lessons from the past allow to optimistically trusting that relatively simple traits might be found that affect yield potential and wide adaptation simultaneously (e.g., [24]). For instance, the incorporation of simple key traits such as reduced height might have such a great impact that may be the basis of a Green Revolution due to its capacity of increasing yield both under potential and most non-potential conditions. Genetically reducing the capacity of the stems to grow through introgression of semidwarfing genes determined firstly an increased partitioning of biomass accumulated during stem elongation to the growing spikes [25, 26]; then the additional availability of resources in the growing spikes allowed floret development to proceed normally in more floret primordia consequently increasing the number of grains [27] and therefore parallel improvements in yield, as cereals are most frequently sink-limited during grain filling even under nonoptimal environments [28, 29]. However, as further reducing height would not keep improving yields [30], it is critical identifying alternative traits that being rather simple were still putatively related to yield across a wide range of conditions. Difficulties in identifying such traits is reflected in the fact that despite continuous breeding efforts in the last decade, current genetic progress in yield potential fall short of both those attained before (see [31] and references therein) and that required to match expected increases in demand [23]. Future improvements in yield potential would largely depend upon the identification of alternative traits that being relatively simple are putatively related to yield in a wide range of conditions representing the target environments of the breeding program. In this context, a thorough examination of GE will be critical both for identifying traits in a top-down approach dissecting yield into physiologically sound traits across conditions representing the target environments, and for determining the stability of the relationship between the identified trait(s) and yield.

In an even more general context, GE has important implications in applied breeding programs [5]. Based on the magnitude and nature of GE, breeders have to decide whether to aim for wide or for specific adaptation. This decision determines the choice of locations for selection, the allocation of limited resources in advanced line testing, and the assessment of the potential trade-off between empirical, molecular, and physiological screening of parents and advanced lines. Related to wide adaptation is the question of breeding sites: Can selection under optimum high-input environments identify genotypes adapted to more stressed environments? Salvatore Ceccarelli and Stefania Grando at ICARDA have produced a significant number of contributions on the issue of wide versus specific adaptation in barley (see [32] and their own references therein for a review). They have strongly advocated the exploitation of specific adaptation for optimum use of resources particularly in marginal environments, arguing that selection for high yield potential has not increased yield under low-input conditions. However, success of the CIMMYT wheat program aiming at wide adaptation is based on a completely different approach. Rather than focusing on any specific environmental

conditions, continuous selection cycles, referred to as shuttle breeding, are carried out in alternative and extremely diverse high yield potential environments differing in altitude, latitude, photoperiod, temperature, rainfall, soil type, and disease spectrum. As a result, CIMMYT wheat genotypes have shown high yield potential and wide adaptation across large geographical regions, perhaps with the exception of very marginal; in fact, poor adaptation of CIMMYT genotypes to specific environments often reflected susceptibility to specific plant diseases.

Field experimentation aims at covering a representative sample of environmental variation. However, the need for adequate resource allocation raises the question of whether multilocation testing in a limited number of years can adequately sample the array of environmental conditions where a variety can be grown. If the MET analysis of variance identifies GY as the most significant term, testing for many crop cycles should be preferred. However, this is not suitable given the increasing pressure to develop new cultivars. Therefore, breeders often substitute temporal for spatial environmental variation, assuming that GL is similar in nature to GY and that GLY is absent. Resource allocation for varietal experimentation schemes depends on the relative magnitude of the variance components for the genotype and GE interaction terms. Given the small number of years available for testing, and the frequently dominant effect of GY and GLY interactions, there is little point in a very extensive series of trials in a given year with a high proportion of genotypes retained throughout. Integrated mixed model analyses for the selected genotypes across the breeding stages can counterweigh for the limited number of years in the later stages of field testing.

A series of papers have suggested the use of reference and probe genotypes to characterize environmental variation and assess GE repeatability [33]. By defining a common reference set of genotypes consistently grown across locations and years, a breeder could define a long-term target environment and weight results from each location in a given year in accordance with its across-year representativeness. Probe genotypes with differential response to known biotic and abiotic conditions could also be used to characterize environments. However, practical application of these two principles is not common. Genetic gains for unidentified biotic and abiotic stresses by direct selection on extensive MET are possible. A more sound approach could be the growing of genotypes in a few key environments with well-characterized levels of the target stress. Manipulation of the breeding environment and selection of key parents for crossing should result in improved genetic gains. However, this second approach requires a clear understanding of the major stress as well as the facilities to reproduce it.

A germplasm strategy is also needed for breeding for wide and specific adaptation. For most crops, there is an important gap between elite and unimproved gene pools as most breeders focus on germplasm reflecting decades of intensive crossing, selection, and recombination [34]. However as genetic gains attained by conventional breeding decrease, more emphasis should be given to the use of new genetic variability both through pre-breeding or through construction of new parent for crosses, incorporating desired traits from local land races and related wild species, or from other unrelated organism through transgenesis.

The first studies on GE were based on standard variety trials across a series of environments. That allowed identification of the wide or narrow adaptation of the checked cultivars, but little could be said on the genetic basis of adaptation. Extensive field testing of biparental crosses (e.g., [35]), either in the form of doubled haploids, or recombinant inbred lines populations, allows for the assessment of the genetic control of plant adaptation based on standard linkage and QTL analyses, but their use is limited by the level of polymorphisms between parents. In contrast, diverse genotypic panels accumulating multiple recombination events provide ample genetic variation for association studies. However, their main limitation is the high incidence of false-positive associations due to the difficulty to distinguish between true and pseudo linkage between molecular markers and traits of interest, due to population substructure and correlated selection [36]. More recently, other more complex crossing systems have been proposed to exploit the advantages of both linkage analysis and association mapping. This is the case, for example, of the so-called MAGIC (multiparent advanced generation intercross) [37], the nested association mapping (NAM) design based on a huge set of recombinant inbred lines derived from

a large number of founder genotypes [38, 39], and AMPRIL (a multiparent recombinant inbred line population) [40].

The use of physiological criteria in analytical breeding is critical for success [41–44]. Breeders develop a deep knowledge of their target environments and of the agroecological adaptation of their genetic materials. However, whereas intensive work is continuously been carried out by crop physiologists in the area of yield potential and adaptation, not many breeders regularly incorporate new physiological criteria in their mainstream-breeding program. In any case, physiological assessment of adaptation is needed to complement breeders' impressions particularly in the first and last stages of a breeding program: selection of parents and assessment of adaptation of new advanced lines. Similarly, despite exciting progress in molecular marker-assisted selection, applied breeding still depends heavily on direct phenotypic selection of advanced genotypes.

In the rest of this entry, two different aspects will be presented: First, an example of the physiological implications of GE through the study of a trait, time to flowering, that has a clear effect on adaptation; second, a series of increasingly complex statistical models to characterize genotypic adaptation, to identify genotypes showing wide or specific adaptation and to dissect the genetic complexity behind this integrative trait. Although these sections may look quite disconnected, a thorough knowledge of crop physiology and/or their genetic control could allow construction of more powerful integrated statistical models incorporating as genetic covariables this information in order to improve the understanding of the nature of GE. Conversely, the statistical models can identify certain genotypes which, if well characterized, could allow for empirical identification of key adaptative traits.

Traits Determining Adaptation

The number of physiological traits with a potential effect in determining yield and adaptation is extraordinarily large. In an excellent *Crop Physiology* manual recently edited by Sadras and Calderini [45], many traits are reviewed and organized according to different criteria from capture and efficiency in the use of resources to crop development and plant architecture. Many trade-off exists between traits that, if ignored, will slow down genetic progress for both potential and actual farmer yields. Araus et al. [43] have also reviewed a number of potentially useful physiological criteria for breeding, particularly, in the framework of C3 cereals under Mediterranean conditions. Crop physiology as a whole is beyond the objectives of this entry. Therefore, the focus is on the single most important crop trait determining plant adaptation, time to flowering, as an example of a key trait to describe the underlying mechanisms and implications for GE.

Time to Flowering

Crop phenology – life cycle as influenced by seasonal variations in climate – has been widely recognized as the most important single factor determining adaptation and thereby crop performance. In determinate species, it allows for matching crop development with availability of resources, avoiding abiotic stresses due to climatic conditions such as late spring frosts and terminal drought. To maximize attainable yield, the most "critical phases" for yield determination have to be match with the most favorable (or least unfavorable) growing conditions. In some cases (Northern Hemisphere), the obvious way to achieve this is sowing cold-tolerant genotypes early enough to have full growth in early spring, but in the warmer Southern Hemisphere similar maximum yields can be achieved sowing in winter with significantly shorter phases, provided the critical phases are ideally timed [46–49]. Crop phenology is, thus, not only a key adaptative trait, but it may also affect yield potential, since different structures are produced throughout the crop cycle, and some of them may be more important than others in determining yield potential [50]. If the pattern of water deficit in the target region is relatively predictable, manipulation of genes responsible for crop phenology is the most sustainable approach to increase attainable yield and plant adaptation.

The importance of flowering time has been shown, for example, with the fast and diverse shifts in heading time, or in vernalization and photoperiod responses, due to natural selection: When the same bulk population is grown under contrasting environments [51]; when comparing different sowing dates [52]; when

studying the contrasting developmental patterns of genotypes adapted to particular regions [53–55]; or in retrospective studies showing changes in heading date over time due to breeding, particularly in areas where the crop was introduced more recently (e.g., bread wheat in Australia; [48]; durum wheat in certain regions of Spain; [56]). Therefore, crop phenology is an important source of GE for yield when testing genotypes from regions differing in climatic conditions [57, 58].

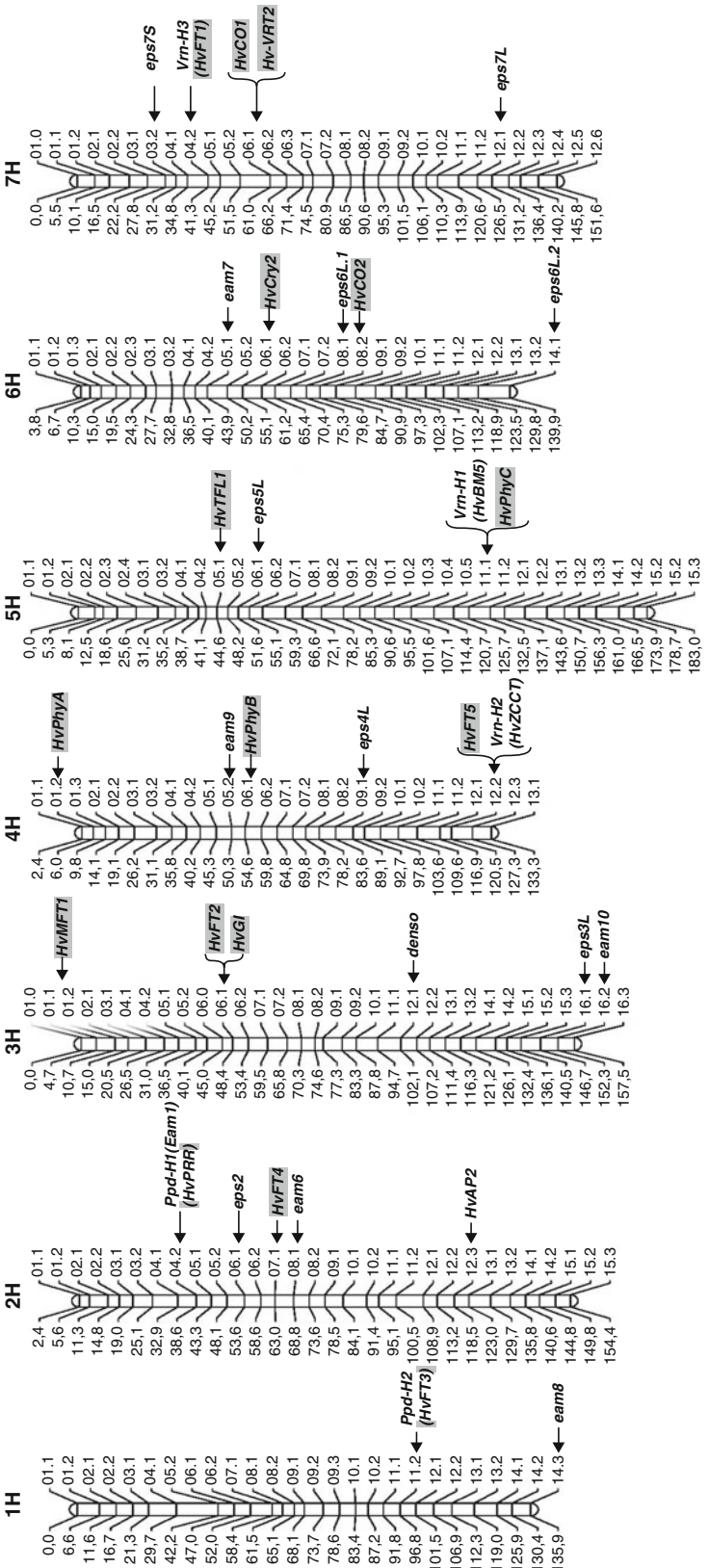
The three major factors determining flowering time are differential responses to photoperiod and vernalization and intrinsic earliness or earliness per se [50]. Further evidence from recent studies in wheat [59–61] support the idea that earliness per se genes represent basically genotypic differences in the response to non-vernalizing temperatures [62, 63]. The wide genotypic differences for these factors are considered as responsible for the spread of winter cereals, worldwide to a wide range of latitudes and altitudes [49, 64].

Genetic Factors Controlling Time to Flowering

At the gene or marker level, the importance of flowering time in crop performance is shown, for example, through the geographical distribution of alleles of major genes such as photoperiod (*Ppd*) and vernalization (*VRN*) responsive genes [49, 64, 65]. The co-location of QTLs for heading with QTLs for yield (e.g., [35, 66–69]), which may help to define an optimal window for heading or combination of alleles in the tested environments [70]. Moreover, in some of these studies, QTLs with strong effects on heading collocated with some of the QTLs for yield that exhibited strongest QTL by environment interactions [35, 69–71]. Recent studies have shown, through factorial regression, that a great part of the effect of these QTLs for heading (underlying QTLxE for yield) can be explained by the different sensitivity of the alleles to environmental conditions such as temperature during different parts of the crop cycle [11, 72].

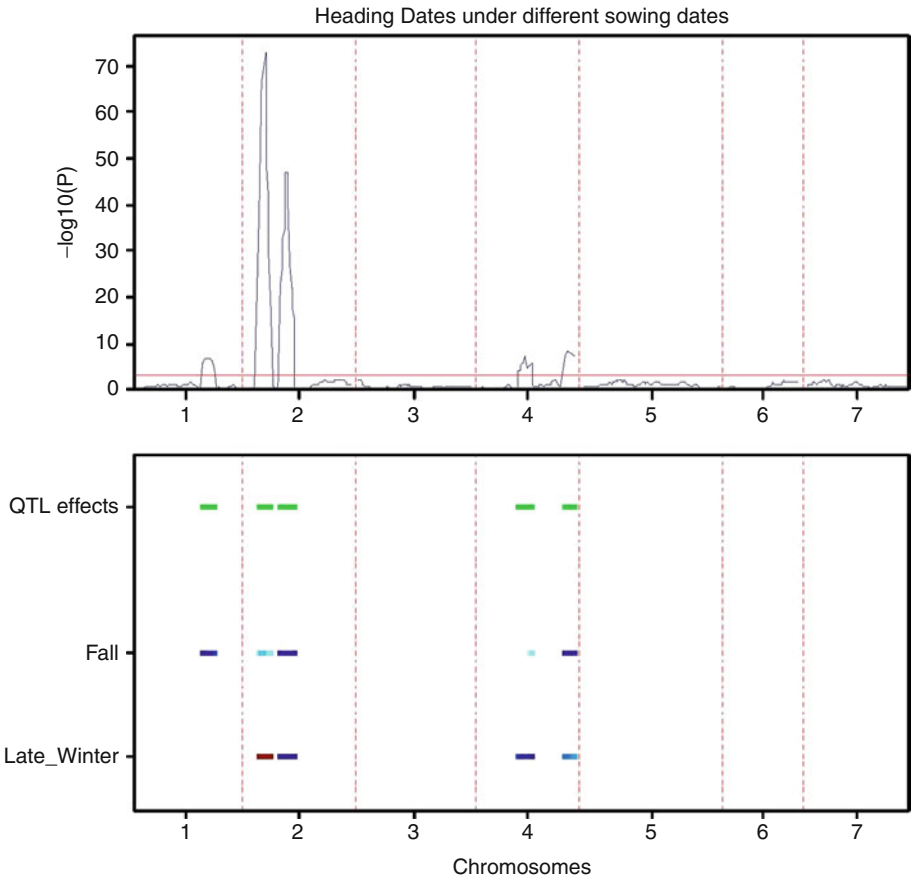
In the last decade, candidate genes have been identified for major loci controlling flowering time in barley and wheat: The photoperiod responsive gene *Ppd*-H1 in barley and its wheat homologues *Ppd*-D1, *Ppd*-B1, and *Ppd*-A1 are *PRR*-like genes [73, 74]. In both species, the photoperiod-responsive allele accelerates flowering under long-day conditions, but in barley, the greatest differences between sensitive and insensitive alleles are found under long-day conditions or high latitudes, while in wheat, under short day conditions or low latitudes [49, 64, 75, 76]. *HvFT3* is the candidate gene for another gene related to photoperiod in barley, *Ppd*-H2, whose active allele is expressed and accelerates flowering only under short photoperiod or low latitudes [75, 77]. The vernalization genes *VRN*-H1 and its homologues *VRN*-A1, *VRN*-B1, and *VRN*-D1 in wheat are MADS-box transcription factors similar to *APETALA1* in *Arabidopsis* [78–80]. *HvZCCT* and *TaZCCT* are the candidate genes for *VRN*-H2 and its wheat homologue *VRN*-Am2, respectively [81, 82]. The alleles at these loci and their interactions determine the sensitivity to vernalization (e.g., [82, 83]). Finally *VRN*-H3 and its homologues *VRN*-A3, *VRN*-B3, and *VRN*-D3 are *FT*-like genes, which also interact with PPD and VRN genes [77, 84, 85]. Other reported genes that determine differences in heading time are the "earliness per se" loci (*eps*) identified in barley by Laurie et al. [75], the series of "early maturing" (*Eam*) loci [86–89], and the gene HvAP2 [90]. However, except for the latter, no candidate genes have been found yet for them and their role is much less clear.

Figure 2 shows the location of the mentioned loci for barley, as well as for some other genes which are homologues to flowering genes in rice and *Arabidopsis* but whose effect on heading is unknown in barley. In wheat, other less characterized loci have also been identified, as the gene *Eps*-2B on 2BS [91, 92]; *Eps*-Am on 1AL sensitive to temperature [59, 60]; *VRN*-D4 close to the centromere in 5D [93], and other earliness per se genes on 5AL [94]. Additionally other loci have been found to have an effect on heading time in different regions than the loci mentioned above, although most of them with smaller effects: by the use of aneuploids in wheat [49, 95] or through QTL mapping both in barley (e.g., [35, 66–69]) and wheat (e.g., [92, 96–98]). These studies would confirm that heading time is under a strong but complex genetic control [49, 95]. Although particular VRN and PPD alleles may be more frequent in some geographical areas, variation has been found between genotypes within regions, so it is possible finding different combinations of VRN and PPD alleles in successful genotypes well adapted to particular regions, which would reinforce the idea that several



Genotype by Environment Interaction and Adaptation. Figure 2

Barley consensus function map showing the location of the vernalization, photoperiod, and earliness per se loci described in the text, as well as some other genes which are homologues to flowering genes in rice and *Arabidopsis*, whose direct effects on heading are still unknown in barley. Distances are given in Kosambi cM and linkage groups are oriented with short arms at the *top*



Genotype by Environment Interaction and Adaptation. Figure 3

Genome scan for heading date for the Steptoe × Morex doubled haploid population grown in fall and late winter sowing in Spain in 2009. *Top*: $-\log_{10}$ (*p* values) for the test on QTL+QTL.E effects are shown. The *red horizontal line* indicates the 5% genome-wide significance threshold. *Bottom*: *Upper most line* in *green* gives all genomic positions for which null hypothesis of no QTL+QTL.E is rejected. For the fall and late winter sowing environment, all positions for which there is environment-specific QTL expression are indicated with colors: *blue* showing that the allele from Steptoe delays heading, while *red*/*brown* shows that the Morex allele delays heading

other genes may be important in the control of flowering time [64]. As sensitivity to vernalization expresses at earlier stages of development than that to photoperiod, the fact that different combinations of VRN and PPD alleles may confer a similar time to heading or anthesis may also open room for fine-tuning developmental partitioning of a certain time to flowering into different lengths of vegetative and reproductive phases, which might be relevant in improving adaptation (see below).

A very simple quantitative genetic analysis of heading date (HD) for the Steptoe × Morex doubled haploid barley population [35] sown in fall and late winter in 2009 in Spain can be deduced from Fig. 3 which also illustrates alternative types of QTLxE interactions. In the top part of the figure there is, for a MET situation, a whole genome scan according to a composite interval mapping strategy [99] as implemented by Biometris, Wageningen University and Research Center, in GenStat (version 13th, [100]). All markers in the seven barley chromosomes are represented in sequential order on the X-axis. On the Y-axis is the *p* value, expressed on a minus logarithmic scale, for the successive regression models,

including not just the marker or position of interest, but additional markers that act as cofactors. With $-\log_{10}$ (p value) increasing, the evidence for a QTL at that position becomes larger. The bottom part of the figure shows firstly, in green, a one-dimensional summary of the profile in the upper panel, that is, all positions for which the joint null hypothesis of no QTL main effect and QTLxE interaction was rejected. Below the overall test for QTL effects across environments, for each individual environment, in this case defined by fall and late winter planting, an approximate test for environment-specific QTL effects is given in yellow-brown-red (QTL allele second parent increases trait) or light blue-dark blue (QTL allele first parent increases trait). Two major QTLs seem to determine heading date for the genotypes in these two trials, both on the short arm of Chromosome 2H, corresponding to two known genes, Ppd-H1 and Eam6, on Fig. 2. A very strong qualitative or crossover interaction QTLxE interaction is shown for Ppd-H1; the Morex allele (yellow-red) in the late winter sowing (under long-day photoperiod) delays heading, whereas the Steptoe allele at this locus (blue) delays heading under short days on the fall sowing. Non-crossover interaction is shown for Eam6. The presence of the Steptoe allele always delays heading, but more under fall sowing (darker blue effect) than under late winter sowing. Other minor QTLs are shown in chromosomes 1H and 4H.

Genetic Factors Controlling Duration of Subphases of Time to Flowering

The effect of these genes or QTLs may vary not only due to different conditions in temperature and photoperiod, or to epistatic interactions with other genes or QTLs, but also they may have different effects on the different phases of the crop cycle. This may be interesting for improving both adaptability and yield potential. Studying the genetic control of different pre-heading phases could bring about a better understanding of crop development patterns and more tools to fine-tuning it. For example, some adaptative characters, such as the avoidance of late frosts in spring, could be better assessed by knowing the duration of the phase from sowing to terminal spikelet rather than total time to anthesis (e.g., [101]). Moreover extending the duration of stem elongation, without modifying total time to anthesis, which is a key trait for adaptability as shown above, has been proposed as a trait to further increase yield potential [102, 103]. This has been proposed because the stem elongation phase is critical for yield determination, as the number of fertile florets at anthesis, which determines the final number of grains, is set during this phase [104, 105].

Several authors have shown that there is partially independent variability between different pre-heading phases (variability in pre-heading phases between genotypes with similar time to heading), both in wheat [106–108] and barley [109–114]. Other authors have shown that responses to vernalization, photoperiod, and temperature can each differ greatly among genotypes and between phases [50, 62, 115, 116]. In some studies using chromosome substitution lines, near isogenic lines and/or single chromosome recombinant lines, hexaploid wheat Ppd-D1 and Ppd-B1 alleles had different effects on the duration of pre-heading phases and on their response to photoperiod, although results seemed to depend on the genetic background and the environmental conditions of each experiment (see results and review by [117]). Recently Lewis et al. [61] found that alleles of a cultivar and a wild line of *Triticum monoccocum* for Eps-Am had different effects on the leaf initiation and the spikelet initiation phases (due to different sensitivity to temperature), but not on stem elongation, while they had little effect on total time to heading. On the other hand, many of the QTLs responsible for a different genetic control between pre-heading phases had little or no effect on total time to heading, so they may be more difficult to detect when assessing only heading time [111, 118]. Some of these differences in the length of pre- and post-heading phases were maintained under different conditions of photoperiod and temperature [119].

Statistical Approaches for GE Characterization

Means across environments are adequate indicators of genotypic performance only in the absence of crossover GE. When present, the use of means across environments ignores the differential reaction of genotypes to environmental changes. In an analysis of variance, introduction of the GE interaction term, $(GE)_{ij}$ for $i = 1$ to g genotypes and $j = 1$ to e environments, creates

as many parameters as there are GE combinations, making predictions of phenotypic responses for environments that were not in the set of trial environments impossible. Most approaches for the study of GE interaction and adaptation depart from ANOVA models with GE interaction terms and are therefore purely empirical descriptions of phenotypic performances of a set of genotypes across a fixed sample of environments. However, if the physiological or environmental underlying causes determining GE interaction can be determined, identification of genotypes better adapted to certain specific environmental conditions would be possible and, thus, larger genetic gains would be achievable. Furthermore, if the traits conferring adaptation and their genetic control are revealed, direct implementation in breeding may be feasible.

This entry reviews three types of statistical approaches used in GE interaction for breeding and variety development: (1) regression on the environmental mean, best known as Finlay–Wilkinson regression, or joint regression analysis; (2) linear-bilinear models, like AMMI and GGE; and (3) factorial regression models (see specific references for these methods below). These methods differ not only on the information they provide, but also in their predictive ability for breeding. A discussion of these three types of models from a common statistical perspective can be found in [120, 121]. The approaches aim at substituting the $(GE)_{ij}$ term by a linear or bilinear approximation using fewer parameters (Table 1). The replacement of double-indexed ANOVA GE interaction parameters by single-indexed regression and bilinear parameters introduces predictive properties.

Regression on the Mean

The most widely used and abused statistical method in breeding programs for characterizing GE has been the regression-on-the-mean analysis first proposed by Yates and Cochran [122] and made popular by Finlay and Wilkinson [123] (FW), and also named joint regression analysis. This method summarizes phenotypic responses to environmental changes as straight lines differing in both intercept (related to genotypic main effect) and slope (which estimates environmental sensitivity); GE interaction is revealed by differences in the slopes of individual genotypes. These straight lines are produced upon regressing individual genotypic means per environment on average site performance across all genotypes in that environment, where the regression is done across the full set of environments.

The rationale behind FW is that in the absence of explicit environmental information, a good estimate of the agronomical value of any environment may be given by the average phenotypic performance of all genotypes in that environment. This method has an important conceptual drawback. Two environments may have a similar low average yield for two completely different agroecological reasons, for example, presence of a disease and an episode of a late spring frost just before flowering. This model assumes the genotypic sensitivity to these two stresses to be approximately the same when the different stresses produce the same environmental means. Therefore, the use of the model is best restricted to those rare cases in which environmental differences are driven by just a single major biotic or abiotic factor; in these cases, the linear regression on the mean model may reflect linear differences in relation to the predominant stress factor. However, if environmental differences are due to a major stress, why not using, rather than the average phenotypic value at every environment, a direct estimate of the genotypic sensitivity to this stress as in the factorial regression method described below?

Regression-on-the-mean models are conceptually simple: The differential genotypic responses are summarized by their slopes, but it is very important to point out that their value and use should depend on the proportion of GE sum of squares that can be described by the differential environmental sensitivities of the genotypes. Figure 4 presents an example for which the Finlay and Wilkinson model should have never been used; however, it has been presented in this entry as similar reports are still too often seen in many publications. It summarizes a small MET consisting of seven barley varieties (Var_1 to Var_7) grown at ten Spanish environments according to model III in Table 1. In the part of this figure, there are the simple linear regression models for the seven varieties. If nothing else is shown, it can be wrongly assumed that there are substantial differences among genotypic slopes. This is also shown on the top table that includes

Genotype by Environment Interaction and Adaptation. Table 1 Overview of statistical models for GE analyses from two-way genotype by environment table of means derived from MET

General model	Specific model	Model	Data required	Statistical models for $E(Y_{ij}) - \mu$	Key information provided[a]
Reference models	*Additive*	I	Phenotypic data[b]	$G_i + E_j + e_{ij}$	Average cultivar yields
	Full interaction	II	Phenotypic data	$G_i + E_j + (GE)_{ij}$	Departures from additivity for each environment
Regression on the mean	*Finlay and Wilkinson*	III	Phenotypic data	$G_i + E_j + \beta_i E_j + e_{ij}$	Cultivar sensitivity (in form of slopes) to changes in environmental productivity
Bilinear models	*AMMI*	IV	Phenotypic data	$G_i + E_j + \sum_{k=1}^{K} a_{ki} b_{kj} + e_{ij}$	Joint adaptation patterns of genotypes to environments
	GGE	V	Phenotypic data	$E_j + \sum_{k=1}^{K} a'_{ki} b'_{kj} + e_{ij}$	Identification of the "winning genotype" for each uniform subset of environments
Factorial regression models	*Factorial regression model*	VI	Phenotypic and environmental data	$G_i + E_j + \beta_i z_j + e_{ij}$	Cultivar sensitivities (β_i) to changes in any environmental variable z
	Genotypic factorial regression model: QTL.E model	VII	Phenotypic and genotypic (marker information) data	$x_i\rho + E_j + x_i\rho_j + e_{ij}$	Marker (x) potentially associated to QTL and to QTL.E and the corresponding QTL (ρ) and the QTL.E (ρ_j) effects[c]
	Integrated factorial regression model	VIII	Phenotypic, genotypic, and environmental data	$x_i\rho + E_j + x_i(\lambda z_j) + e_{ij}$	QTL sensitivity to changes in environmental variable z[d]

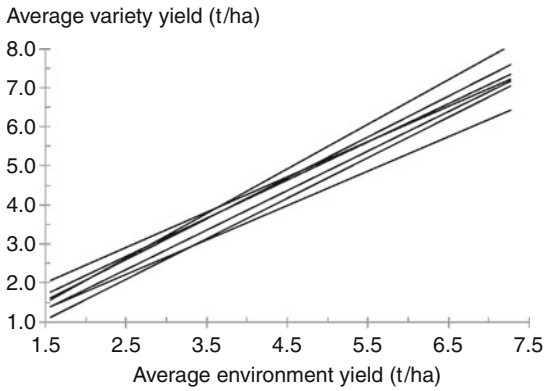
[a]See text for a more detailed discussion of each model

[b]Phenotypic response of the $i = 1...g$ genotype at the $j = 1...e$ environment

[c]In the presence of QTL.E, ρ_j adjusts the average QTL expression across environments, ρ, to a more appropriate level for the individual environment j. This model can be easily extended to x_s markers throughout all the genome

[d]λ is a constant that determines the extent to which a unit change in z, an environmental covariable, influences the effect of a QTL allele substitution. This model can be easily extended to x_s markers and z_t environmental variables

regression estimates. When independent simple linear regression analyses are fitted for the seven genotypes, the slopes varied from 0.88 to 1.14 and the individual straight lines were very significant (R^2 from 84% to 98%; p values from 1.8×10^{-04} to 7.1×10^{-08}). However, these R^2s do not mean anything in the GE context. They simply confirm that the genotypic yield increases with the mean environmental yield, which is obvious in the way that this model is built. Based on these estimates, it can be wrongly stated, for example, that Var_3 (slope equal to 1.14) apparently benefits more to improvements in the overall productivity of the environment than Var_6 (1.01) and particularly than Var_2 (0.88) which, with the lowest sensibility, does worst than expected. However, this model is completely inadequate for this MET and the previous estimates are useless and misleading and should have never been determined. The standard errors of the slopes, which can be used to assess the significance of the differences among slopes, ranges from 0.06 to 0.14, with an average standard error of the difference equal to 0.16. They are too large for detecting significant



Estimates	Var_1	Var_2	Var_3	Var_4	Var_5	Var_6	Var_7
Mean (t/ha)	4.21	3.6	4.41	4.32	3.71	3.92	4.23
Slope (s.e.)	0.98 (0.08)	0.88 (0.11)	1.14 (0.06)	0.90 (0.14)	1.04 (0.14)	1.01 (0.09)	1.05 (0.08)
R^2	0.95	0.88	0.98	0.84	0.88	0.94	0.95

Source of variation	Degrees of freedom	Sum of Squares	Semipartial R^2 (%)	Mean Squares	F-value	*p*-value
E	9	210.58	89.0	23.40	63.08	<0.0001
G	6	5.97	2.5	1.00	2.68	0.0237
GE	54	20.03	8.5	0.37		
Heterogenity of slopes	*6*	*1.42*	*7.1*	*0.24*	*0.61*	*0.7207*
Residual	*48*	*18.61*	*92.9*	*0.39*		

Genotype by Environment Interaction and Adaptation. Figure 4
Inappropriate use of the Finlay and Wilkinson analysis for a MET consisting of seven barley genotypes grown in ten environments in Spain

differences between genotypic slopes. Furthermore, joint regression analysis of variance table (bottom part of Fig. 4) shows that the observed differences among the genotypic slopes (Heterogeneity of slopes) only explains 7.1% of the GE sum of squares, which is not statistically significant (p value = 0.721).

Bilinear Models (AMMI and GGE)

The usefulness of the integration of ecophysiological and statistical tools in the interpretation of GE interaction is examined based upon the joint application of two multiplicative models for interaction: the additive main effects and multiplicative interaction (AMMI) model [6], and the factorial regression model [120, 124]. Both provide information and insight beyond the classical analysis of variance of two-way genotype by environment tables. AMMI represents an empirical approach (based on yield itself) to analyze GE interaction. Factorial regression attempts to describe interaction by including external genetic, phenotypic, and environmental information (e.g., morphophysiological traits, climatic data, etc.) on the levels of the genotypic and environmental factors. It implies a more analytical approach to the understanding of GE.

The Finlay and Wilkinson model belongs to a wider class of statistical models named linear-bilinear which estimate genotypic sensitivities to one or more environmental characterizations that are just linear functions of the phenotypic data [124–127]. However, the additive main effects and multiplicative interaction (AMMI) model [128–131] and the GGE models [132, 133] represent more powerful, and thus, useful examples of linear-bilinear models in plant breeding. These two model classes generate for every genotype and for every environment a series of K scores, which

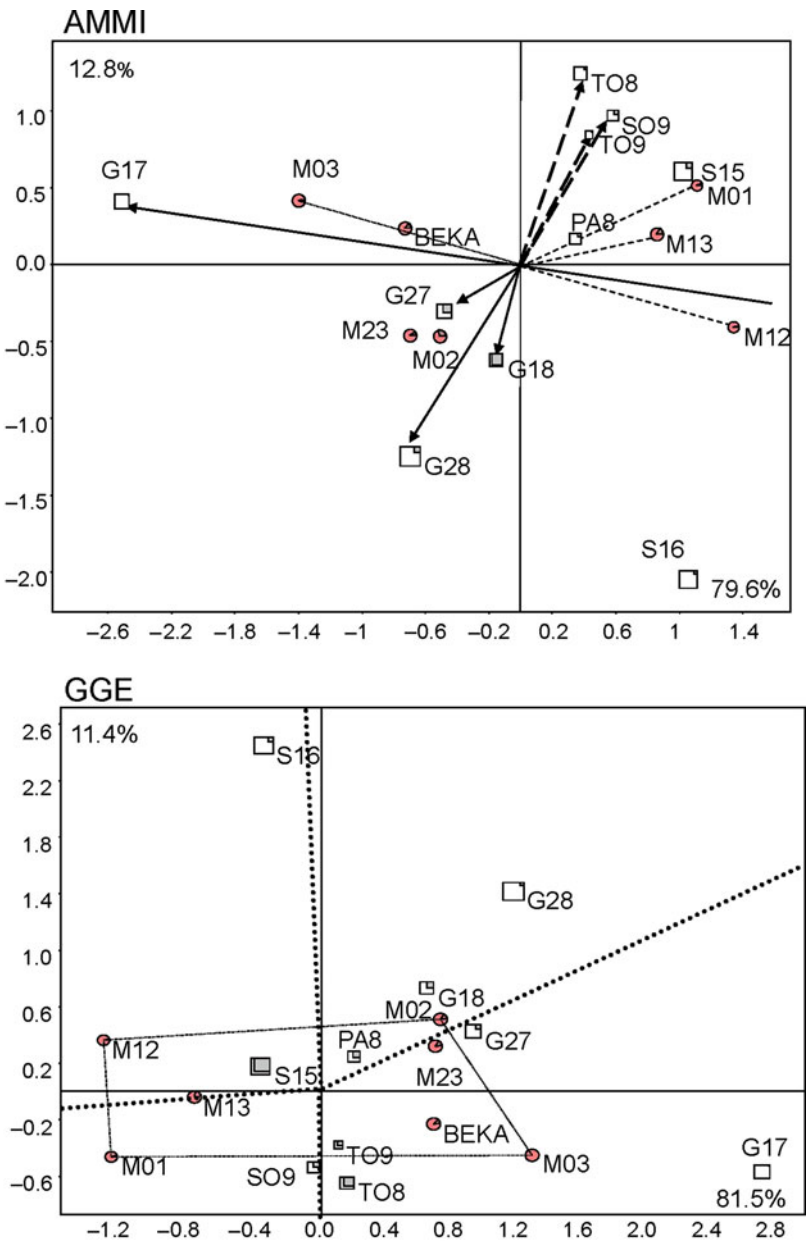
summarize the differential sensitivity of the genotypes to the prevalent, and typically unknown, stresses present in the analyzed MET.

The AMMI model successively partitions the $(GE)_{ij}$ interaction term from the basic ANOVA reference model into a series of K multiplicative terms or products of the form $a_{ki}b_{kj}$, where, for the kth term, a_{ki} refers to the genotypic sensitivity of genotype i to an hypothetical environmental variable b_k, which has value b_{kj} in environment j (Table 1, model IV). Alternatively, b_{kj} refers also to the environmental potentiality of environment j to an hypothetical genotypic variable a_k, which takes value a_{ki} for genotype i. The K hypothetical environmental (genotypic) variables have the property of discriminating maximally between genotypes (environments). The number of multiplicative terms to be retained for an appropriate estimate of the GE interaction, K, can be estimated in various ways, see, for example, Gollob [130], Gauch [6], and Cornelius [134]. From a practical point of view, the AMMI model is fitted in two steps. First, an additive ANOVA model is fitted containing the main effects for G and E and then the residuals from the additive model are used to construct the GE interaction matrix. This interaction matrix is then subjected to a singular value decomposition that generates the above-introduced genotypic and environmental scores [128, 130, 131].

Key outputs of the AMMI analysis are the genotypic and environmental scores for the K retained axes, along with the proportions of the interaction sum of squares explained by the multiplicative terms. The output of the $K = 2$ AMMI model, retaining just the first two interaction axes (IPCA1 and IPCA2), can be directly visualized by means of a biplot [5, 128, 135]. If both axes together explain most of the GE interaction, interpretation of the biplot is very simple and potentially extremely useful for understanding GE interaction. The ith genotype is placed in the biplot according to the (a_{1i}, a_{2i}) genotypic scores; similarly, the jth environment is defined by its two IPCA environmental scores (b_{1j}, b_{2j}). Distance of a genotype or environment to the origin is proportional to the GE interaction generated by that genotype or environment, respectively. Genotypes placed close together show similar adaptation patterns. Close environments generate similar GE interactions.

The actual interaction of genotype i in environment j can be estimated by the projection of the genotype position (a_{1i}, a_{2i}) on the jth environmental vector that goes from the origin (0,0) to (b_{1j}, b_{2j}), that is the line that goes through the origin with slope equal to b_{2j}/b_{1j}. The distance between the genotype projection on the line to the origin also provides information about the absolute magnitude of the interaction of genotype i in environment j. Genotype i will be well adapted to environment j, that is, positive interaction, if the projection is in the direction of the environmental vector and negative otherwise. The sign of the interaction of the genotype i in environment j can be estimated by the cosine between the ith genotypic and the jth environmental vector. It will be positive if both vectors form acute (close to 0°) angles, negative if the angle is obtuse (close to 180°), and nonexistent (no interaction) if they form a right angle (close to 90°). In a similar way, two environments whose vectors form an acute angle generate a similar type of GE interaction across genotypes, the environments have positive genetic correlation. If the two environmental vectors form an angle close to 180°, whichever genotype is well adapted in one environment will be poorly adapted to the other, the environments have a negative environmental interaction. Finally if both environmental vectors form a right angle, the genotypic behavior at one environment will be independent of the behavior at the other site, the genetic correlation is zero.

The upper part of Fig. 5 shows an AMMI biplot generated by a set of seven genotypes grown at ten environments. The genotypes are shown by circles and they represent a barley variety Beka, three derived single nonallelic mutants, M01, M02, M03, and the three binary mutant combinations, M12, M13, M23. The environments are shown in the biplot by squares which represent location by year combinations across Spain. Production of these mutants and analysis of these data was presented elsewhere [136, 137]. In this MET, the GE interaction is well described by the AMMI $K = 2$ model, as both axes explain together more than 90% of the GE sum of squares. The average yield of each environment and genotype is shown proportional to the area of its corresponding symbol. Within each symbol there is a, generally small, darker sector that represents the proportion of its sum of squares not



Genotype by Environment Interaction and Adaptation. Figure 5

AMMI and GGE biplots for a MET consisting of seven barley genotypes grown at ten environments in Spain (Data taken from [105]). See text for a detailed description of genotypes and environments

explained by this model. In this case all environments are well represented except for G27 y G18, which generate GE interactions not correctly described by the AMMI $K = 2$ model. Beka is placed close to the origin and, thus, it is the genotype that interacts least with the ten environments; on the contrary, M12 and M03 are the two genotypes that interact most with the environments. G17 and S16 are the two environments which showed the largest GE interaction, that is, whose genotypic yields depart most from their averages. PA8, near the origin, produced yields close to the average across all environments.

The relative position of both genotypes and environments can provide some clarification on the nature of the GE interactions in this MET. The first IPCA seems to be associated with differential behavior of genotypes carrying the first mutation, M01, M12, and M13, with positive scores in comparison to the other genotypes. These mutants are particularly poorly adapted to Granada (G in the biplot, especially G17). The second axis, which is quantitatively less important, seems associated with mutant 2 (M02, M12 y M23), which shows negative scores on this axis, whereas the other genotypes have positive scores; the specific adaptation of this mutant to the environments is not as clear.

The angle formed by any two environmental vectors is related to the relative similarity among environments, say, the genetic correlation, as determined by the genotypic yields. In this case, the relative yields of the genotypes in Toledo (TO8 y TO9) seem very similar to Soria (SO8). They all form acute angles with cosine and correlation close to 1. T09, with a smaller size square, had lower yields that the others. By comparing the angle of these three environmental vectors with the vector determined by G28 (very obtuse angle closed to 180° and cosine and correlation close to −1), it can be deduced that those genotypes that behave relatively well in G28 perform poorly in the other three sites and vice versa. The analysis of the genotypic projection on environmental vectors gives clues about specific adaptation patterns. For example for G17, M03 showed a good adaptation to this environment, whereas M12 was particularly poorly adapted there. This AMMI analysis was done on the MET data used for the analysis in Fig. 4. Whereas the Finlay and Wilkinson method was able to explain only 7% of the GE sum of squares, the AMMI model for $K = 2$ retained 90% of the GE sum of squares. Furthermore, as described in the previous paragraphs, the known structure of the seven genotypes developed through artificial mutagenesis, suggested a model with a plausible genetic meaning.

The environmental and genetic scores are simple statistical estimates derived from MET phenotypic data, without any direct physiological meaning. However, these empirical estimates can be associated to physiological processes by correlating the environmental scores to explicit environmental measurements, such as soil or meteorological variables; these correlations can often provide meaningful agroecological information about the nature of GE interactions [11, 14, 138–140].

Another member of the linear-bilinear model class is the GGE model [132, 133], in which single value decomposition is done on the sum of the G and GE components by just subtracting the environmental means (environmental centered) on the two-way table of means (Table 1, model V) rather than on GE interactions alone, as done in AMMI. A GGE biplot for $K = 2$ provides additional information of potential interest to breeders, as it allows for the direct identification of the "winning" genotype in any potentially uniform subset of environments. To do so, the most extreme genotypic scores are connected delimiting an irregular polygon enclosing all other genotypes, that is, a convex hull is constructed. In the previous example (Fig. 5, bottom) this is an irregular quadrilateral defined by M12, M02, M03 y M01. Next, lines perpendicular to each side of the polygon/convex hull are drawn (thicker lines in Fig. 5, bottom) up to the boundaries of the biplot. In this way sectors are created, called mega environments, which contain environments that behave relatively uniform with respect to the genotypes. The "winning genotype" in a mega environment is the genotype that is placed at the vertex of the polygon inside that mega environment. For example, M12 is the best-adapted genotype in the mega environment defined by S15 and, particularly, S16. Mutant M03 is the most productive genotype in G18 and G28. Of course, this interpretation is subjected to the condition that most of G+GE variability is retained in the first two GGE axes.

Factorial Regression Models

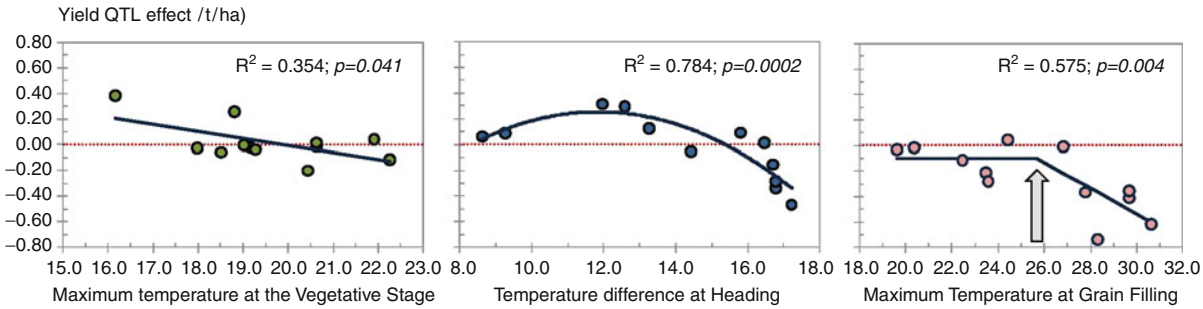
Factorial regression models were developed to incorporate additional explicit environmental information (variable z in Table 1 model VI) into a model [120, 121] for GE interaction and estimate the genotypic sensitivity of each of g genotypes (β_i in Table 1 model VI) to these independent variables (regressors, covariables). The regression on the mean or FW analyses reported before may be seen as a specific case of factorial regression, in which the average yield in each environment is used as an explicit environmental characterization. In the general form, any explicit agroecological variable

individually recorded for each environment could be used as independent explanatory variable. Average yield can be a reflection of a certain meteorological variable, such as available soil water. In this situation, this variable recorded for each environment could be used as explanatory independent variable to describe GE interaction (variable z in model VI Table 1). The genotypic slopes will have a more direct physiological meaning when they estimate, for example, sensitivity to changes in available soil water, which is an approximation to water use efficiency. In a triticale MET, GE interaction for grain yield was regressed on soil pH and the genotypic slopes directly assessed the sensitivities to changes in soil pH [141]. Extension to multiple environmental variables and complex response curves is conceptually simple and easily computable using standard statistical packages. As for any multiple regression models, a central question is the choice of variables for description of GE interaction. Continuous monitoring of the environment generates huge numbers of environmental covariables, which will complicate identification of the most relevant ones. Purely statistical selection procedures often lead to physiologically incomprehensible models. Therefore, agroecological insights of genotypes and environments should augment and prevail over purely statistical considerations. A helpful prescreening of environmental covariables can sometimes be done by correlating covariables to scores derived from AMMI or GGE analyses [11].

Factorial Regression Models Incorporating Explicit Genotypic Information Genotypic covariables can also be used to partition the G and GE terms. Molecular markers such as DNA polymorphisms for anonymous sequences or for functional genes are the most useful and readily available genetic covariables. For a codominant marker in a diploid species with potential genotypes *AA*, *Aa*, and *aa*, the number of *A* alleles (2, 1, and 0 to represent genotypes *AA*, *Aa*, and *aa*, respectively) could be used as a genetic covariable, x, in a factorial regression model (Table 1, model VII). If multiple markers across the whole genome are sequentially used, factorial regression has the ability to detect, locate, and estimate QTL main effects and QTL by environment interactions. For marker positions adjacent to a QTL, the ρ slope in model VII (Table 1) estimates directly the effect of a QTL allele substitution. Similarly, the $(GE)_{ij}$ interaction can be further partitioned into a term for differential QTL expression across environments, ρ_j, and a residual GE interaction. For a full genome scan, factorial regression models can be fitted on grid of genomic positions, on markers and in between markers, when necessary. Virtual markers, in between observed markers, can be easily generated from flanking marker information (see [142]). Factorial regression models which include genetic covariables can be potentially used for any set of genotypes for which genetic predictors can be constructed, from standard biparental offspring populations and unrelated diverse association panels, to more complicated intercross systems, such as MAGIC [37], NAM [38, 39], and AMPRIL [40] described before. The QTL.E interaction model shown in model VII (Table 1) is based on application of a simple marker regression to our data. To construct multiple QTL models, a composite interval mapping approach can be followed by incorporating cofactors, or markers that correct for QTL elsewhere, on the genome.

Factorial Regression Models Incorporating Explicit Environmental and Genotypic Information The final goal of any MET is to understand the nature of GE interaction in terms of differential sensitivity of the different QTLs or genes to external environmental variables. This is also possible by means of factorial regression models [11, 13, 72, 92]. Differential QTL expression for environments, ρ_j, can be regressed on any environmental covariable, z, to relate the differential QTL expression directly to key environmental variables responsible for GE. This is done by substituting the QTL.E term, $x_i\rho_j$, with a linear regression $x_i(\lambda z_j)$ and a residual term. λ is a constant that determines the extent to which a unit change in z, the environmental covariable, influences the effect of a QTL allele substitution. The statistical model used is listed as model VIII in Table 1 which can be easily extended to multiple markers (x_{si}) and various environmental variables (z_{tj}).

Van Eeuwijk et al. [143, 144] and Boer et al. [99] provide examples of differential QTL expression in maize data to environmental variables; by incorporating marker information and environmental covariables describing the environment,



Genotype by Environment Interaction and Adaptation. Figure 6

Differential sensitivities of three major QTLs to temperature, recorded at three different growing periods for the Steptoe × Morex doubled haploid population (Data taken from the North American Barley Genome Project). Twelve sites with environmental characterizations were available. Three different models were used: a straight-line regression model, a second-degree polynomial, and a "broken-stick" factorial regression model

these models allow for prediction of differential genotypic sensitivities to environmental changes. An example of the output of these fully integrated genotypic and environmental models is shown in Fig. 6, which shows an analysis for the "Steptoe × Morex" double haploid population data from the North American Barley Genome Project, grown at 12 sites and with environmental covariables at hand. Three main QTLs were responsible of GE interaction [71]. Differential QTL effects across environments could be associated to three different environmental variables related to temperature taken at three different growth periods and according to three alternative models: a simple linear regression model, a second degree response, and a "broken-stick" model (Fig. 6). Furthermore, two out of three QTL.E interactions showed a "crossover" type interaction: The sign of the QTL effect changed according to the value of the environmental external variable. This figure clearly illustrates the importance of QTL.E interaction for complex traits such as grain yield in barley.

The Mixed Model Framework: Modeling Variance-Covariance Structures

Table 1 shows different alternatives for modeling the expected responses of a genotype to environmental changes, without any specific concern about the implicit assumptions of the analyses of variance. Standard linear models take for granted that error terms are independent and have constant variance. However for MET, these assumptions are overly simplistic as variances within environments and correlations between environments tend to be heterogeneous. For the sake of brevity and simplicity, how the mixed model framework also allows for modeling of the variance-covariance component of the data has not been described. However, the optimal statistical modeling for MET data should focus first in finding an adequate variance-covariance model for the random terms and then, as discussed above, search for a parsimonious model for the expected responses. Choice of variance-covariance model can have strong implications. In the case of QTL modeling, QTL may erroneously be declared significant or nonsignificant because of over or under estimation of effect sizes and standard errors [72, 145]. The mixed model framework, which combines modeling of means and variances, provides a more appropriate modeling environment for GE and QTL.E interactions offering flexibility with regard to assumptions on heterogeneity of variances and on correlations across environments [17].

Computer Software for GE Analyses

Annicchiarico [3] lists a series of user-friendly computer software available for many GE analyses. CROPSTAT is a freely available package developed by the International Rice Research Institute [146] that has specific modules for FW and AMMI analysis. MATMODEL available in a free version [147] also provides AMMI and joint regression modeling and it

is particularly useful for handling missing data. INFOGEN [148] within the INFOSTAT system [149] also includes most described tools for the analysis of MET. At the same time, there are also dedicated commercial softwares, such as GGE BIPLOT [132], useful for joint regression, AMMI, and GGE. Obviously, all general statistical packages can easily be programmed to fit all linear-bilinear models described in this entry in a fixed model context, whereas some like GenStat, ASREML, and SAS also allow fitting mixed bilinear models. SAS instructions for many GE analyses are presented in Kang [100]. GenStat [150] includes specific procedures for FW, AMMI, and GGE analyses. Version 13 of GenStat (2010) also includes dedicated menus for QTL and QTL.E analyses for segregating crosses and for association analyses. GenStat has a policy of free licensing of older versions to institutions in developing countries and for educational purposes in the form of the GenStat Discovery version.

Future Directions

Plant breeding research experiences fast changes. Nowadays, at the genomic side, sequencing and single-nucleotide polymorphism (SNP) technology is becoming increasingly cheap for not only model species, but also for crop species. Besides information at the DNA level, genomic information at RNA, protein, and metabolite level starts to become common. As a consequence, huge amounts of data start to become available for characterizing genotypes at various genomic levels. Similar developments can be observed for monitoring the environment. Environmental characterizations can be stored over the growing season for all environmental factors that are believed to be relevant.

In the past, genotypic and environmental information was the bottleneck; however, the current focus has shifted to access to the right plant material and their correct phenotyping. High-throughput phenotyping techniques are being developed that facilitate monitoring of individual plants at arbitrary small intervals over the growing season. However, high-throughput phenotyping schemes taken in individual cell/tissue/organ/single plant level may not mean anything at the crop level. Up-scaling from processes taking place in a fraction of a second and in a fraction of space to relevant crop traits (produced in a hectare through several months) has consistently failed in the past and remains a challenge. Crop physiology can play a key role in understanding multi-trait interactions for up-scaling from gene to crop.

The strongly increased availability of phenotypic, genomic, and environmental information begs for new statistical techniques that allow the increased information to be used in an effective way. Various requirements can be defined. First, phenotypic information will increasingly concern a wide array of traits that are repeatedly measured over time. Correlations between these traits will need to be explicitly modeled, as will be the correlations between the repeated measurements for the same trait. Information from multiple environments can be treated in the same way as information from multiple traits, although correlations between the same trait in different environments may ask for other models than the correlations between different traits in the same environment and different traits in different environments. Standard mixed model procedures will fail, as too many variances and covariances/correlations will require estimation. A way out may be too regularize the pattern of variances and covariances by inserting biological information in the estimation in the form of alternative statistical tools, such as priors (Bayesian methods) or penalties (penalized multivariate regressions). One popular way of reducing the number of correlation parameters is by imposing network structures on sets of trait by environment combinations, thereby effectively fitting sparse matrices to the inverses of the correlation matrices. The graphical lasso is an example of such an approach [151].

Turning to increased marker numbers and selecting meaningful genotype to phenotype models in the face of 100,000s of SNP markers demand new statistical approaches. As identification of individually contributing SNPs in such conditions is very difficult, an alternative strategy emphasizing prediction from markers above identification of markers is rapidly gaining popularity. In genomic selection, the idea is to use all markers simultaneously for predicting marker-based breeding values that help in ranking individuals on genetic merit [152–154]. Bayesian and penalized regression techniques help to regularize the estimates for individual marker contributions, as it will be evident that with standard regression techniques it is impossible to estimate hundreds of thousands of

marker effects. Mixed models can in this context be interpreted as an example of a Bayesian technique in which the prior for the marker effects is a normal distribution. Equivalently, mixed models can be seen as penalized regressions in which the ratios of variance components determine the penalties (shrinkage factors). As an illustration, one may regress a phenotypic trait on a large set of markers, assuming the effects of the markers of individual chromosomes to follow normal distributions with chromosome-specific variances. The predicted values for the genotypes from such a mixed model represent the genomic breeding value. This breeding value can be used for selection purposes. Examples of genomic selection for multiple environments are still hard to find.

The increased information from intensive environmental monitoring can be used to improve prediction of genotypic performance by integrating it with other types of genotype-specific information in crop growth models [16, 17, 155–157]. The environmental information is fed into a suitable crop growth model and when physiological parameters of the crop growth model can be specified at genotype-specific level, the crop growth model can produce predictions for individual genotypes in any environment for which a full environmental characterization is given. An integration of crop growth modeling with genomic selection is possible when the values for the genotype-specific physiological parameters in the crop growth model are inserted from Bayesian or mixed genomic selection models.

The increased amounts of phenotypic, genomic, and environmental data pose strong demands on our statistical ingenuity, but interesting solutions start to appear on the horizon. In this forthcoming scenario, elaborations of mixed models, Bayesian techniques and penalized methods will play a major role in the analysis of GE interactions.

Bibliography

Primary Literature

1. Student (1908) The probable error of a mean. Biometrika 6:1–25
2. Annicchiarico P (2002) Genotype × environment interactions: challenges and opportunities for plant breeding and cultivar recommendations. FAO plant production and protection paper no. 174. FAO, Rome
3. Annicchiarico P (2009) Coping with and exploiting genotype-by-environment interactions. In: Ceccarelli S, Guimaraës EP, Weltzien E (eds) Participatory plant breeding. FAO, Rome, pp 519–564
4. Cooper M, Hammer GL (eds) (1996) Plant adaptation and crop improvement. CAB International, Wallingford
5. Fox PN, Crossa J, Romagosa I (1997) Multi-environment testing and genotype by environment interaction. In: Kempton RA, Fox PN (eds) Statistical methods for plant variety evaluation. Chapman and Hall, London, pp 117–137
6. Gauch HG (1992) Statistical analysis of regional yield trials. Elsevier, Amsterdam
7. Kang MS (1998) Using genotype-by-environment interaction for crop cultivar development. Adv Agron 62:199–252
8. Kang MS, Gauch HG (1996) Genotype by environment interaction: new perspectives. CRC Press, Boca Raton
9. Kempton RA, Fox PN (1997) Statistical methods for plant variety evaluation. Chapman and Hall, London
10. Romagosa I, Fox PN (1993) Genotype-environment interaction and adaptation. In: Hayward MD, Bosemark NO, Romagosa I (eds) Plant breeding, principles and prospects. Chapman and Hall, London, pp 373–390
11. Romagosa I, van Eeuwijk FA, Thomas WTB (2009) Statistical analyses of genotype by environment data. In: Carena M (ed) Handbook of plant breeding, vol 3, Cereals. Springer, New York, pp 291–331
12. van Eeuwijk FA (2006) Genotype by environment interaction: basics and beyond. In: Lamkey K, Lee M (eds) Plant breeding: the Arnell Hallauer international symposium. Blackwell, Oxford, pp 155–170
13. van Eeuwijk FA, Malosetti M, Yin X, Struik PC, Stam P (2005) Statistical models for genotype by environment data: from conventional ANOVA models to eco-physiological QTL models. Aust J Agr Res 56:883–894
14. Voltas J, van Eeuwijk FA, Igartua E, Garcia del Moral LF, Molina-Cano JL, Romagosa I (2002) Genotype by environment interaction and adaptation in barley breeding: basic concepts and methods of analysis. In: Slafer GA, Molina-Cano JL, Savin R, Araus JL, Romagosa I (eds) Barley science: recent advances from molecular biology to agronomy of yield and quality. Haworth Pres, Binghamton, pp 205–241
15. Paterson AH (1998) Molecular dissection of complex traits. CRC Press, Boca Raton
16. Cooper M, van Eeuwijk FA, Hammer GL, Podlich DW, Messina C (2009) Modeling QTL for complex traits: detection and context for plant breeding. Curr Opin Plant Biol 12:231–240
17. van Eeuwijk FA, Bink MCAM, Chenu K, Chapman SC (2010) Detection and use of QTL for complex traits in multiple environments. Curr Opin Plant Biol 13:193–205
18. Calderini DF, Slafer GA (1999) Has yield stability changed with genetic improvement of wheat yield? Euphytica 107:51–59
19. Foulkes MJ, Sylvester-Bradley R, Weightman R, Snape J (2007) Identifying physiological traits associated with improved drought resistance in winter wheat. Field Crop Res 103:11–24

20. Reynolds MP, Borlaug NE (2006) Impacts of breeding on international collaborative wheat improvement. J Agric Sci 144:3–17, Cambridge
21. Slafer GA, Araus JL (2007) Physiological traits for improving wheat yield under a wide range of conditions. In: Spiertz JHJ, Struik PC, van Laar HH (eds) Scale and complexity in plant systems research: gene-plant-crop relations. Springer, Dordrecht, pp 147–156
22. Fischer RA, Edmeades GO (2010) Breeding and cereal yield progress. Crop Sci 50:S85–S98
23. Reynolds MP, Foulkes J, Slafer GA, Berry P, Parry MJ, Snape JW, Angus WJ (2009) Raising yield potential in wheat. J Exp Bot 60:1899–1918
24. Slafer GA (2003) Genetic basis of yield as viewed from a crop physiologist's perspective. Ann Appl Biol 142:117–128
25. Siddique KHM, Belford RK, Perry MW, Tennant D (1989) Growth, development and light interception of old and modern wheat cultivars in a Mediterranean environment. Aust J Agr Res 40:473–487
26. Slafer GA, Andrade FH (1993) Physiological attributes related to the generation of grain yield in bread wheat cultivars released at different eras. Field Crop Res 31:351–367
27. Miralles DF, Katz SD, Colloca A, Slafer GA (1998) Floret development in near isogenic wheat lines differing in plant height. Field Crop Res 59:21–30
28. Miralles DJ, Slafer GA (1995) Yield, biomass and yield components in dwarf, semidwarf and tall isogenic lines of spring wheat under recommended and late sowing dates. Plant Breed 114:392–396
29. Richards RA (1996) Increasing yield potential in wheat – source and sink limitations. In: Reynolds MP, Rajaram S, McNab A (eds) Increasing yield potential in wheat: breaking the barriers. CIMMYT, Mexico, pp 134–149
30. Foulkes J, Slafer GA, Davies WJ, Berry P, Sylvester-Bradley R, Martre P, Calderini DF, Griffiths S, Reynolds M (2011) Raising yield potential of wheat. III. Optimizing partitioning to grain while maintaining lodging resistance. J Exp Bot 62:469–486
31. Denison RF (2009) Darwinian agriculture: real, imaginary and complex trade-offs as constraints and opportunities. In: Sadras VO, Calderini D (eds) Crop physiology. Applications for genetic improvement and agronomy. Academic, Burlington, pp 215–234
32. Grando S, Ceccarelli S (2009) Breeding for quantitative variables. Part 3: breeding for resistance to abiotic stress. In: Ceccarelli S, Guimaraës EP, Weltzien E (eds) Participatory plant breeding. FAO, Rome, pp 391–417
33. Cooper M, Fox PN (1996) Environmental characterization based on probe and reference genotypes. In: Cooper M, Hammer GL (eds) Plant adaptation and crop improvement. CAB International, Wallingford, pp 529–547
34. Rasmusson DC (1996) Germplasm is paramount. In: Reynolds MP, Rajaram S, McNab A (eds) Increasing yield potential in wheat: breaking the barriers. CIMMYT, Mexico, pp 28–37
35. Hayes PM, Liu BH, Knapp SJ, Chen F, Jones B, Blake T, Franckowiak J, Rasmusson D, Sorrells M, Ullrich SE, Wesenberg D, Kleinhofs A (1993) Quantitative trait locus effects and environmental interaction in a sample of North American barley germplasm. Theor Appl Genet 87:392–401
36. Comadran J, Thomas WTB, van Eeuwijk FA, Ceccarelli S, Grando S, Stanca AM, Pecchioni N, Akar T, Al-Yassin A, Benbelkacem A, Ouabbou H, Bort J, Romagosa I, Hacket CA, Russell JR (2009) Patterns of genetic diversity and linkage disequilibrium in a highly structured Hordeum vulgare association mapping population for the Mediterranean basin. Theor Appl Genet 119:175–187
37. Cavanagh C, Morell M, Mackay I, Powell P (2008) From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. Curr Opin Plant Biol 11:215–221
38. Buckler ES, Holland JB, Bradbury PJ et al (2009) The genetic architecture of maize flowering time. Science 325:714–718
39. Yu J, Holland JB, McMullen MD, Buckler ES (2008) Genetic design and statistical power of nested association mapping in maize. Genetics 178:539–551
40. Huang X, Paulo MJ, Boer M, Effgen S, Keizer P, Koornneef M, van Eeuwijk FA (2011) Analysis of natural allelic variation in Arabidopsis using a multiparent recombinant inbred line population. Proc Natl Acad Sci 108:4488–4493
41. Araus JL, Bort J, Steduto P, Villegas D, Royo C (2003) Breeding cereals for Mediterranean conditions: ecophysiological clues for biotechnology application. Ann Appl Biol 142:129–141
42. Araus JL, Slafer GA, Royo C, Serret MD (2008) Breeding for yield potential and stress adaptation in cereals. Crit Rev Plant Sci 27:377–412
43. Araus JL, Slafer GA, Reynolds MO, Royo C (2009) Breeding for quantitative variables. Part 5: breeding for yield potential. In: Ceccarelli S, Guimaraës EP, Weltzien E (eds) Participatory plant breeding. FAO, Rome, pp 449–477
44. Cattivelli L, Ceccarelli S, Romagosa I, Stanca M (2011) Abiotic stresses in barley: problems and solutions. In: Ullrich SE (ed) Barley: improvement, production, and uses. Wiley-Blackwell, Harrisongburg, pp 282–306
45. Sadras VO, Calderini D (2009) Crop physiology. Applications for genetic improvement and agronomy. Academic, Burlington
46. Loss SP, Siddique KHM (1994) Morphological and physiological traits associated with wheat yield increases in Mediterranean environments. Adv Agron 52:229–276
47. Passioura JB (2002) Environmental biology and crop improvement. Funct Plant Biol 29:537–546
48. Richards RA (1991) Crop improvement for temperate Australia, future opportunities. Field Crop Res 26:141–169
49. Worland AJ (1996) The influence of flowering time genes on environmental adaptability in European wheats. Euphytica 89:49–57
50. Slafer GA, Rawson HM (1994) Sensitivity of wheat phasic development to major environmental factors: a re-examination of some assumptions made by physiologists and modellers. Aust J Plant Physiol 21:393–426

51. Goldringer I, Prouin C, Rousset M, Galic N, Bonnin I (2006) Rapid differentiation of experimental populations of wheat for heading time in response to local climatic conditions. Ann Bot 98:805–817
52. Young KJ, Elliott GA (1994) An evaluation of barley accessions for adaptation to the cereal growing regions of western Australia, based on time to ear emergence. Aust J Agr Res 45:75–92
53. Hoogendoorn J (1985) A reciprocal F_1 monosomic analysis of the genetic control of time of ear emergence, number of leaves and number of spikelets in wheat (Triticum aestivum L.). Euphytica 34:545–558
54. Lasa JM, Igartua E, Ciudad FJ, Codesal P, Garcia EV, Gracia MP, Medina B, Romagosa I, Molina-Cano JL, Montoya JL (2001) Morphological and agronomical diversity patterns in the Spanish barley core collection. Hereditas 135:217–225
55. van Oosterom EJ, Acevedo E (1992) Adaptation of barley (Hordeum vulgare L.) to harsh Mediterranean environments. Euphytica 62:15–27
56. Álvaro F, Isidro J, Villegas D, García del Moral LF, Royo C (2008) Breeding effects on grain filling, biomass partitioning, and remobilization in Mediterranean durum wheat. Agron J 100:361–370
57. Jackson PA, Byth DE, Fischer KS, Johnston RP (1994) Genotype × environment interactions in progeny from a barley cross: II. Variation in grain yield, yield components and dry matter production among lines with similar times to anthesis. Field Crop Res 37:11–23
58. Van Oosterom EJ, Kleijn DM, Ceccarelli S, Nachit MM (1993) Genotype-by-environment interactions of Barley in the Mediterranean region. Crop Sci 33:669–674
59. Appendino ML, Slafer GA (2003) Earliness per se and its dependence upon temperature in diploid wheat lines differing in the major gene *Eps-A^{m}1* alleles. J Agric Sci 141:149–154
60. Bullrich L, Appendino ML, Tranquilli G, Lewis S, Dubcovsky J (2002) Mapping of a thermo-sensitive earliness per se gene on *Triticum monococcum* chromosome 1A^m. Theor Appl Genet 105:585–593
61. Lewis S, Faricelli ME, Appendino ML, Valárik M, Dubcovsky J (2008) The chromosome region including the earliness per se locus *Eps-A^{m}1* affects the duration of early developmental phases and spikelet number in diploid wheat. J Exp Bot 59:3595–3607
62. Slafer GA (1996) Differences in phasic development rate amongst wheat cultivars independent of responses to photoperiod and vernalization. A viewpoint of the intrinsic earliness hypothesis. J Agric Sci 126:403–419
63. Slafer GA, Rawson HM (1995) Base and optimum temperatures vary with genotype and stage of development in wheat. Plant Cell Environ 18:671–679
64. Cockram J, Jones H, Leigh FJ, O'Sullivan D, Powell W, Laurie DA, Greenland A (2007) Control of flowering time in temperate cereals, genes, domestication, and sustainable productivity. J Exp Bot 58:1231–1244
65. Eagles HA, Cane K, Vallance N (2009) The flow of alleles of important photoperiod basically and vernalisation genes through Australian wheat. Crop Pasture Sci 60:646–657
66. Baum M, Grando S, Backes G, Jahoor A, Sabbagh A, Ceccarelli S (2003) QTLs for agronomic traits in the Mediterranean environment identified in recombinant inbred lines of the cross 'Arta' × *H. spontaneum* 41-1. Theor Appl Genet 107:1215–1225
67. Bezant J, Laurie D, Pratchett N, Chojecki J, Kearsey M (1996) Marker regression mapping of QTL controlling flowering time and plant height in a spring barley (*Hordeum vulgare* L.) cross. Heredity 77:64–73
68. Li JZ, Huang XQ, Heinrichs F, Ganal MW, Röder MS (2006) Analysis of QTLs for yield components, agronomic traits and disease resistance in an advanced backcross population of spring barley. Genome 49:454–466
69. Tinker NA, Mather DE, Blake TK, Briggs KG, Choo TM, Dahleen L, Dofing SM, Falk DE, Ferguson T, Franckowiak JD, Graf R, Hayes PM, Hoffman D, Irvine RB, Kleinhofs A, Legge W, Rossnagel BG, Saghai Maroof MA, Scoles GJ, Shugar LP, Steffenson B, Ullrich S, Kasha KJ (1996) Regions of the genome that affect agronomic performance in two-row barley. Crop Sci 36:1053–1062
70. Cuesta-Marcos A, Casas AM, Hayes PM, Gracia MP, Lasa JM, Ciudad F, Codesal P, Molina-Cano JL, Igartua E (2009) Yield QTL affected by heading date in Mediterranean grown barley. Plant Breeding 128:46–53
71. Romagosa I, Ullrich SE, Han F, Hayes PM (1996) Use of the AMMI model in QTL mapping for adaptation in barley. Theor Appl Genet 93:30–37
72. Malosetti M, Voltas J, Romagosa I, Ullrich SE, van Eeuwijk FA (2004) Mixed models including environmental variables for studying QTL by environment interaction. Euphytica 137:139–145
73. Beales J, Turner A, Griffiths S, Snape JW, Laurie DA (2007) A *Pseudo-Response Regulator* is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). Theor Appl Genet 115:721–733
74. Wilhelm EP, Turner AS, Laurie DA (2009) Photoperiod insensitive Ppd-A1a mutations in tetraploid wheat (Triticum durum Desf.). Theor Appl Genet 118:285–294
75. Laurie DA, Pratchett N, Bezant JH, Snape JW (1995) RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley (Hordeum vulgare L.) cross. Genome 38:575–585
76. Jones H, Leigh FJ, Mackay I, Bower MA, Smith LMJ, Charles MP, Jones G, Jones MJ, Brown TA, Powell W (2008) Population-based resequencing reveals that the flowering time adaptation of cultivated barley originated East of the Fertile Crescent. Mol Biol Evol 25:2211–2219
77. Faure S, Higgins J, Turner A, Laurie DA (2007) The flowering locus T-like gene family in barley (Hordeum vulgare). Genetics 176:599–609
78. Fu D, Szűcs P, Liuling Y, Helguera M, Skinner JS, Zitzewitz J, Hayes PM, Dubcovsky J (2005) Large deletions within the first intron in *Vrn-1* are associated with spring growth habit in barley and wheat. Mol Genet Genomics 273:54–65

79. Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, Dennis ES (2003) MADS box genes control vernalization-induced flowering in cereals. Proc Natl Acad Sci 22:13099–13104
80. Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene Vrn1. Proc Natl Acad Sci 100:6263–6268
81. Distelfeld A, Tranquilli G, Chengxia L, Yan L, Dubcovsky J (2009) Genetic and molecular characterization of the Vrn2 loci in tetraploid wheat. Plant Physiol 149:245–257
82. Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, San Miguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat *Vrn2* gene, a flowering repressor down-regulated by vernalization. Science 303:1640–1644
83. Casao MC, Igartua E, Karsai I, Bhat PR, Cuadrado N, Gracia MP, Lasa JM, Casas AM (2011) Introgression of an intermediate VRNH1 allele in barley (Hordeum vulgare L.) leads to reduced vernalization requirement without affecting freezing tolerance. Mol Breed. doi:10.1007/s11032-010-9497
84. Bonnin I, Rousset M, Madur D, Sourdille P, Dupuits C, Brunel D, Goldringer I (2008) FT genome A and D polymorphisms are associated with the variation of earliness components in hexaploid wheat. Theor Appl Genet 116:383–394
85. Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene *Vrn3* is an orthologue of *FT*. Proc Natl Acad Sci 103:19581–19586
86. Börner A, Buck-Sorlin GH, Hayes PM, Malyshev S, Korzun V (2002) Molecular mapping of major genes and quantitative trait loci determining flowering time in response to photoperiod in barley. Plant Breed 121:129–132
87. Franckowiak JD (1997) Revised linkage maps for morphological markers in barley, *Hordeum vulgare*. Barley Genet Newsl 26:9–21
88. Lundqvist U, Franckowiak JD, Konishi T (1997) New and revised descriptions of barley genes. Barley Genet Newsl 26:22–516
89. Stracke S, Börner A (1998) Molecular mapping of the photoperiod response gene ea7 in barley. Theor Appl Genet 97:797–800
90. Chen A, Baumann U, Fincher GB, Collins NC (2009) Flt-2L, a locus in barley controlling flowering time, spike density, and plant height. Funct Integr Genomics 9:243–254
91. Scarth R, Law CN (1983) The location of the photoperiodic gene, Ppd2, and an additional factor for ear-emergence time on chromosome 2B of wheat. Heredity 51:607–619
92. Shindo C, Tsujimoto H, Sasakuma T (2003) Segregation analysis of heading traits in hexaploid wheat utilizing recombinant inbred lines. Heredity 90:56–63
93. Yoshida T, Nishida H, Zhu J, Nitcher R, Distelfed A, Akashi Y, Kato K, Dubcovsky J (2010) Vrn-D4 is a vernalization gene located on the centromeric region of chromosome 5D in hexaploid wheat. Theor Appl Genet 120:543–552
94. Kato K, Miura H, Sawada S (2002) Characterization of QEet.ocs-5A.1, a quantitative trait locus for ear emergence time on wheat chromosome 5AL. Plant Breed 121:389–393
95. Law CN, Worland AJ (1997) Genetic analysis of some flowering time and adaptative traits in wheat. New Phytol 137:19–28
96. Griffiths S, Simmonds J, Leverington M, Wang Y, Fish L, Sayers L, Alibert L, Orford S, Wingen L, Herry L, Faure S, Laurie D, Bilham L, Snape J (2009) Meta-QTL analysis of the genetic control of ear emergence in elite European winter wheat germplasm. Theor Appl Genet 119:383–395
97. Kuchel H, Hollamby G, Langridge P, Williams K, Jefferies SP (2006) Identification of genetic loci associated with ear-emergence in bread wheat. Theor Appl Genet 113:1103–1112
98. Sourdille P, Snape JW, Charmet G, Nakata N, Bernard S, Bernard M (2000) Detection of QTLs for heading time and photoperiod response in wheat using a doubled-haploid population. Genome 43:487–494
99. Boer M, Wright D, Feng L, Podlich D, Luo L, Cooper M, van Eeuwijk FA (2007) A mixed model QTL analysis for multiple environment trial data using environmental covariables for QTLxE, with an example in maize. Genetics 177:1801–1813
100. Payne RW, Murray DA, Harding SA, Baird DB, Soutar DM (2010) GenStat for windows (13th edition) introduction. VSN International, Hemel Hempstead
101. Limin A, Corey A, Hayes PM, Fowler DB (2007) Low-temperature acclimation of barley cultivars used as parents in mapping populations: response to photoperiod, vernalization and phenological development. Planta 226:139–146
102. Slafer GA, Abeledo LG, Miralles DJ, González FG, Whitechurch EM (2001) Photoperiod sensitivity during stem elongation as an avenue to raise potential yield in wheat. Euphytica 119:191–197
103. Slafer GA, Araus JL, Royo C, García del Moral LF (2005) Promising eco-physiological traits for genetic improvement of cereal yields in Mediterranean environments. Ann Appl Biol 146:61–70
104. Fischer RA (2007) Understanding the physiological basis of yield potential in wheat. J Agric Sci 145:99–113
105. Miralles DJ, Slafer GA (2007) Sink limitations to yield in wheat, how could it be reduced? J Agric Sci 145:139–149
106. Halloran GM, Pennell AL (1982) Duration and rate of development phases in wheat in two environments. Ann Bot 49:115–121
107. Whitechurch EM, Slafer GA, Miralles DJ (2007) Variability in the duration of stem elongation in wheat and barley genotypes. J Agron Crop Sci 193:138–145
108. Borràs-Gelonch G, Rebetzke G, Richards R, Romagosa I (2011b) Genetic control of duration of pre-anthesis phases in wheat (Triticum aestivum L.) and relationships to leaf appearance, tillering and dry matter accumulation. Journal of Experimental Botany, doi: 10.1093/jxb/err230
109. Appleyard M, Kirby EJM, Fellowes G (1982) Relationships between the duration of phases in the pre-anthesis life cycle of spring barley. Aust J Agr Res 33:917–925
110. Borràs G, Romagosa I, van Eeuwijk F, Slafer GA (2009) Genetic variability in the duration of pre-heading phases and relationships with leaf appearance and tillering dynamics in a barley population. Field Crop Res 113:95–104
111. Borràs-Gelonch G, Slafer GA, Casas A, van Eeuwijk F, Romagosa I (2010) Genetic control of pre-heading phases and other traits related to development in a double haploid barley population (Hordeum vulgare L.). Field Crop Res 119:36–47

112. Kernich GC, Halloran GM, Flood RG (1995) Variation in development patterns of wild barley (*Hordeum spontaneum* L) and cultivated barley (*H vulgare* L). Euphytica 82:105–115
113. Kernich GC, Halloran GM, Flood RG (1997) Variation in duration of pre-anthesis phases of development in barley (*Hordeum vulgare*). Aust J Agr Res 48:59–66
114. Kitchen BM, Rasmusson DC (1983) Duration and inheritance of leaf initiation, spike initiation and spike growth in barley. Crop Sci 23:939–943
115. González FG, Slafer GA, Miralles DJ (2002) Vernalization and photoperiod responses in wheat pre-flowering reproductive phases. Field Crop Res 74:183–195
116. Miralles DJ, Richards RA (2000) Responses of leaf and tiller emergence and primordium initiation in wheat and barley to interchanged photoperiod. Ann Bot 85:655–663
117. González FG, Slafer GA, Miralles DJ (2005) Pre-anthesis development and number of fertile florets in wheat as affected by photoperiod sensitivity genes *Ppd-D1* and *Ppd-B1*. Euphytica 146:253–269
118. Zhou Y, Li W, Wu W, Chen Q, Mao D, Worland AJ (2001) Genetic dissection of heading time and its components in rice. Theor Appl Genet 102:1236–1242
119. Borràs-Gelonch G, Denti M, Thomas WTB, Romagosa I (2011a) Genetic control of pre-heading phases in the Steptoe x Morex barley population under different conditions of photoperiod and temperature. Euphytica doi: 10.1007/s10681-011-0526-7
120. Denis JB (1988) Two-way analysis using covariates. Statistics 19:123–132
121. van Eeuwijk FA, Denis JB, Kang MS (1996) Incorporating additional information on genotypes and environments in models for two-way genotype by environment tables. In: Kang MS, Gauch HG (eds) Genotype-by-environment interaction. CRC Press, Boca Raton, pp 15–50
122. Yates F, Cochran WG (1938) The analysis of groups of experiments. J Agric Sci 28:556–580
123. Finlay KW, Wilkinson GN (1963) The analysis of adaptation in a plant breeding programme. Aust J Agr Res 14:742–754
124. van Eeuwijk FA (1995) Linear and bilinear models for the analysis of multi-environment trials: I. An inventory of models. Euphytica 84:1–7
125. Denis JB, Gower JC (1996) Asymptotic confidence regions for biadditive models: interpreting genotype-environment interactions. Appl Stat 45:479–492
126. Gabriel KR (1998) Generalised bilinear regression. Biometrika 85:689–700
127. van Eeuwijk FA (1995) Multiplicative interaction in generalized linear models. Biometrics 51:1017–1032
128. Gabriel KR (1978) Least squares approximation of matrices by additive and multiplicative models. J Roy Stat Soc Ser B 40:186–196
129. Gauch HG (1988) Model selection and validation for yield trials with interaction. Biometrics 44:705–715
130. Gollob HF (1968) A statistical model which combines features of factor analysis and analysis of variance techniques. Psychometrika 33:73–115
131. Mandel J (1969) The partitioning of interaction in analysis of variance. J Res NBS 73B:309–328
132. Yan W, Kang MS (2003) GGE biplot analysis: a graphical tool for breeders, geneticists, and agronomists. CRC Press, Boca Raton
133. Yan W, Hunt LA, Sheng Q, Szlavnics Z (2000) Cultivar evaluation and mega-environment investigation based on the GGE biplot. Crop Sci 40:597–605
134. Cornelius PL (1993) Statistical tests and retention of terms in the additive main effects and multiplicative interaction model for cultivar trials. Crop Sci 33:1186–1193
135. Kempton RA (1984) The use of biplots in interpreting variety by environment interactions. J Agric Sci 103:123–135
136. Molina-Cano JL, García del Moral LF, Ramos JM, García del Moral MB, Romagosa I, Roca de Togores F (1990) Quantitative phenotypical expression of three mutant genes in barley and the basis for defining an ideotype for Merditerranean environments. Theor Appl Genet 80:762–768
137. Romagosa I, Fox PN, del Moral G, Ramos JM, García del Moral B, Roca de Togores F, Molina-Cano JL (1993) Integration of statistical and physiological analyses of adaptation of near-isogenic barley lines. Theor Appl Genet 86:822–826
138. Vargas M, Crossa J, van Eeuwijk FA, Ramírez ME, Sayre K (1999) Using AMMI, factorial regression, and partial least squares regression models for interpreting genotype × environment interaction. Crop Sci 39:955–967
139. Voltas J, van Eeuwijk FA, Sombrero A, Lafarga A, Igartua E, Romagosa I (1999) Integrating statistical and ecophysiological analysis of genotype by environment interaction for grain filling of barley in Mediterranean areas I. Individual grain weight. Field Crop Res 62:63–74
140. Voltas J, van Eeuwijk FA, Araus JL, Romagosa I (1999) Integrating statistical and ecophysiological analysis of genotype by environment interaction for grain filling of barley in Mediterranean areas II. Grain growth. Field Crop Res 62:75–84
141. Royo C, Rodríguez A, Romagosa I (1993) Differential adaptation of complete and substituted triticale to acid soils. Plant Breed 111:113–119
142. Lynch M, Walsh JB (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland
143. van Eeuwijk FA, Crossa J, Vargas M, Ribaut JM (2001) Variants of factorial regression for analysing QTL by environment interaction. In: Gallais A, Dillmann C, Goldringer I (eds) Eucarpia, quantitative genetics and breeding methods: the way ahead, vol 96, INRA Editions Versailles Les Colloques series. INRA, Paris, pp 107–116
144. van Eeuwijk FA, Crossa J, Vargas M, Ribaut JM (2002) Analysing QTL by environment interaction by factorial regression, with an application to the CIMMYT drought and low nitrogen stress programme in maize. In: Kang MS (ed) Quantitative genetics, genomics and plant breeding. CAB International, Wallingford, pp 245–256
145. Piepho HP, Pillen K (2004) Mixed modeling for QTL × environment interaction analysis. Euphytica 137:147–153
146. IRRI (2008) CropStat for windows, version 5. Biometrics and Bioinformatics Unit, International Rice Research Institute, Los

Baños, Philippines. http://www.irri.org/science/software/irristat.asp. Accessed 27 Feb 2008
147. Gauch HG (2007) MATMODEL version 3.0: Open source software for AMMI and related analyses. Crop and Soil Sciences, Cornell University, Ithaca. http://www.css.cornell.edu/staff/gauch. Accessed 27 Feb 2008
148. Balzarini MG, Bruno C, Peña A, Teich I, Di Rienzo JA (2010) Estadística en Biotecnología. Aplicaciones en InfoGen. Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina
149. Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW (2008) InfoStat, versión 2008. Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina
150. Kang MS (2003) Handbook of formulas and software for plant geneticists and breeders. Haworth Press, Binghamton
151. Friedman J, Hastie T, Tibshirani R (2008) Sparse inverse covariance estimation with the graphical lasso. Biostatistics 9:432–441
152. Bernardo R, Yu J (2007) Prospects for genomewide selection for quantitative traits in maize. Crop Sci 47:1082–1090
153. Heffner EL, Sorrells ME, Jannink JL (2009) Genomic selection for crop improvement. Crop Sci 49:1–12
154. Jannink JL, Lorenz AJ, Iwata H (2010) Genomic selection in plant breeding: from theory to practice. Brief Funct Genomics 9:166–177
155. Bertin N, Martre P, Génard M, Quilot B, Salon C (2010) Under what circumstances can process-based simulation models link genotype to phenotype for complex traits? Case-study of fruit and grain quality traits. J Exp Bot 61: 955–967
156. Chenu K, Chapman SC, Tardieu F, McLean G, Welcker C, Hammer GL (2009) Simulating the yield impacts of organ-level quantitative trait loci associated with drought response in maize – a 'gene-to-phenotype' modeling approach. Genetics 183:1507–1523
157. Hammer G, Cooper M, Tardieu F, Welch S, Walsh B, van Eeuwijk FA, Chapman S, Podlich D (2006) Models for navigating biological complexity in breeding improved crop plants. Trends Plant Sci 11:587–593

Books and Reviews

Ceccarelli S, Guimaraës EP, Weltzien E (eds) (2009) Participatory plant breeding. FAO, Rome

Global Economic Impact of Transgenic/Biotech Crops (1996–2008)

Graham Brookes
Agricultural Economist, PG Economics Ltd, Dorset, UK

Article Outline

Glossary

Direct farm income benefit Improvements in income arising from changes in yield and production levels or associated with cost reductions/productivity enhancements associated with the use of transgenic crops.

Herbicide tolerance Tolerance to a herbicide (e.g., glyphosate) delivered by genetic modification techniques. This allows a crop to be sprayed with the "tolerant herbicide" without harming the crop but providing good weed control.

Insect resistance Resistance to a pest (e.g., corn-boring pests) delivered by genetic modification techniques. This allows a crop to be grown without having to use alternative methods of pest control, notably the use of insecticides.

Nonpecuniary benefit Additional farm-level benefits to direct farm income benefits that are more intangible and difficult to measure in monetary terms (e.g., additional management flexibility).

No tillage agriculture The use of a production technique in which the soil is not tilled/plowed. It is in contrast to traditional plow-based production systems and allows farmers to save on fuel use and contributes to improved soil water retention and reduced soil erosion.

Second crop soybeans The planting of a crop of soybeans after another crop (often wheat) in the same growing season. This allows a farmer to obtain two crops from the same piece of land in one season.

Definition of the Subject

The application of biotechnology to commercial agriculture on a widespread basis has occurred since 1996. The extent of this adoption in terms of crops and (biotechnology) traits is explored and the associated economic impacts for the period 1996–2008 are assessed, to help identify some of the main reasons why farmers have adopted the technology.

Introduction

This article examines specific global socioeconomic impacts on farm income over the 13-year period 1996–2008. It also quantifies the production impact of the technology on the key crops in areas where it has been used. The analysis concentrates on farm income effects because this is a primary driver of adoption among farmers (both large commercial and small-scale subsistence). It also considers more indirect farm income or nonpecuniary benefits, and quantifies the (net) production impact of the technology. More specifically, it covers the following main issues:

- Impact on crop yields
- Effect on key costs of production, notably seed cost and crop protection expenditure
- Impact on other costs such as fuel and labor
- Effect on profitability
- Other impacts such as crop quality, scope for planting a second crop in a season and impacts that are often referred to as intangible impacts such as convenience, risk management, and husbandry flexibility
- Production effects

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

The contribution is based largely on extensive analysis of existing farm-level impact data for biotech crops. While primary data for impacts of commercial cultivation were not available for every crop, in every year and for each country, a substantial body of representative research and analysis is available and this has been used as the basis for the analysis presented.

As the economic performance and impact of this technology at the farm level varies widely, both between, and within regions/countries (as applies to any technology used in agriculture), the measurement of performance and impact is considered on a case-by-case basis in terms of crop and trait combinations. The analysis presented is based on the average performance and impact recorded in different crops by the studies reviewed; the average performance being the most common way in which the identified literature has reported impact. Where several pieces of relevant research (e.g., on the impact of using a GM trait on the yield of a crop in one country in a particular year) have been identified, the findings used have been largely based on the average of these findings.

This approach may both, overstate, or understate, the real impact of GM technology for some trait, crop and country combinations, especially in cases where the technology has provided yield enhancements. However, as impact data for every trait, crop, location, and year is not available, the authors have had to extrapolate available impact data from identified studies to years for which no data are available. Therefore, the authors acknowledge that this represents a weakness of the research. To reduce the possibilities of over/understating impact, the analysis:

- Directly applies impacts identified from the literature to the years that have been studied. As a result, the impacts used vary in many cases according to the findings of literature covering different years. Hence, the analysis takes into account the variation in the impact of the technology on the yield based on its effectiveness in dealing with (annual) fluctuations in pest and weed infestation levels as identified by research.
- Uses current farm-level crop prices and bases any yield impacts on (adjusted – see below) current average yields. In this way, some degree of dynamic has been introduced into the analysis that would, otherwise, be missing if constant prices and average yields indentified in year-specific studies had been used.
- Includes some changes and updates to the impact assumptions identified in the literature based on consultation with local sources (analysts, industry representatives) so as to better reflect prevailing/changing conditions (e.g., pest and weed pressure, cost of technology).
- Adjusts downward the average base yield (in cases where GM technology has been identified as having delivered yield improvements) on which the yield enhancement has been applied. In this way, the impact on total production is not overstated.

Other aspects of the methodology used to estimate the impact on direct farm income are as follows:

- Impact is quantified at the trait and crop level, including where stacked traits are available to farmers. Where stacked traits have been used, the individual trait components were analyzed separately to ensure estimates of all traits were calculated.
- All values presented are nominal for the year shown and the base currency used is the US dollar. All financial impacts in other currencies have been converted to US dollars at prevailing annual average exchange rates for each year.
- The analysis focuses on the changes in farm income for each year, arising from the impact of GM technology on yields, key costs of production, notably seed cost and crop protection expenditure and also the impact on costs such as fuel and labor (inclusion of impact on these categories of cost are, however, more limited than the impacts on seed and crop protection costs because only a few of the papers reviewed have included consideration of such costs in their analyses). Therefore, in most cases the analysis relates to impact of crop protection and seed cost only.
- Crop quality (e.g., improvements in quality arising from less pest damage or lower levels of weed impurities that result in price premia being obtained from buyers) and the scope for facilitating the planting of a second crop in a season (e.g., second crop soybeans in Argentina following wheat that would, in the absence of the GM herbicide-tolerant

(GM HT) seed, probably not have been planted). Thus, the farm income effect measured is essentially a gross margin impact (impact on gross revenue less variable costs of production) rather than a full net cost of production assessment. Through the inclusion of yield impacts and the application of actual (average) farm prices for each year, the analysis also indirectly takes into account the possible impact of biotech crop adoption on global crop supply and world prices.

This article also examines some of the more intangible (more difficult to quantify) economic impacts of GM technology. The literature in this area is much more limited and in terms of aiming to quantify these impacts, largely restricted to the US-specific studies. The findings of this research (notably relating to the USA, and drawing on Marra and Piggot [1, 2] are summarized and extrapolated to the cumulative biotech crop planted areas in the USA over the period 1996–2008.

Lastly, this article includes estimates of the production impacts of GM technology at the crop level. These have been aggregated to provide the reader with a global perspective of the broader production impact of the technology. These impacts derive from the yield impacts (where identified), but also from the facilitation of additional cropping within a season (notably in relation to soybeans in South America).

Economic Impact of Transgenic/Biotech Crops

The section below is structured on a trait and country basis highlighting the key farm-level impacts.

Herbicide-Tolerant Soybeans

The USA

In 2008, 92% of the total US soybean crop was planted to genetically modified herbicide-tolerant cultivars (GM HT). The farm-level impact of using this technology since 1996 is summarized in Table 1.

The key features are as follows:

- The primary impact has been to reduce the soybean cost of production. In the early years of adoption, these savings were between $25/ha and $34/ha. In recent years, estimates of the cost savings have been in the range of $30–$85/ha (based on a comparison of conventional herbicide regimes in the early 2000s that would be required to deliver a comparable level of weed control to the GM HT soybean system). In 2008, the cost savings declined relative to earlier years because of the significant increase in the global price of glyphosate relative to increases in the price of other herbicides (commonly used on conventional soybeans). The main savings have come from lower herbicide costs (while there were initial cost savings in herbicide expenditure, these increased when glyphosate came off-patent in 2000. Growers of GM HT soybeans initially applied Monsanto's Roundup herbicide but over time, and with the availability of low-cost generic glyphosate alternatives, many growers switched to using these generic alternatives (the price of Roundup also fell significantly post 2000) plus a $6–$10/ha savings in labor and machinery costs.
- Against the background of underlying improvements in average yield levels over the 1996–2008 period (via improvements in plant breeding), the specific yield impact of the GM HT technology used up to 2008 has been neutral (some early studies of the impact of GM HT soybeans in the USA, suggested that GM HT soybeans produced lower yields than conventional soybean varieties. Where this may have occurred, it applied only in early years of adoption when the technology was not present in all leading varieties suitable for all of the main growing regions of the USA. By 1998/1999, the technology was available in leading varieties and no statistically significant average yield differences have been found between GM and conventional soybean varieties.
- The annual total national farm income benefit from using the technology rose from $5 million in 1996 to $1.42 billion in 2007. In 2008, the farm income was about $1.2 billion. The cumulative farm income benefit over the 1996–2008 period (in nominal terms) was $11 billion.
- In added value terms, the increase in farm income in recent years has been equivalent to an annual increase in production of between +5% and +10%.

Argentina

As in the USA, GM HT soybeans were first planted commercially in 1996. Since then, use of the technology

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 1 Farm-level income impact of using GM herbicide-tolerant (GM HT) soybeans in the USA 1996–2008

Year	Cost savings ($/ha)	Net cost saving/increase in gross margins, inclusive of cost of technology ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1996	25.2	10.39	5.0	0.03
1997	25.2	10.39	33.2	0.19
1998	33.9	19.03	224.1	1.62
1999	33.9	19.03	311.9	2.5
2000	33.9	19.03	346.6	2.69
2001	73.4	58.56	1,298.5	10.11
2002	73.4	58.56	1,421.7	9.53
2003	78.5	61.19	1,574.9	9.57
2004	60.1	40.33	1,096.8	4.57
2005	69.4	44.71	1,201.4	6.87
2006	57.0	32.25	877.1	4.25
2007	85.2	60.48	1,417.2	6.01
2008	68.6	43.88	1,219.5	4.25

Sources and notes:

1. Impact data 1996–1997 based on Marra et al [3], 1998–2000 based on Carpenter and Gianessi [4] and 2001 [5] onward based on Sankala and Blumenthal [6, 7] and Johnson and Strom [8] plus updated 2008 to reflect recent changes in herbicide prices
2. Cost of technology: $14.82/ha 1996–2002, $17.3/ha 2003, $19.77/ha 2004, $24.71/ha 2005 onward
3. The higher values for the cost savings in 2001 onward reflect the methodology used by Sankala and Blumenthal, which was to examine the conventional herbicide regime that would be required to deliver the same level of weed control in a low/reduced till system to that delivered from the GM HT no/reduced till soybean system. This is a more robust methodology than some of the more simplistic alternatives used elsewhere. In earlier years, the cost savings were based on comparisons between GM HT soy growers and/or conventional herbicide regimes that were commonplace prior to commercialization in the mid-1990s when conventional tillage systems were more important

has increased rapidly and almost all soybeans grown in Argentina are GM HT (99%). Not surprisingly, the impact on farm income has been substantial, with farmers deriving important cost saving and farm income benefits both similar and additional to those obtained in the USA (Table 2). More specifically, it covers the following main issues:

- The impact on yield has been neutral (i.e., no positive or negative yield impact).
- The cost of the technology to Argentine farmers has been substantially lower than in the USA (about $1–$4/ha compared to $15–$25/ha in the USA: see Table 1) mainly because the main technology provider (Monsanto) was not able to obtain patent protection for the technology in Argentina. As such, Argentine farmers have been free to save and use biotech seed without paying any technology fees or royalties (on farm-saved seed) for many years and estimates of the proportion of total soybean seed used that derives from a combination of declared saved seed and uncertified seed in 2008 were about 75% (i.e., 25% of the crop was planted to certified seed).
- The savings from reduced expenditure on herbicides, fewer spray runs, and machinery use have been in the range of $24–$30/ha, although in

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 2 Farm-level income impact of using GM HT soybeans in Argentina 1996–2008

Year	Cost savings ($/ha)	Net saving on costs (inclusive of cost of technology ($/ha)	Increase in farm income at a national level ($ millions)	Increase in farm income from facilitating additional second cropping ($ millions)
1996	26.10	22.49	0.9	0
1997	25.32	21.71	42	25
1998	24.71	21.10	115	43
1999	24.41	20.80	152	118
2000	24.31	20.70	205	143
2001	24.31	20.70	250	273
2002	29.00	27.82	372	373
2003	29.00	27.75	400	416
2004	30.00	28.77	436	678
2005	30.20	28.96	471	527
2006	28.72	26.22	465	699
2007	28.61	26.11	429	1,134
2008	16.37	13.87	233	765

Sources and notes:

1. The primary source of information for impact on the costs of production is Qaim and Traxler [9, 10]. This has been updated in recent years to reflect changes in herbicide prices
2. All values for prices and costs denominated in Argentine pesos have been converted to US dollars at the annual average exchange rate in each year
3. The second cropping benefits are based on the gross margin derived from second crop soybeans multiplied by the total area of second crop soybeans (less an assumed area of second crop soybeans that equals the second crop area in 1996 – this was discontinued from 2004 because of the importance farmers attach to the GM HT system in facilitating them remaining in no tillage production systems). The source of gross margin data comes from Grupo CEO
4. Additional information is available in Appendix 1
5. The net savings to costs understate the total gains in recent years because two thirds to 80% of GM HT plantings have been to farm-saved seed on which no seed premium was payable (relative to the $3-$4/ha premium charged for new seed)

2008, savings fell back to about $16/ha because of the significant increase in the price of glyphosate relative to other herbicides. Net income gains have been in the range of $21–$29/ha, although in 2008 a lower average level of about $14/ha has occurred.

- The price received by farmers for GM HT soybeans in the early years of adoption was, on average, marginally higher than for conventionally produced soybeans because of lower levels of weed material and impurities in the crop. This quality premia was equivalent to about 0.5% of the baseline price for soybeans.
- The net income gain from the use of the GM HT technology at a national level was $233 million in 2008. Since 1996, the cumulative benefit (in nominal terms) has been $3.57 billion.
- An additional farm income benefit that many Argentine soybean growers have derived comes from the additional scope for second cropping of soybeans. This has arisen because of the simplicity, ease, and weed management flexibility provided by the (GM) technology, which has been an important factor facilitating the use of no and reduced tillage production systems. In turn, the adoption of low/no tillage

production systems has reduced the time required for harvesting and drilling subsequent crops and hence has enabled many Argentine farmers to cultivate two crops (wheat followed by soybeans) in one season. As such, 20% of the total Argentine soybean crop was second crop in 2008 (3.4 million hectares), compared to 8% in 1996. Based on the additional gross margin income derived from second crop soybeans (see Appendix 1), this has contributed a further boost to national soybean farm income of $765 billion in 2008 and $5.19 billion cumulatively since 1996.

- The total farm income benefit inclusive of the second cropping was $998 million in 2008 and $8.76 billion cumulatively between 1996 and 2008.
- In added value terms, the increase in farm income from the direct use of the GM HT technology (i.e., excluding the second crop benefits) in the last 3 years has been equivalent to an annual increase in production of between +2% and +7%. The additional production from second soybean cropping facilitated by the technology in 2008 was equal to 20% of total output.

Brazil

GM HT soybeans were probably first planted in Brazil in 1997. Since then, the area planted has increased to 62% of the total crop in 2008 (until 2003 all plantings were technically illegal).

The impact of using GM HT soybeans has been similar to that identified in the USA and Argentina. The net savings on herbicide costs have been larger in Brazil due to higher average costs of weed control. Hence, the average cost saving arising from a combination of reduced herbicide use, fewer spray runs, labor and machinery savings were between $30/ha and $81/ha in the period 2003–2008 (Table 3). The net cost saving after deduction of the technology fee (assumed to be about $20/ha in 2008) has been between $9/ha and $61/ha in recent years. At a national level, the adoption of GM HT soybeans increased farm income levels by $592 million in 2008. Cumulatively over the period 1997–2008, farm incomes have risen by $2.74 billion (in nominal terms).

In added value terms, the increase in farm income from the use of the GM HT technology in 2008 was equivalent to an annual increase in production of +2.6% (about 1.54 million tons).

Paraguay and Uruguay

GM HT soybeans have been grown since 1999 and 2000 respectively in Paraguay and Uruguay. In 2008, they accounted for 90% of total soybean plantings in Paraguay and 99% of the soybean plantings in Uruguay (as in Argentina, the majority of plantings are to farm saved or uncertified seed). Using the farm-level impact data obtained from the Argentine research [9, 10] – we are not aware of any published country-specific impact research having been conducted in these two countries) and applying this to production in these two countries, Fig. 1 summarizes the national farm-level income benefits that have been derived from using the technology. In 2008, the respective national farm income gains were $58.8 million in Paraguay and $7.9 million in Uruguay.

Canada

GM HT soybeans were first planted in Canada in 1997. In 2008, the share of total plantings accounted for by GM HT soybeans was 73% (0.88 million hectares).

At the farm level, the main impacts of use have been similar to the impacts in the USA. The average farm income benefit has been within a range of $14–$40/ha and the increase in farm income at the national level was $12.6 million in 2008 (Table 4). The cumulative increase in farm income since 1997 has been $116 million (in nominal terms). In added value terms, the increase in farm income from the use of the GM HT technology in 2008 was equivalent to an annual increase in production of about 1% (34,500 tons).

South Africa

In 2001, GM HT soybeans were planted commercially in South Africa. In 2008, 184,000 ha (80%) of total soybean plantings were to varieties containing the GM HT trait. In terms of impact at the farm level, net cost savings of between $5/ha and $9/ha have been achieved through reduced expenditure on herbicides (Table 5), although in 2008, with the significant increase in glyphosate prices relative to other herbicides, this has fallen back to $2/ha. At the national level, the increase in farm income was $0.32 million in 2008. Cumulatively, the farm income gain since 2001 has been $4.13 million.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 3 Farm-level income impact of using GM HT soybeans in Brazil 1997–2008

Year	Cost savings ($/ha)	Net cost saving after inclusion of technology cost ($/ha)	Impact on farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1997	38.8	35.19	3.8	0.06
1998	42.12	38.51	20.5	0.31
1999	38.76	35.15	43.5	0.96
2000	65.32	31.71	43.7	0.85
2001	46.32	42.71	58.7	1.02
2002	40.00	36.39	66.7	1.07
2003	77.00	68.00	214.7	1.62
2004	76.66	61.66	320.9	2.95
2005	73.39	57.23	534.6	5.45
2006	81.09	61.32	730.6	6.32
2007	29.85	8.74	116.3	0.68
2008	64.07	44.44	591.9	2.63

Sources and notes:

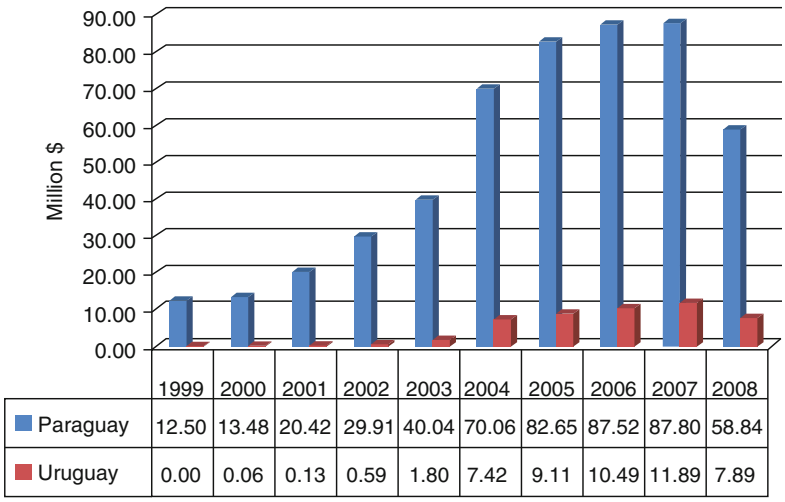
1. Impact data based on 2004 comparison data from the Parana Department of Agriculture [11] Cost of production comparison: biotech and conventional soybeans, in USDA GAIN report BR4629 of 11 November 2004. www.fas.usad.gov/gainfiles/200411/146118108.pdf for the period to 2006 [11]. From 2007 based on Galveo [12]
2. Cost of the technology from 2003 is based on the royalty payments officially levied by the technology providers. For years up to 2002, the cost of technology is based on costs of buying new seed in Argentina (the source of the seed). This probably overstates the real cost of the technology and understates the cost savings
3. All values for prices and costs denominated in Brazilian Real have been converted to US dollars at the annual average exchange rate in each year

Romania

In 2008, Romania was not officially permitted to plant GM HT soybeans, having joined the EU at the start of 2007 (the EU has not permitted the growing of GM HT soybeans to date). The impact data presented below therefore covers the period 1999–2006.

The growing of GM HT soybeans in Romania had resulted in substantially greater net farm income gains per hectare than any of the other countries using the technology:

- Yield gains of an average of 31% have been recorded [14]. This yield gain has arisen from the substantial improvements in weed control (weed infestation levels, particularly of difficult to control weeds such as Johnson grass have been very high in Romania. This is largely a legacy of the economic transition during the 1990s, which resulted in very low levels of farm income, abandonment of land, and very low levels of weed control. As a result, the weed bank developed substantially and has been subsequently very difficult to control, until the GM HT soybean system became available [glyphosate has been the key to controlling difficult weeds like Johnson grass]). In recent years, as fields have been cleaned up of problem weeds, the average yield gains have decreased and were reported at +13% in 2006 (source: farmer survey conducted in 2006 on behalf of Monsanto Romania).
- The cost of the technology to farmers in Romania tended to be higher than other countries, with seed being sold in conjunction with the herbicide.



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 1
National farm income benefit from using GM herbicide-tolerant (GM HT) soybeans in Paraguay and Uruguay 1999–2008 (million $)

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 4 Farm-level income impact of using GM HT soybeans in Canada 1997–2008

Year	Cost savings ($/ha)	Net cost saving/increase in gross margin (inclusive of technology cost: $/ha)	Impact on farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1997	64.28	41.17	0.041	0.01
1998	56.62	35.05	1.72	0.3
1999	53.17	31.64	6.35	1.29
2000	53.20	31.65	6.71	1.4
2001	49.83	29.17	9.35	3.4
2002	47.78	27.39	11.92	2.79
2003	49.46	14.64	7.65	1.47
2004	51.61	17.48	11.58	1.48
2005	55.65	18.85	13.30	2.26
2006	59.48	23.53	17.99	2.22
2007	61.99	24.52	16.87	1.57
2008	56.59	14.33	12.61	1.03

Sources and notes:
1. Impact data based on George Morris Centre Report [13] and updated in recent years to reflect changes in herbicide prices
2. All values for prices and costs denominated in Canadian dollars have been converted to US dollars at the annual average exchange rate in each year

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 5 Farm-level income impact of using GM HT soybeans in South Africa 2001–2008

Year	Cost savings ($/ha)	Net cost saving/increase in gross margin after inclusion of technology cost ($/ha)	Impact on farm income at a national level ($ millions)
2001	26.72	7.02	0.042
2002	21.82	5.72	0.097
2003	30.40	7.90	0.24
2004	34.94	9.14	0.46
2005	36.17	9.12	1.42
2006	33.96	5.17	0.83
2007	32.95	5.01	0.72
2008	25.38	1.77	0.32

Sources and notes:
1. Impact data (Data source: Monsanto South Africa – data provision not a reference)
2. All values for prices and costs denominated in South African Rand have been converted to US dollars at the annual average exchange rate in each year

For example, in the 2002–2006 period, the average cost of seed and herbicide per hectare was $120–$130/ha. This relatively high cost however, did not deter adoption of the technology because of the major yield gains, improvements in the quality of soybeans produced (less weed material in the beans sold to crushers that resulted in price premia being obtained in the early years – no longer relevant post 2005), and cost savings derived.

- The average net increase in gross margin in 2006 was $59/ha (an average of $105/ha over the 8 years of commercial use: Table 6).
- At the national level, the increase in farm income amounted to $7.6 million in 2006. Cumulatively in the period 1999–2006, the increase in farm income was $44.6 million (in nominal terms).
- The yield gains in 2006 were equivalent to an 9% increase in national production (the annual average increase in production over the 8 years was equal to 10.1%).
- In added value terms, the combined effect of higher yields, improved quality of beans, and reduced cost of production on farm income in 2006 was equivalent to an annual increase in production of 9.3% (33,230 tons).

Mexico

GM HT soybeans were first planted commercially in Mexico in 1997 (on a trial basis) and in 2008, a continued trial area of 7,330 ha (out of total plantings of 88,000 ha) were varieties containing the GM HT trait.

At the farm level, the main impacts of use have been a combination of yield increase (+9.1% in 2004 and 2005, +3.64% in 2006, +3.2% 2007, and +2.4% 2008) and (herbicide) cost savings. The average farm income benefit has been within a range of $54–$89/ha (inclusive of yield gain, cost savings, and after payment of the technology fee/seed premium of $34.5/ha) and the increase in farm income at the national level was $0.04 million in 2008 (Table 7). The cumulative increase in farm income since 2004 has been $3.35 million (in nominal terms). In added value terms, the increase in farm income from the use of the GM HT technology in 2008 was equivalent to an annual increase in production of about 0.5%.

Bolivia

GM HT soybeans were officially permitted to be planted in 2008, although "illegal" plantings have occurred for several years. For the purposes of analysis

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 6 Farm-level income impact of using herbicide-tolerant soybeans in Romania 1999–2006

Year	Cost saving ($/ha)	Cost savings net of cost of technology ($/ha)	Net increase in gross margin ($/ha)	Impact on farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1999	162.08	2.08	105.18	1.63	4.0
2000	140.30	−19.7	89.14	3.21	8.2
2001	147.33	−0.67	107.17	1.93	10.3
2002	167.80	32.8	157.41	5.19	14.6
2003	206.70	76.7	219.01	8.76	12.7
2004	63.33	8.81	135.86	9.51	13.7
2005	64.54	9.10	76.16	6.69	12.2
2006	64.99	9.10	58.79	7.64	9.3

Sources and notes:

1. Impact data (Sources: Brookes [14] and Monsanto Romania [15]. Average yield increase 31% applied to all years to 2003 and reduced to +25% 2004, +19% 2005 and +13% 2006. Average improvement in price premia from high quality 2% applied to years 1999–2004
2. All values for prices and costs denominated in Romanian Lei have been converted to US dollars at the annual average exchange rate in each year
3. Technology cost includes cost of herbicides
4. The technology was not permitted to be planted from 2007 – due to Romania joining the EU

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 7 Farm-level income impact of using GM HT soybeans in Mexico 2004–2008

Year	Cost savings ($/ha)	Net cost saving/increase in gross margin (inclusive of technology cost and yield gain: $/ha)	Impact on farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
2004	49.44	82.34	1.18	3.07
2005	51.20	89.41	0.94	2.13
2006	51.20	72.98	0.51	1.05
2007	51.05	66.84	0.33	0.9
2008	33.05	54.13	0.40	0.5

Sources and notes:

1. Impact data based on Monsanto, 2005, 2007, and 2008 [16–18]. Reportes final del programa Soya Solución Faena en Chiapas. Monsanto Comercial
2. All values for prices and costs denominated in Mexican pesos have been converted to US dollars at the annual average exchange rate in each year

in this section, impacts have been calculated back to 2005, when an estimated 0.3 million hectares of soybeans used GM HT technology. In 2008, an estimated 453,000 ha (63% of total crop) used GM HT technology.

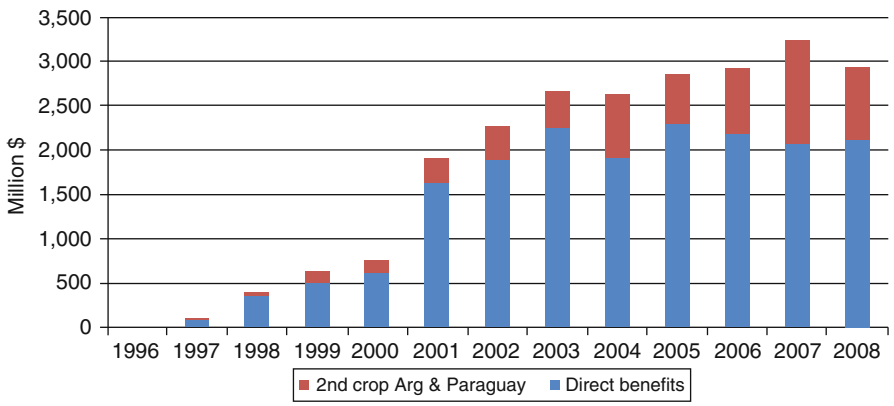
The main impacts of the technology are as follows (Table 8):

- An increase in yield arising from improved yield control. The research work conducted by

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 8 Farm-level income impact of using GM HT soybeans in Bolivia 2005–2008

Year	Cost savings excluding seed cost premium ($/ha)	Net cost saving/increase in gross margin (inclusive of technology cost and yield gain: $/ha)	Impact on farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
2005	9.28	39.73	12.08	4.09
2006	9.28	36.60	15.55	6.35
2007	9.28	44.40	19.45	7.37
2008	9.28	80.09	36.33	7.24

Sources and notes:
1. Impact data based on Fernandez et al. [19]. Average yield gain assumed +15%, cost of technology $3.32/ha



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 2
Global farm-level income benefits derived from using GM HT soybeans 1996–2008 (million $)

Fernandez et al. [19] estimated a 30% yield difference between GM HT and conventional soybeans although some of the yield gain reflected the use of poor-quality conventional seed by some farmers. In the analysis presented, a more conservative yield gain of +15% has been used.

- GM HT soybeans are assumed to trade at a price discount to conventional soybeans of −2.7%, reflecting the higher price set for conventional soybeans by the Bolivian government in 2008.
- The cost of the technology to farmers has been about $3.3/ha and the cost savings equal to about $9.3/ha, resulting in a net cost of production change of +$6/ha.
- Overall, in 2008, the average farm income gain from using GM HT soybeans was about $80/ha, resulting in a total farm income gain of $36.3 million. Cumulatively since 2005, the total farm income gain is estimated at $83.4 million.

Summary of Global Economic Impact

In global terms, the farm-level impact of using GM HT technology in soybeans was $2.12 billion in 2008 (Fig. 2). If the second crop benefits arising in Argentina are included, this impact rises to $2.92 billion. Cumulatively since 1996, the farm income benefit has been

(in nominal terms) $17.9 billion ($23.3 billion if second crop gains in Argentina and Paraguay are included).

In terms of the total value of soybean production from the countries growing GM HT soybeans in 2008, the additional farm income (inclusive of Argentine second crop gains) generated by the technology is equal to a value-added equivalent of 4.3%. Relative to the value of global soybean production in 2008, the farm income benefit added the equivalent of 4.1%.

These economic benefits should be placed within the context of a significant increase in the level of soybean production in the main GM adopting countries since 1996 (a 63% increase in the area planted in the leading soybean producing countries of the USA, Brazil, and Argentina).

These economic benefits mostly derive from cost savings although farmers in Mexico, Bolivia, and Romania also obtained yield gains (from significant improvements in weed control levels relative to levels applicable prior to the introduction of the technology). If it is also assumed that all of the second crop soybean gains are effectively additional production that would not have otherwise occurred without the GM HT technology (the GM HT technology facilitated major expansion of second crop soybeans in Argentina and to a lesser extent in Paraguay) then these gains are de facto "yield" gains. Under this assumption, of the total cumulative farm income gains from using GM HT soy, $5.56 billion (24%), is due to yield gains/second crop benefits and the balance, 76%, is due to cost savings.

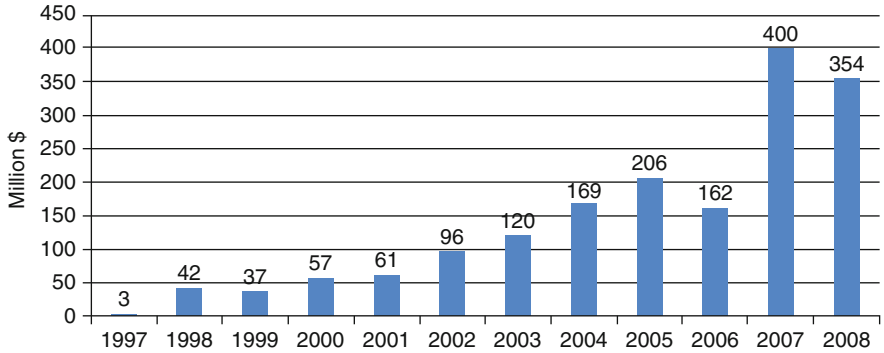
Herbicide-Tolerant Maize

The USA

Herbicide-tolerant maize has been used commercially in the USA since 1997 and in 2008 was planted on 63% of the total US maize crop. The impact of using this technology at the farm level is summarized in Fig. 3. As with herbicide-tolerant soybeans, the main benefit has been to reduce costs, and hence improve profitability levels. Average profitability improved by $20–$25/ha in most years ($17.6/ha in 2008 – affected by the significant increase in glyphosate prices relative to other herbicides). The net gain to farm income in 2008 was $354 million and cumulatively, since 1997 the farm income benefit has been $1.7 billion. In added value terms, the effect of reduced costs of production on farm income in 2008 was equivalent to an annual increase in production of 0.71% (2.17 million tons).

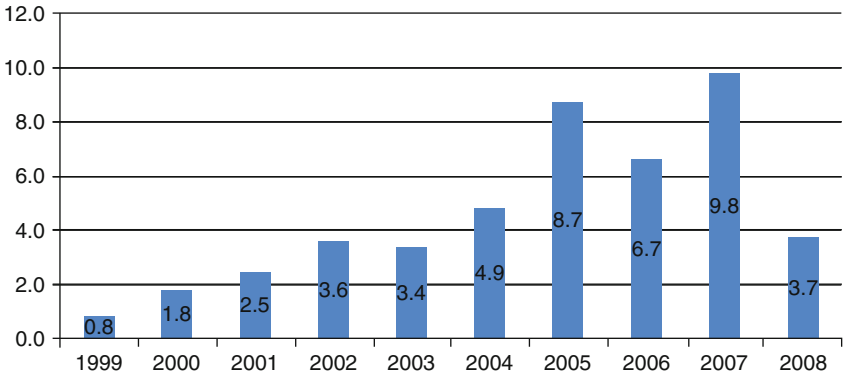
Canada

In Canada, GM HT maize was first planted commercially in 1997. By 2008, the proportion of total plantings accounted for by varieties containing a GM HT trait was 51%. As in the USA, the main benefit has been to reduce



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 3

National farm income impact of using GM HT maize in the USA 1997–2008 (Source and notes: Impact analysis based on Sankala and Blumenthal [6, 7] and Johnson and Strom [8] and updated for 2008 to reflect changes in herbicide prices. Estimated cost of the technology $14.83/ha in years up to 2004, $17.3/ha in 2005, $24.71/ha 2006 onward. Cost savings (mostly from lower herbicide use) $33.47/ha in 2004, $38.61/ha 2005, $29.27/ha 2006, $42.28/ha 2007, and $40.87/ha 2008)



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 4
National farm income impact of using GM HT maize in Canada 1999–2008 ($ million) (Source and notes: Impact analysis based on data supplied by Monsanto Canada. Estimated cost of the technology $18-$32/ha, cost savings (mostly from lower herbicide use) $31–$45/ha)

costs and to improve profitability levels. Average annual profitability has improved by between $12/ha and $18/ha up to 2007, but fell to about $6/ha in 2008 (due to the higher price increases for glyphosate relative to other herbicides). In 2008, the net increase in farm income was $3.7 million and cumulatively since 1999 the farm income benefit has been $45.8 million. In added value terms, the effect of reduced costs of production on farm income in 2008 was equivalent to an annual increase in production of 0.22% (23,500 tons: Fig. 4).

Argentina

GM HT maize was first planted commercially in Argentina in 2004 and in 2008, varieties containing a GM HT trait were planted on 805,000 ha (35% of the total maize area). It has been adopted in two distinct types of area, the majority (80%) in the traditional "corn production belt" and 20% in newer maize-growing regions, which have been traditionally known as more marginal areas that surround the "Corn Belt." The limited adoption of GM HT technology in Argentina up to 2006 was mainly due to the technology only being available as a single gene, not stacked with the GM IR trait, which most maize growers have also adopted. Hence, faced with an either GM HT or GM IR trait available for use, most farmers have chosen the GM IR trait because the additional returns derived from adoption have tended to be (on average) greater from the GM IR trait than the GM HT trait (see below for further details of returns from the GM HT trait). Stacked traits became available in 2007 and contributed to the significant increase in the GM HT maize area relative to 2006.

In relation to impact on farm income, the following observations were made:

- In all regions, the cost of the technology (about $20/ha) has been broadly equal to the saving in herbicide costs.
- In the Corn Belt area, use of the technology has resulted in an average 3% yield improvement via improved weed control. In the more marginal areas, the yield impact has been much more significant (+22%) as farmers have been able to significantly improve weed control levels.
- In 2008, the additional farm income at a national level from using GM HT technology has been +$61.6 million, and cumulatively since 2004, the income gain has been $113.8 million.

South Africa

Herbicide-tolerant maize has been grown commercially in South Africa since 2003, and 6,46,000 ha out of total plantings of 2.43 million hectares were herbicide tolerant in 2008. Farmers using the technology have found that small net savings in the cost of production have occurred (i.e., the cost saving from reduced expenditure on herbicides has been greater

than the cost of the technology), although in 2008, due to the significant rise in the global price of glyphosate relative to their herbicides, the net farm income balance was negative, at about −$2/ha. This resulted in a total net farm loss arising from using GM HT technology of $1.43 million, though since 2003, there has been a net cumulative income gain of $3.77 million.

Philippines

GM HT maize was first grown commercially in 2006, and 2008 was planted on 270,000 ha. Information about the impact of the technology is limited, although industry sources estimate that, on average farmers using it have derived a 15% increase in yield. Based on a cost of the technology of $24–$27/ha (and assuming no net cost savings), the net national impact on farm income was +$15.9 million in 2008. Cumulatively, since 2006, the total farm income gain has been $27.1 million

Summary of Global Economic Impact

In global terms, the farm-level economic impact of using GM HT technology in maize was $433.5 million in 2008 (82% of which was in the USA). Cumulatively since 1997, the farm income benefit has been (in nominal terms) $1.9 billion. Of this, 92% has been due to cost savings and 8% to yield gains (from improved weed control relative to the level of weed control achieved by farmers using conventional technology).

In terms of the total value of maize production in the main countries using this technology in 2008, the additional farm income generated by the technology is equal to a value-added equivalent of 0.3% of global maize production.

Herbicide-Tolerant Cotton

The USA

GM HT cotton was first grown commercially in the USA in 1997 and in 2008 was planted on 68% of total cotton plantings.

The farm income impact of using GM HT cotton is summarized in Table 9. The primary benefit has been to reduce costs, and hence improve profitability levels, with annual average profitability increasing by between $21/ha and $49/ha (the only published source that has examined the impact of HT cotton in the USA is the work by Sankala and Blumenthal [6, 7], and Johnson and Strom [8]. In the 2001 study, the costs saved were based on historic patterns of herbicides used on conventional cotton in the mid/late 1990s. The latter studies estimated cost savings on the basis of the conventional herbicide treatment that would be required to deliver the same level of weed control as GM HT cotton. Revised analysis has, however, been conducted for 2008 to reflect changes in the costs of production (notably cost of the technology (in particular "Roundup Ready Flex technology"), higher prices for glyphosate relative to other herbicides in 2008 and additional costs incurred to control weeds resistant to glyphosate in some regions) in the years up to 2004. Since then, net income gains have fallen to between $1/ha and $5/ha. The relatively small positive impact on direct farm income in 2008 (and in the last few years) reflects a combination of reasons, including the higher cost of the technology, significant price increases for glyphosate relative to price increases for other herbicides, and additional costs incurred for management of weeds resistant to glyphosate (notably *Palmer Amaranth*). Overall, the net direct farm income impact in 2008 is estimated to be $2.5 million (this does not take into consideration any nonpecuniary benefits associated with adoption of the technology: see Section 3.9). Cumulatively, since 1997, there has been a net farm income benefit from using the technology of $799 million.

Other Countries

Australia, Argentina, South Africa, and Mexico are the other countries where GM HT cotton is commercially grown; from 2000 in Australia, 2001 in South Africa, 2002 in Argentina, and 2005 in Mexico. In 2008, 79% (50,460 ha), 38% (124,000 ha), 75% (9,750 ha), and 40% (50,000 ha) respectively of the total Australian, Argentine, South African, and Mexican cotton crops were planted to GM HT cultivars.

We are not aware of any published research into the impact of GM HT cotton in South Africa, Argentina, or Mexico. In Australia, although research has been conducted into the impact of using GM HT cotton (e.g., Doyle et al. [20]) this does not provide

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 9 Farm-level income impact of using GM HT cotton in the USA 1997–2008

Year	Cost savings ($/ha)	Net cost saving/increase in gross margins, inclusive of cost of technology ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1997	34.12	21.28	12.56	0.2
1998	34.12	21.28	30.21	0.58
1999	34.12	21.28	53.91	1.29
2000	34.12	21.28	61.46	1.22
2001	65.59	45.27	161.46	4.75
2002	65.59	45.27	153.18	3.49
2003	65.59	45.27	129.75	2.33
2004	83.35	48.80	154.72	2.87
2005	71.12	2.89	9.57	0.18
2006	73.66	3.31	13.29	0.22
2007	76.01	5.40	16.56	0.32
2008	72.76	1.20	2.50	0.08

Source and notes:

1. Impact analysis based on Sankala and Blumenthal [6, 7] and Johnson and Strom [8] and own analysis for 2008
2. Estimated cost of the technology $12.85/ha (1997–2000) and $21.32/ha 2001–2003, $34.55 2004, $68.22/ha 2005, $70.35/ha 2006, $70.61/ha 2007, and £71.56/ha 2008

quantification of the impact. Drawing on industry source estimates, the main impacts are as follows:

- *Australia*: No yield gain and cost of the technology in the range of $30–$45/ha up to 2007. The cost of the technology increased with the availability of "Roundup Ready Flex" and in 2008 was about $63/ha. The cost savings from the technology (after taking into consideration the cost of the technology have delivered small net gains of $5–$7/ha, although estimates relating to the net average benefits from Roundup Ready Flex are about $25/ha in 2008 [20]. Overall, in 2008, the total farm income from using the technology was about $3 million and cumulatively, since 2000, the total gains have been $8.3 million.
- *Argentina*: No yield gain and a cost of technology in the range of $30–$40/ha, although with the increasing availability of stacked traits in recent years, the "cost" part of the HT technology has fallen to $24/ha. Net farm income gains (after deduction of the cost of the technology) have been $8–$18/ha and in 2008 were just under $10/ha. Overall, in 2008, the total farm income from using GM HT cotton technology was about $7.4 million, and cumulatively since 2002, the farm income gain has been $34.2 million.
- *South Africa*: No yield gain and a cost of technology in the range of $15–$25/ha. Net farm income gains from cost savings (after deduction of the cost of the technology) have been $30–$60/ha. In 2008, the average net gain was $33.6/ha and the total farm income benefit of the technology was $0.37 million. Cumulatively since 2001, the total farm income gain from GM HT cotton has been $2.2 million.
- *Mexico*: Average yield gains of +3.6% from improved weed control have been reported in the first 3 years of use, although no yield gain was recorded in 2008. The average cost of the technology has been in the range of $60–$66/ha and typical net farm income gains of about $80/ha, though in 2008, with no yield gains this fell back to $16/ha.

Overall, in 2008, the total farm income gain from using GM HT cotton was about $1.35 million and cumulatively since 2005, the total farm income gain has been $11.7 million.

Summary of Global Economic Impact

Across the five countries using GM HT cotton in 2008, the total farm income impact derived from using GM HT cotton was +$14.6 million. Cumulatively since 1997, there have been net farm income gains of $855.8 million (93% of this benefit has been in the USA). Of this, 96% has been due to cost savings and 4% to yield gains (from improved weed control relative to the level of weed control achieved using conventional technology).

Herbicide-Tolerant Canola

Canada

Canada was the first country to commercially use GM HT canola in 1996. Since then, the area planted to varieties containing GM HT traits has increased significantly, and in 2008 was 83% of the total crop (5.43 million hectares).

The farm-level impact of using GM HT canola in Canada since 1996 is summarized in Table 10. The key features are as follows:

- The primary impact in the early years of adoption was increased yields of almost 11% (e.g., in 2002 this yield increase was equivalent to an increase in total Canadian canola production of nearly 7%). In addition, a small additional price premia was achieved from crushers through supplying cleaner crops (lower levels of weed impurities). With the development of hybrid varieties using conventional technology, the yield advantage of GM HT canola relative to conventional alternatives (the main one of which is "Clearfield" conventionally derived herbicide-tolerant varieties. Also, hybrid canolas now account for the majority of plantings (including some GM hybrids) with the hybrid vigor delivered by conventional breeding techniques (even in the GM HT [to glyphosate] varieties) has been eroded. As a result, our analysis has applied the yield advantage of +10.7% associated with the GM HT technology in its early years of adoption (source: Canaola Council study of 2001) to 2003. From 2004, the yield gain has been based on differences between average annual variety trial results for "Clearfield" (conventional herbicide-tolerant varieties) and biotech alternatives. The biotech alternatives have also been differentiated into glyphosate tolerant and glufosinate tolerant. This resulted in the following observation: for GM glyphosate-tolerant varieties no yield difference for 2004, 2005, and 2008 and +4% 2006 and 2007. For GM glufosinate-tolerant varieties, the yield differences were +12% 2004 and 2008, +19% 2005, +10% 2006 and 2007. The quality premia associated with cleaner crops (see above) has not been included in the analysis from 2004.
- Cost of production (excluding the cost of the technology) has fallen, mainly through reduced expenditure on herbicides and some savings in fuel and labor. These savings have annually been between about $25/ha and $36/ha. The cost of the technology to 2003 was however marginally higher than these savings resulting in a net increase in costs of $3– $5/ha. On the basis of comparing GM HT canola with "Clearfield" HT canola (from 2004), there has been a net cost saving of between $5/ha and $10/ha, although in 2008 this was $17/ha.
- The overall impact on profitability (inclusive of yield improvements and higher quality) has been an increase of between $22/ha and $48/ha up to 2003. On the basis of comparing GM HT canola with "Clearfield" HT canola (from 2004), the net increase in profitability has been between $23/ha and $66/ha.
- The annual total national farm income benefit from using the technology has risen from $6 million in 1996 to $364 million in 2008. The cumulative farm income benefit over the 1996–2008 period (in nominal terms) was $1.64 billion.
- In added value terms, the increase in farm income in 2008 has been equivalent to an annual increase in production of 6.3%.

The USA

GM HT canola has been planted on a commercial basis in the USA since 1999. In 2008, 95% of the US canola crop was GM HT (380,230 ha).

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 10 Farm-level income impact of using GM HT canola in Canada 1996–2008

Year	Cost savings ($/ha)	Cost savings inclusive of cost of technology ($/ha)	Net cost saving/ increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1996	28.59	−4.13	45.11	6.23	0.4
1997	28.08	−4.05	37.11	21.69	1.17
1998	26.21	−3.78	36.93	70.18	3.43
1999	26.32	−3.79	30.63	90.33	5.09
2000	26.32	−3.79	22.42	59.91	5.08
2001	25.15	−1.62	23.10	53.34	5.69
2002	24.84	−3.59	29.63	61.86	6.17
2003	28.04	−4.05	41.42	132.08	6.69
2004	21.42	+4.44	19.09	70.72	4.48
2005	23.11	+4.50	32.90	148.12	6.56
2006	34.02	+16.93	50.71	233.13	8.09
2007	35.44	+17.46	66.39	341.44	7.54
2008	36.36	+17.56	66.63	364.23	6.35

Sources and notes:

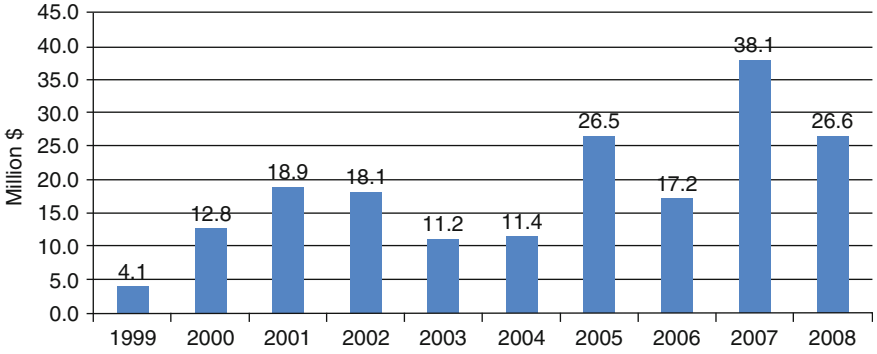
1. Impact data based on Canola Council study [21] to 2003 and Gusta et al. [22]. Includes a 10.7% yield improvement and a 1.27% increase in the price premium earned (cleaner crop with lower levels of weed impurities) until 2003. After 2004, the yield gain has been based on differences between average annual variety trial results for Clearfield and biotech alternatives. The biotech alternatives have also been differentiated into glyphosate tolerant and glufosinate tolerant. This resulted in the following observation: for GM glyphosate-tolerant varieties no yield difference for 2004, 2005, and 2008 and +4% 2006 and 2007. For GM glufosinate-tolerant varieties, the yield differences were +12% 2004 and 2008, +19% 2005, +10% 2006 and 2007

2. Negative values denote a net increase in the cost of production (i.e., the cost of the technology was greater than the other cost (e.g., on herbicides) reductions

3. All values for prices and costs denominated in Canadian dollars have been converted to US dollars at the annual average exchange rate in each year

The farm-level impact has been similar to the impact identified in Canada. More specifically, the following observations were noted:

- Average yields increased by about 6% in the initial years of adoption. As in Canada (see above) the availability of high-yielding hybrid conventional varieties has eroded some of this yield gain in recent year relative to conventional alternatives. As a result, the positive yield impacts post 2004 have been applied on the same basis as in Canada (comparison with Clearfields: see Canada above).
- The cost of the technology has been $12–$17/ha for glufosinate-tolerant varieties and $12–$33/ha for glyphosate-tolerant varieties. Cost savings (before inclusion of the technology costs) have been $35–$45/ha ($22/ha in 2008) for glufosinate-tolerant canola and $40–$79/ha for glyphosate-tolerant canola.
- The net impact on gross margins has been between +$22/ha and +$90/ha ($5/ha in 2008) for glufosinate-tolerant canola, and +$28/ha and +$61/ha for glyphosate-tolerant canola.
- At the national level, the total farm income benefit in 2008 was $26.6 million (Fig. 5) and the cumulative benefit since 1999 has been $185 million.
- In added value terms, the increase in farm income in 2008 has been equivalent to an annual increase in production of about 10.3%.



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 5
National farm income impact of using GM HT canola in the USA 1999–2008 (Source and notes: Impact analysis based on Sankala and Blumenthal [6, 7] and Johnson and Strom [8]. Decrease in total farm income impact 2002–2004 is due to decline in total plantings of canola in the USA (from 612,000 in 2002 to 316,000 ha in 2004). Positive yield impact applied in the same way as Canada from 2004)

Australia

GM HT canola was permitted for commercial use in the two states of Victoria and New South Wales in 2008, and was planted on 10,100 ha in that year (2008/09). Ninety-five percent of these plantings had tolerance to the herbicide glyphosate and the balance were tolerant to glufosinate.

A fairly comprehensive farm survey-based analysis of impact of the glyphosate-tolerant canola was commissioned by Monsanto, which involved interviews with 92 of the 108 farmers using this technology in 2008/09 [23, 24]. Key findings from this survey are as follows:

- The technology was made available in both open-pollinated and hybrid varieties, with the open-pollinated varieties representing the cheaper end of the seed market, where competition was mainly with open-pollinated varieties containing herbicide tolerance (derived conventionally) to herbicides in the triazine (TT) group. The hybrid varieties containing glyphosate tolerance competed with nonherbicide-tolerant conventional hybrid varieties and herbicide-tolerant "Clearfield" hybrids (tolerant to the imidazolinone group of herbicides), although, were used in 2008, all of the 33 farmers in the survey using GM HT hybrids did so mainly in competition and comparison with "Clearfield" varieties.
- The GM HT open-pollinated varieties sold to farmers at a premium of about $Aus3/ha (about $2.5 US/ha) relative to the TT varieties. The GM HT hybrids sold at a seed premium of about $Aus 9/ha ($7.55 US/ha) compared to "Clearfield" hybrids. In addition, farmers using the GM HT technology paid a "technology" fee in two parts; one part was a set fee of $Aus500 per farm plus $Aus 10.2/ton of output of canola. On the basis that there were 108 farmers using GM HT (glyphosate tolerant) technology in 2008, the average "up front" fee paid for the technology was $Aus5.62/ha. On the basis of average yields obtained for the two main types of GM HT seed used, those using open-pollinated varieties paid $11.83/ha (basis: average yield of 1.16 tons/ha) and those using GM HT hybrids paid $Aus12.95/ha (basis: average yield of 1.27 tons/ha). Therefore, the total seed premium and technology fee paid by farmers for the GM HT technology in 2008–2009 was $Aus20.45/ha ($17.16 US/ha) for open-pollinated varieties and $Aus 27.57/ha ($23.13 US/ha) for hybrid varieties. After taking into consideration, the seed premium/technology fees, the GM HT system was marginally more expensive by $Aus3/ha ($2.5 US/ha) and $Aus4/ha ($US 3.36/ha) respectively for weed control than the TT and Clearfield varieties.
- The GM HT varieties delivered higher average yields than their conventional counterparts: +22.11% compared to the TT varieties and +4.96% compared to the "Clearfield" varieties.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 11 Farm-level income impact of using GM HT canola in Australia 2008 ($US)

Year	Average cost saving ($/ha)	Average cost savings (net after cost of technology ($/ha)	Average net increase in gross margins ($/ha)	Increase in farm income at a national level ($)
2008	19.18	−20.77	93.37	943,054

Source derived from and based on Monsanto survey of license holders 2008

Notes:

1. The average values shown are weighted averages
2. Other weighted average values derive include yield +21.1% and quality (price) premium of 2.1% applied on the basis of this level of increase in average oil content

In addition, the GM HT varieties produced higher oil contents of +2% and +1.8% respectively compared to TT and "Clearfield" varieties.

- The average reduction in weed control costs from using the GM HT system (excluding seed premium/technology fee) was $Aus 17/ha for open-pollinated varieties (competing with TT varieties) and $Aus 24/ha for hybrids (competing with Clearfield varieties).

In the analysis summarized below in Table 11, these research findings have been applied to the total GM HT crop area on a weighted basis in which the results of GM HT open-pollinated varieties that compete with TT varieties were applied to 64% of the total area and the balance of area used the results from the GM HT hybrids competing with "Clearfield" varieties. This weighting reflects the distribution of farms in the survey, in which 59 (64%) of the farmers indicated they grew open-pollinated varieties and 33 (34%) grew hybrids. The findings show an average farm income gain of $US 93/ha and a total farm income gain of $0.93 million in 2008.

Summary of Global Economic Impact

In global terms, the farm-level impact of using GM HT technology in canola in Canada, the USA, and Australia was $392 million in 2008. Cumulatively, since 1996, the farm income benefit has been (in nominal terms) $1.83 billion. Within this, 79% has been due to yield gains and the balance (21%) has been from cost savings.

In terms of the total value of canola production in these three countries in 2008, the additional farm income generated by the technology is equal to a value-added equivalent of 6.9%. Relative to the value of global canola production in 2008, the farm income benefit added the equivalent of 1.5%.

GM Herbicide-Tolerant (GM HT) Sugar Beet

GM HT sugar beet was first grown commercially in the USA in 2007 (under 1,000 ha), although it was 2008 before sufficient quantities of seed were available for widespread commercial cultivation. In 2008, just under 258,000 ha of GM HT sugar beet were planted, equal to about 63.5% of the total US crop. The highest levels of penetration of the technology (85% plus of total crop) occurred in Idaho, Wyoming, Nebraska, and Colorado, with about 50% of the crops in the largest sugar beet growing states of North Dakota and Michigan being GM HT.

Impact of the technology in these early years of adoption has been identified as follows:

(a) *Yield*: Analysis by Kniss [25] covering a limited number of farms in Wyoming (2007) identified positive yield impacts of +8.8% in terms of additional root yield (from better weed control) and +12.6% in terms of sugar content relative to conventional crops (i.e., the GM HT crop had about a 3.8% higher sugar content, which amounts to a 12.8% total sucrose gain relative to conventional sugar beet once the root yield gain was taken into consideration). In contrast, Khan [26] found similar yields reported between conventional and GM HT sugar beet in the Red River Valley region (North Dakota) and Michigan. These contrasting results probably reflect a combination of factors including:

- The sugar beet growing regions in Wyoming can probably be classified as high weed problem areas, and as such, are regions where obtaining effective weed control is difficult using conventional technology (timing of application is key to weed control in sugar beet, with optimal time for application being when weeds are small). Also some weeds (e.g., Kochia) are

resistant to some of the commonly used ALS inhibitor herbicides like chlorsulfuron. The availability of GM HT sugar beet with its greater flexibility on application timing has therefore potentially delivered important yield gains for such growers.

- The GM HT trait was not available in all leading varieties suitable in all growing regions in 2008, hence the yield benefits referred to above from better weed control have to some extent been counterbalanced by only being available in poorer performing germ plasm in states like Michigan and North Dakota (notably not being available in 2008 in leading varieties with rhizomania resistance). It should be noted that the authors of the research cited in this section both perceive that yield benefits from using GM HT sugar beet will be a common feature of the technology in most regions once the technology is available in leading varieties.
- The year 2008 was reported to have been, in the leading sugar beet growing states, a reasonable year for controlling weeds through conventional technology (i.e., it was possible to get good levels of weed control through timely applications), hence the similar performance reported between the two systems.

(b) Costs of production

- Kniss's work in Wyoming identified weed control costs (comprising herbicides, application, cultivation, and hand labor) for conventional beet of $437/ha compared to $84/ha for the GM HT system. After taking into consideration the $131/ha seed premium/technology fee for the GM HT trait, the net cost differences between the two systems was $222/ha in favor of the GM HT system. Kniss did, however, acknowledge that the conventional costs associated with this sample were high relative to most producers (reflecting application of maximum dose rates for herbicides and use of hand labor), with a more typical range of conventional weed control costs being between $171/ha and $319/ha (average $245/ha).
- Khan's analysis puts the typical weed control costs in the Red River region of North Dakota to be about $227/ha for conventional compared to $91/ha for GM HT sugar beet. After taking into consideration the seed premium/technology fee (assumed by Khan to be $158/ha), the total weed control costs were $249/ha for the GM HT system, $22/ha higher than the conventional system. Despite this net increase in average costs of production, most growers in this region used (and planned to continue using), the GM HT system because of the convenience and weed control flexibility benefits associated with it (which research by Marra and Piggot [1]) estimated in the corn, soybean, and cotton sectors to be valued at between $12/ha and $25/ha to US farmers). It is also likely that Khan's analysis may understate the total cost savings from using the technology by not taking into account savings on application costs and labor for hand weeding.

For the purposes of our analysis, we have drawn on both these pieces of work, as summarized in Table 12. This shows a net farm income gain in 2008 of over $21 million to US sugar beet farmers (average gain per hectare of just under $83/ha). With the availability of GM HT technology in more of the leading varieties, it is expected that the farm income gains associated with yield gains will be greater in subsequent years.

GM Insect-Resistant (To Corn-Boring Pests: GM IR) Maize

The USA

GM IR maize was first planted in the USA in 1996 and in 2008, seed containing GM IR traits was planted on 57% (18.14 million hectares) of the total US maize crop.

The farm-level impact of using GM IR maize in the USA since 1996 is summarized in Table 13:

- The primary impact has been increased average yields of about 5% (in 2008 this additional production is equal to an increase in total US maize production of +2.41%).
- The net impact on cost of production has been a small increase of between $1/ha and $9/ha (additional cost of the technology being higher than the estimated average insecticide cost savings of $15–$16/ha).
- The annual total national farm income benefit from using the technology has risen from $8.76 million in

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 12 Farm-level income impact of using GM HT sugar beet in the USA 2007–2008

Year	Average cost saving ($/ha)	Average cost savings (net after cost of technology ($/ha)	Average net increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
2007	353.35	222.39	584.00	472,680	0.03
2008	142.50	−8.58	82.88	21,380,290	1.83

Sources derived from and based on Kniss [25] and Khan [26]

Notes:

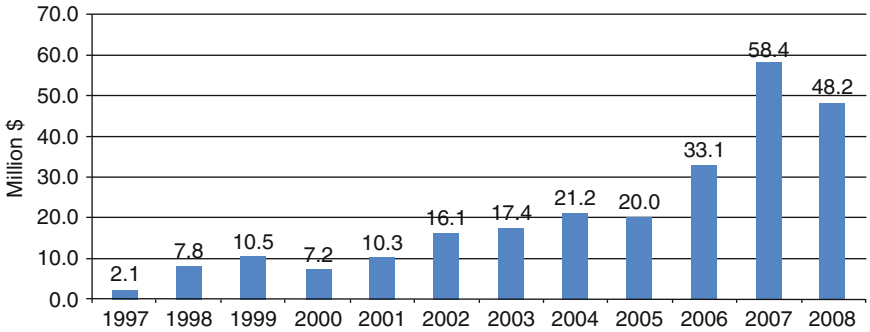
1. The yield gains identified by Kniss have been applied to the 2007 GM HT plantings in total and to the estimated GM HT plantings in the states of Idaho, Wyoming, Nebraska, and Colorado, where penetration of plantings in 2008 was 85% (these states account for 26% of the total GM HT crop in 2008), and which are perceived to be regions of above average weed problems. For all other regions, no yield gain is assumed. Across the entire GM HT area in 2008, this equates to a net average yield gain of +3.28%
2. The seed premium of $131/ha, average costs of weed control respectively for conventional and GM HT systems of $245/ha and $84/ha, from Kniss were applied to the crop in Idaho, Wyoming, Nebraska, and Colorado. The seed premium of $158/ha, weed control costs of $227/ha and $249/ha respectively for conventional and GM HT sugar beet, identified by Khan were applied to all other regions using the technology. These states account for 26% of the total GM HT crop in 2008. The resulting average values for seed premium/cost of technology across the entire 2008 GM HT crop was therefore $151.08/ha and the average weed control cost saving associated with the GM HT system (before taking into consideration the seed premium) was $142.5/ha

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 13 Farm-level income impact of using GM IR maize in the USA 1996–2008

Year	Cost saving ($/ha)	Cost savings (net after cost of technology ($/ha)	Net increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1996	24.71	−9.21	29.20	8.76	0.03
1997	24.71	−9.21	28.81	70.47	0.27
1998	20.30	−4.8	27.04	167.58	0.77
1999	20.30	−4.8	25.51	206.94	1.04
2000	22.24	−6.74	24.32	148.77	0.71
2001	22.24	−6.74	26.76	155.87	0.72
2002	22.24	−6.74	30.74	240.45	0.96
2003	22.24	−6.74	31.54	291.00	1.14
2004	15.88	−6.36	33.82	363.41	1.32
2005	15.88	−1.42	34.52	399.91	1.60
2006	15.88	−1.42	55.78	707.23	1.86
2007	15.88	−1.42	61.22	1,136.21	2.28
2008	24.71	−8.83	67.51	1,224.59	2.40

Sources and notes:

1. Impact data based on a combination of studies including the ISAAA (James) review [27], Marra et al. [3], Sankala and Blumenthal [6, 7], and Johnson and Strom [8], Gianessi and Carpenter [28]
2. Yield impact +5% based on average of findings of above studies
3. Insecticide cost savings based on the above references
4. – (minus) value for net cost savings means the cost of the technology is greater than the other cost savings



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 6

National farm income impact of using GM IR maize in Canada 1996–2008

Notes:

1. Yield increase of 5% based on industry assessments (consistent with US analysis). Cost of technology and insecticide cost savings based on US analysis,

2. GM IR area planted in 1996 = 1,000 ha,

3. All values for prices and costs denominated in Canadian dollars have been converted to US dollars at the annual average exchange rate in each year

1996 to $1.22 billion in 2008. The cumulative farm income benefit over the 1996–2008 period (in nominal terms) was $5.12 billion.

- In added value terms, the increase in farm income in 2008 was equivalent to an annual increase in production of 2.4%.

Canada

GM IR maize has also been grown commercially in Canada since 1996. In 2008, it accounted for 62% of the total Canadian maize crop of 1.2 million hectares. The impact of GM IR maize in Canada has been very similar to the impact in the USA (similar yield and cost of production impacts). At the national level, in 2008 the additional farm income generated from the use of GM IR maize was $48.2 million and cumulatively since 1996 the additional farm income (in nominal terms) was $252 million (Fig. 6).

Argentina

In 2008, GM IR maize traits were planted on 75% of the total Argentine maize crop (GM IR varieties were first planted in 1998).

The main impact of using the technology on farm profitability has been via yield increases. Various studies (e.g., see ISAAA review in James [27]) and Trigo and Cap [29] have identified an average yield increase in the region of 8–10%, hence an average of 9% has been used in the analysis up to 2004. More recent trade source estimates provided to the authors put the average yield increased in the last 2–3 years to be between 5% and 6%. Accordingly, our analysis uses a yield increase value of 5.5% for the years from 2004.

No savings in costs of production have arisen for most farmers because very few maize growers in Argentina have traditionally used insecticides as a method of control for corn-boring pests. As such, average costs of production have increased by $20–$22/ha (the cost of the technology).

The net impact on farm profit margins (inclusive of the yield gain) has, in recent years, been an increase of about $20/ha. In 2008, the national level impact on profitability was an increase of $41 million (an added value equal to 2.15% of the total value of production). Cumulatively, the farm income gain since 1997 has been $269.7 million.

South Africa

GM IR maize has been grown commercially in South Africa since 2000. In 2008, 56% of the country's total maize crop of 2.42 million hectares used GM IR cultivars.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 14 Farm-level income impact of using GM IR maize in South Africa 2000–2008

Year	Cost savings ($/ha)	Net cost savings inclusive of cost of technology ($/ha)	Net increase in gross margin ($/ha)	Impact on farm income at a national level ($ millions)
2000	13.98	1.87	43.77	3.31
2001	11.27	1.51	34.60	4.46
2002	8.37	0.6	113.98	19.35
2003	12.82	0.4	63.72	14.66
2004	14.73	0.46	20.76	8.43
2005	15.25	0.47	48.66	19.03
2006	14.32	−2.36	63.75	63.05
2007	13.90	0.22	182.90	225.70
2008	11.74	−4.55	87.07	117.73

Sources and notes:

1. Impact data (Sources: Gouse [30–32] and Van der Weld [33])
2. Negative value for the net cost savings = a net increase in costs (i.e., the extra cost of the technology was greater than the other (e.g., less expenditure on insecticides) cost savings
3. All values for prices and costs denominated in South African Rand have been converted to US dollars at the annual average exchange rate in each year

The impact on farm profitability is summarized in Table 14. The main impact has been an average yield improvement of between 5% and 32% in the years 2000–2004, with an average of about 15% (used as the basis for analysis 2005–2007). In 2008, the estimated yield impact was +10.6% (source: Van der Weld [33]). The cost of the technology $8–$17/ha has broadly been equal to the average cost savings from no longer applying insecticides to control corn-boring pests.

At the national level, the increase in farm income in 2008 was $117.7 million and cumulatively since 2000 it has been $476 million. In terms of national maize production, the use of GM IR technology on 56% of the planted area has resulted in a net increase in national maize production of 5.9% in 2008. The value of the additional income generated was also equivalent to an annual increase in production of about 5.1%.

Spain

Spain has been commercially growing GM IR maize since 1998 and in 2008, 22% (79,270 ha) of the country's maize crop was planted to varieties containing a GM IR trait.

As in the other countries planting GM IR maize, the main impact on farm profitability has been increased yields (an average increase in yield of 6.3% across farms using the technology in the early years of adoption). With the availability and widespread adoption of the Mon 810 trait from 2003, the reported average positive yield impact is about +10%. There has also been a net annual average saving on cost of production (from lower insecticide use) of between $37/ha and $61/ha (Table 15). At the national level, these yield gains and cost savings have resulted in farm income being boosted, in 2008 by $17.9 million and cumulatively since 1998 the increase in farm income (in nominal terms) has been $77.9 million.

Relative to national maize production, the yield increases derived from GM IR maize were equivalent to a 2.2% increase in national production (2008). The value of the additional income generated from Bt maize was also equivalent to an annual increase in production of 2.1%.

Other EU countries

A summary of the impact of GM IR technology in other countries of the EU is presented in Table 16. This shows

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 15 Farm-level income impact of using GM IR maize in Spain 1998–2008

Year	Cost savings ($/ha)	Net cost savings inclusive of cost of technology ($/ha)	Net increase in gross margin ($/ha)	Impact on farm income at a national level ($ millions)
1998	37.40	3.71	95.16	2.14
1999	44.81	12.80	102.20	2.56
2000	38.81	12.94	89.47	2.24
2001	37.63	21.05	95.63	1.10
2002	39.64	22.18	100.65	2.10
2003	47.50	26.58	121.68	3.93
2004	51.45	28.79	111.93	6.52
2005	52.33	8.72	144.74	7.70
2006	52.70	8.78	204.5	10.97
2007	57.30	9.55	274.59	20.63
2008	61.49	10.25	225.36	17.86

Sources and notes:

1. Impact data (based on Brookes [34] and Brookes [35]). Yield impact +6.3% to 2004 and 10% used thereafter (originally Bt 176, latterly Mon 810). Cost of technology based on €18.5/ha to 2004 and €35/ha from 2005
2. All values for prices and costs denominated in Euros have been converted to US dollars at the annual average exchange rate in each year

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 16 Farm-level income impact of using GM IR maize in other EU countries 2005–2008

	Year first planted GM IR maize	Area 2008 (hectares)	Yield impact (%)	Cost of technology 2008 ($/ha)	Cost savings 2008 (before deduction of cost of technology: $/ha)	Net increase in gross margin 2008 ($/ha)	Impact on farm income at a national level 2008 (million $)
France	2005	Nil	N/p	N/p	N/p	N/p	N/p
Germany	2005	3,173	+4	58.57	73.21	78.64	0.25
Portugal	2005	4,851	+12.5	51.24	0	75.60	0.37
Czech Republic	2005	8,380	+10	51.24	26.35	101.95	0.85
Slovakia	2005	1,930	+12.3	51.24	0	228.31	0.44
Poland	2006	3,000	+12.5	51.24	0	133.08	0.40
Romania	2007	7,146	+7.1	46.85	0	26.59	0.19
Total other EU (excluding Spain)		28,480					2.5

Source and notes:

1. Source: Based on Brookes [35]
2. All values for prices and costs denominated in Euros have been converted to US dollars at the annual average exchange rate in each year
3. N/p – planting not permitted in France in 2008

that in 2008, the additional farm income derived from using GM IR technology in these six countries was +$2.5 million, and cumulatively over the 2005–2008 period, the total income gain was $11.1 million.

Other Countries

GM IR maize has been grown commercially in the following countries:

- *The Philippines* since 2003. In 2008, 280,000 ha out of total plantings of 2.6 million (7%) were GM IR. Estimates of the impact of using GM IR (Sources: Gonsalves [36], Yorobe [37], and Ramon [38]) show annual average yield increases in the range of 14.3–34%. Taking the midpoint of this range (+24.15%), coupled with a small average annual insecticide cost saving of about $12–$13/ha and average cost of the technology of about $33/ha, the net impact on farm profitability has been between $37/ha and $109/ha. In 2008, the national farm income benefit derived from using the technology was $33.5 million and cumulative farm income gain since 2003 has been $61.2 million.
- *Uruguay* since 2004, and in 2008, 110,000 ha (73% of the total crop) were GM IR. Using Argentine data as the basis for assessing impact, the cumulative farm income gain over the 3 years has been $3.9 million.
- *Brazil* starting in 2008, when 1.45 million hectares were planted to varieties containing a GM IR trait. Based on analysis from Galveo [12], the average yield impact was +4.66%, the cost of the technology was $21.6/ha, insecticide cost savings were $42/ha, and the average improvement to farm income equal to $48.12/ha. Overall, the increase in farm profitability associated with the adoption in 2008 was $69.8 million;
- *Honduras*. Here farm-level "trials" have been permitted since 2003, and in 2008, an estimated 9,000 ha used GM IR traits. Evidence from Falck Zepeda et al. [39] indicated that the primary impact of the technology has been to increase average yields (in 2008 +24%). As insecticides have not traditionally been used by most farmers, no costs of production savings have arisen, coupled with no additional cost for use of the technology (which has been provided free of charge for the trials). In our analysis, we have, however assumed a cost of the technology of $30/ha, and based on this, the estimated farm income benefit derived from the technology was $1.1 million in 2008 and cumulatively since 2003 the income gain has been $2 million.

Summary of Economic Impact

In global terms, the farm-level impact of using GM IR maize was $1.56 billion in 2008. Cumulatively since 1996, the benefit has been (in nominal terms) $6.34 billion. This farm income gain has mostly derived from improved yields (less pest damage) although in some countries farmers have derived a net cost saving associated with reduced expenditure on insecticides.

In terms of the total value of maize production from the countries growing GM IR maize in 2008, the additional farm income generated by the technology is equal to a value-added equivalent of 2.2%. Relative to the value of global maize production in 2008, the farm income benefit added the equivalent of 1.2%.

Insect-Resistant (Bt) Cotton (GM IR)

The USA

GM IR cotton has been grown commercially in the USA since 1996 and by 2008, was used in 63% (1.93 million hectares) of total cotton plantings.

The farm income impact of using GM IR cotton is summarized in Table 17. The primary benefit has been increased yields (by 9–11%), although small net savings in costs of production have also been obtained (reduced expenditure on insecticides being marginally greater than the cost of the technology). Overall, average profitability levels increased by $53–$115/ha with Bollgard I cotton (with a single Bt gene) between 1996 and 2002 and by between $87/ha and $118/ha in 2003–2008 with Bollgard II (containing two Bt genes and offering a broader spectrum of control). This resulted in a net gain to farm income in 2008 of $189 million. Cumulatively, since 1996, the farm income benefit has been $2.44 billion. In added value terms, the effect of the increased yields and reduced costs of production on farm income in 2008 was equivalent to an annual increase in production of 6.3% (165,400 tons).

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 17 Farm-level income impact of using GM IR cotton in the USA 1996–2008

Year	Cost savings (net after cost of technology ($/ha)	Net increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1996	4.98	115.32	94.69	1.19
1997	4.98	103.47	87.28	1.30
1998	4.98	88.54	80.62	1.47
1999	4.98	65.47	127.29	2.89
2000	4.98	74.11	162.88	3.10
2001	4.98	53.04	125.22	3.37
2002	4.98	69.47	141.86	3.11
2003	5.78	120.49	239.98	4.27
2004	5.78	107.47	261.23	4.82
2005	24.48	117.81	332.41	5.97
2006	−5.77	86.61	305.17	4.86
2007	−2.71	114.50	296.00	5.49
2008	−2.71	98.22	189.50	5.89

Sources and notes:

1. Impact data based on Gianessi and Carpenter [28], Sankala and Blumenthal [6, 7], Johnson and Strom [8], Marra et al. [3], and Mullins and Hudson [40]
2. Yield impact +9% 1996–2002 Bollgard I and +11% 2003 onward Bollgard II
3. Cost of technology: 1996–2002 Bollgard I $58.27/ha, 2003–2004 Bollgard II $68.32/ha, $49.62/ha 2005, $46.95/ha 2006, $25.7/ha 2007 and 2008
4. Insecticide cost savings $63.26/ha 1996–2002, $74.10/ha 2003–2005, $41.18/ha 2006, $28.4/ha 2007 and 2008

China

China first planted GM IR cotton in 1997, since when the area planted to GM IR varieties has increased to 64% of the total 5.95 million hectares crop in 2008.

As in the USA, a major farm income impact has been via higher yields of 8–10% on the crops using the technology, although there have also been significant cost savings on insecticides used and the labor previously used to undertake spraying. Overall, annual average costs have fallen by about $145–$200/ha and annual average profitability improved by $123–$472/ha. In 2008, the net national gain to farm income was $859 million (Table 18). Cumulatively, since 1997, the farm income benefit has been $7.6 billion. In added value terms, the effect of the increased yields and reduced costs of production on farm income in 2008 was equivalent to an annual increase in production of 17.1% (1.38 million tons).

Australia

Australia planted 83% of its 2008 cotton crop (total crop of 146,000 ha) to varieties containing GM IR traits (Australia first planted commercial GM IR cotton in 1996).

Unlike the other main countries using GM IR cotton, Australian growers have rarely derived yield gains from using the technology (reflecting the effective use of insecticides for pest control prior to the availability of GM IR cultivars), with the primary farm income benefit being derived from lower costs of

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 18 Farm-level income impact of using GM IR cotton in China 1997–2008

Year	Cost savings (net after cost of technology ($/ha)	Net increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1997	194	333	11.33	0.13
1998	194	310	80.97	1.15
1999	200	278	181.67	4.62
2000	−14	123	150.18	2.61
2001	378	472	1,026.26	20.55
2002	194	327	687.27	11.19
2003	194	328	917.00	12.15
2004	194	299	1,105.26	16.89
2005	145	256	845.58	13.57
2006	146	226	792.28	16.86
2007	152	248	942.7	14.46
2008	148	224	858.6	17.14

Sources and notes:

1. Impact data based on Pray et al. [41, 42], which covered the years 1999–2001. Other years based on average of the 3 years, except 2005 onward based on Shachuan (2006) – personal communication
2. Negative cost savings in 2000 reflect a year of high pest pressure (of pests not the target of GM IR technology), which resulted in above average use of insecticides on GM IR using farms
3. Yield impact +8% 1997–1999 and +10% 2000 onward
4. Negative value for the net cost savings in 2000 = a net increase in costs (i.e., the extra cost of the technology was greater than the savings on insecticide expenditure – a year of lower than average bollworm problems
5. All values for prices and costs denominated in Chinese Yuan have been converted to US dollars at the annual average exchange rate in each year

production (Table 19). More specifically, the following observations were made:

- In the first 2 years of adoption of the technology (Ingard, single gene Bt cotton), small net income losses were derived, mainly because of the relatively high price charged for the seed. Since this price was lowered in 1998, the net income impact has been positive, with cost saving of between $54/ha and $90/ha, mostly derived from lower insecticide costs (including application) more than offsetting the cost of the technology.
- For the last few years of use, Bollgard II cotton (containing 2 Bt genes) has been available offering effective control of a broader range of cotton pests. Despite the higher costs of this technology, users have continued to make significant net cost savings of $186–$212/ha.
- At the national level in 2008, the net farm income gains were $24.2 million and cumulatively since 1996 the gains have been $214.9 million.
- In added value terms, the effect of the reduced costs of production on farm income in 2008 was equivalent to an annual increase in production of 37% (105,000 tons).

Argentina

GM IR cotton has been planted in Argentina since 1998. In 2008, it accounted for 73% of total cotton plantings.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 19 Farm-level income impact of using GM IR cotton in Australia 1996–2008

Year	Cost of technology ($/ha)	Net increase in gross margins/cost saving after cost of technology ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1996	−191.7	−41.0	−1.63	−0.59
1997	−191.7	−35.0	−2.04	−0.88
1998	−97.4	91.0	9.06	0.43
1999	−83.9	88.1	11.80	4.91
2000	−89.9	64.9	10.71	4.38
2001	−80.9	57.9	7.87	5.74
2002	−90.7	54.3	3.91	3.43
2003	−119.3	256.1	16.3	11.49
2004	−179.5	185.8	45.7	21.33
2005	−229.2	193.4	47.9	23.75
2006	−225.9	190.7	22.49	26.01
2007	−251.33	212.1	11.73	40.90
2008	−264.26	199.86	24.23	37.40

Sources and notes:

1. Impact data based on Doyle [43], Taylor [44], CSIRO [45] for bollgard II since 2004
2. All values for prices and costs denominated in Australian dollars have been converted to US dollars at the annual average exchange rate in each year

The main impact in Argentina has been yield gains of 30% (which has resulted in a net increase in total cotton production (2008) of 22%). This has more than offset the cost of using the technology. In terms of gross margin, cotton farmers have gained annually between $25/ha and $249/ha during the period 1998–2007. At the national level, the annual farm income gains in the last 5 years have been in the range of $2–$27 million (Fig. 7). Cumulatively since 1998, the farm income gain from use of the technology has been $95.4 million. In added value terms, the effect of the yield increases (partially offset by higher costs of production) on farm income in 2008 was equivalent to an annual increase in production of 14.6%.

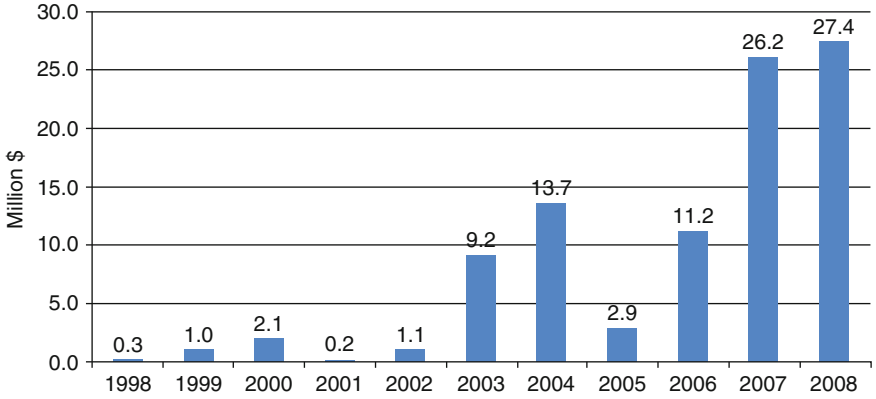
Mexico

GM IR cotton has been planted commercially in Mexico since 1996. In 2008, GM IR cotton was planted on 70,000 ha (56% of total cotton plantings).

The main farm income impact of using the technology has been yield improvements of between 6% and 9% over the last 6 years. In addition, there have been important savings in the cost of production (lower insecticide costs). Overall, the annual net increase in farm profitability has been within the range of $104/ha and $354/ha between 1996 and 2008 (Table 20). At the national level, the farm income benefit in 2008 was $10.5 million and the impact on total cotton production was an increase of 5.2%. Cumulatively since 1996, the farm income benefit has been $76.4 million. In added value terms, the combined effect of the yield increases and lower cost of production on farm income in 2008 was equivalent to an annual increase in production of 5.4%.

South Africa

In 2008, GM IR cotton (first planted commercially in 1998) was planted on 7,750 ha in South Africa (84% of the total crop).



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 7
National farm income impact of using GM IR cotton in Argentina 1998–2008
Sources and notes:
1. Impact data (Sources: Qaim and De Janvry [46, 47]), Elena [48] and for 2005 and 2006 Monsanto LAP, although cost of technology in 2005 from Monsanto Argentina. Area data: source ArgenBio
2. Yield impact +30%, cost of technology $86/ha ($40/ha 2005), cost savings (reduced insecticide use) $17.47/ha
3. All values for prices and costs denominated in Argentine Pesos have been converted to US dollars at the annual average exchange rate in each year

The main impact on farm incomes has been significantly higher yields (an annual average increase of about 24%). In terms of cost of production, the additional cost of the technology (between $17/ha and $24/ha for Bollgard I and $40–$50/ha for Bollgard II (2006 onward) has been greater than the insecticide cost and labor (for water collection and spraying) savings ($12–$23/ha), resulting in an increase in overall cost of production of $2–$32/ha. Combining the positive yield effect and the increase in cost of production, the net effect on profitability has been an annual increase of between $27/ha and $232/ha.

At the national level, farm incomes, over the last 5 years have annually increased by between $1.2 million and $1.7 million (Fig. 8). Cumulatively since 1998, the farm income benefit has been $21 million. The impact on total cotton production was an increase of 20.1% in 2008. In added value terms, the combined effect of the yield increases and lower costs of production on farm income in 2008 was equivalent to an annual increase in production of 14.5% (based on 2008 production levels).

India

GM IR cotton has been planted commercially in India since 2002. In 2008, 6.97 million hectares were planted to GM IR cotton, which is equal to 77% of total plantings.

The main impact of using GM IR cotton has been major increases in yield [54] found average yield increases of 45% in 2002 and 63% in 2003 (average over the 2 years of 54%) relative to conventionally produced cotton. More recent survey data from Monsanto [16] confirm this high-yield impact (+58% reported in 2004) as do data from IMRB [55], which found an average yield increase of 64% in 2005, and IMRB [56], which found a yield impact of +50% in 2006. With respect to cost of production, the average cost of the technology (seed premium: $49–$54/ha) up to 2006 was greater than the average insecticide cost savings of $31–$58/ha resulting in a net increase in costs of production. Following the reduction in the seed premium in 2006 to about $20/ha, farmers have, on average made a net cost saving of about $25/ha. Coupled with the yield gains, important net gains to levels of profitability have been achieved of between $82/ha and $356/ha. At the national level, the farm income gain in 2008 was $1.79 billion and cumulatively since 2002 the farm income gains have been $5.14 billion (Table 21).

The impact on total cotton production was an increase of 31% in 2008 and in added value terms, the combined effect of the yield increases and higher costs of production on farm income in 2008 was equivalent

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 20 Farm-level income impact of using GM IR cotton in Mexico 1996–2008

Year	Cost savings (net after cost of technology ($/ha)	Net increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
1996	58.1	354.5	0.32	0.1
1997	56.1	103.4	1.72	0.5
1998	38.4	316.4	11.27	2.71
1999	46.5	316.8	5.27	2.84
2000	47.0	262.4	6.85	5.76
2001	47.6	120.6	3.04	3.74
2002	46.1	120.8	1.84	3.81
2003	41.0	127.7	3.33	3.67
2004	39.3	130.4	6.24	4.51
2005	40.8	132.3	10.4	7.64
2006	20.4	124.4	6.44	4.06
2007	20.5	139.7	8.38	4.74
2008	19.9	150.4	10.52	5.44

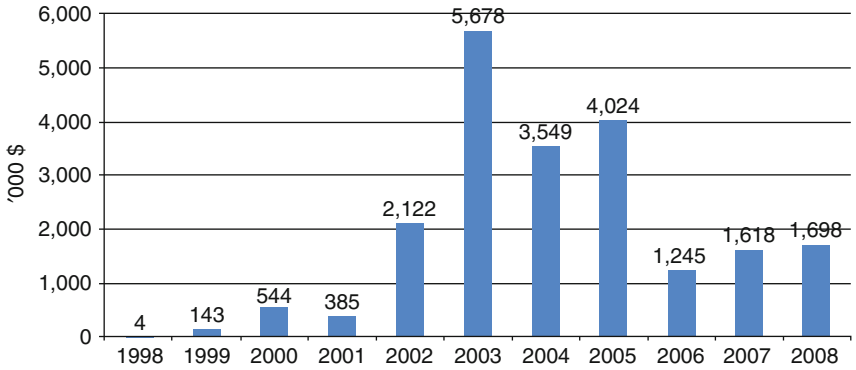
Sources and notes:

1. Impact data based on Traxler et al. [49] covering the years 1997 and 1998. Yield changes data in other years based on official reports submitted to the Mexican Ministry of Agriculture by Monsanto Comercial (Mexico). Also, Martinez-Carillo and Diaz-Lopez [50]
2. Yield impacts: 1996 +37%, 1997 +3%, 1998 +20%, 1999 +27%, 2000 +17%, 2001 +9%, 2002 +7%, 2003 +6%, 2004 +7.6%, 2005 onward +9.25%
3. All values for prices and costs denominated in Mexican Pesos have been converted to US dollars at the annual average exchange rate in each year

to an annual increase in production of 24% (based on the 2008 production level that is inclusive of the GM IR related yield gains).

Brazil

GM IR cotton was planted commercially in Brazil for the first time in 2006, and in 2008 was planted on 178,000 ha (20% of the total crop). This represents a fall in the share of total plantings relative to 2007, when GM IR traits were planted on 32% of the crop. This decline in plantings largely reflects the relative performance of the seed containing the GM IR traits compared to the leading conventional varieties, in which the GM IR trait has not been available. In 2006, on the basis of industry estimates of impact of GM IR cotton relative to similar varieties, an average yield gains of +6% and a net cost saving (reduced expenditure on insecticides after deduction of the premium paid for using the technology) of about +$25/ha were realized. In 2007 and 2008, however, analysis by Galveo [12] and Monsanto Brazil [57] suggests that the yield performance of the varieties containing GM IR traits has been lower (by −3.6% and −2.7% respectively for 2007 and 2008). As a result, the net farm income of using the technology was (after taking into consideration insecticide cost savings and the seed premium), on average, −$34.5/ha in 2007 and a small net gain of about $2/ha in 2008. At a national level in 2008, GM IR cotton technology delivered a net gain of about $0.35 million (a net loss of $12.3 million in 2007). Cumulatively, the total farm income impact has been positive at about $5 million.



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 8
National farm income impact of using GM IR cotton in South Africa 1998–2008
Sources and notes:
1. Impact data based on Ismael et al. [51], Kirsten et al. [52], Morse et al. [53]
2. Yield impact +24%, cost of technology $14–$24/ha for Bollgard I and about $50/ha for Bollgard II, cost savings (reduced insecticide use) $12–$23/ha
3. All values for prices and costs denominated in South African Rand have been converted to US dollars at the annual average exchange rate in each year
4. The decline in the total farm income benefit post 2003 relative to earlier years reflects the decline in total cotton plantings. This was caused by relatively low farm-level prices for cotton in 2004 and 2005 (reflecting a combination of relatively low world prices and a strong South African currency). In more recent years, cotton has become less competitive relative to alternatives such as corn because of higher world grain prices

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 21 Farm-level income impact of using GM IR cotton in India 2002–2008

Year	Cost savings (net after cost of technology ($/ha)	Net increase in gross margins ($/ha)	Increase in farm income at a national level ($ millions)	Increase in national farm income as % of farm-level value of national production
2002	−12.42	82.66	3.69	0.26
2003	−16.2	209.85	20.98	0.47
2004	−13.56	193.36	96.68	1.86
2005	−22.25	255.96	332.74	5.26
2006	3.52	221.02	839.89	14.04
2007	26.41	356.85	2,093.97	22.84
2008	24.28	256.73	1,790.16	24.27

Sources and notes:
1. Impact data based on Bennett et al. [54] and IMRB [55, 56]. As 2008 was reported to be a year of below average pest pressure, the average yield gain used was reduced to +40%
2. All values for prices and costs denominated in Indian Rupees have been converted to US dollars at the annual average exchange rate in each year

Other Countries

- *Colombia*: GM IR cotton has been grown commercially in Colombia since 2002 (20,000 ha planted in 2008 out of a total cotton crop of 40,000 ha). Drawing on recent analysis of impact by Zambrano et al. [58], this shows the main impact has been through a significant improvement in yields of +32%. On the cost impact side, this analysis shows that farmers using GM IR cotton tend to have substantially higher expenditures on pest control than their conventional counterparts, which when taking into consideration the approximate $70/ha cost of the technology results in a net addition to costs of between $200/ha and $280/ha each year (relative to typical expenditures by conventional cotton growers). Nevertheless, after taking into consideration the positive yield effects, the net impact on profitability has been positive. In 2008, the average improvement in profitability was about $33/ha and the total net gain from using the technology was $0.91 million. Cumulatively, since 2002, the net farm income gain has been $13.9 million.
- *Burkino Faso*: GM IR cotton was grown commercially first in 2008. Based on analysis of precommercial trials by Vitale et al. [59, 60], the main impact of the technology is improved yields (by +20%) and savings in insecticide expenditure of about $62/ha. Based on a cost of technology of about $42/ha, the net cost savings are about $20/ha, and inclusive of the yield gains, the estimated net income gain in 2008 was $124/ha. The total aggregate farm income gain in 2008 was therefore $1 million.

Summary of Global Impact

In global terms, the farm-level impact of using GM IR cotton was $2.9 billion in 2008. Cumulatively, since 1996, the farm income benefit has been (in nominal terms) $15.61 billion. Within this, 65% of the farm income gain has derived from yield gains (less pest damage) and the balance (35%) from reduced expenditure on crop protection (spraying of insecticides).

In terms of the total value of cotton production from the countries growing GM IR in 2008, the additional farm income generated by the technology is equal to a value-added equivalent of 19.3% (based on the 2008 production level inclusive of the GM IR related yield gains). Relative to the value of global cotton production in 2008, the farm income benefit added the equivalent of 11.1%.

Other Biotech Crops

Maize/Corn Rootworm Resistance

GM rootworm-resistant (CRW) corn has been planted commercially in the USA since 2003. In 2008, there were 13.7 million hectares of CRW corn (43% of the total US crop).

The main farm income impact (Impact data based on Sankala and Blumenthal [6, 7], Johnson and Strom [8], Rice [61]), and Alston et al. [62]) has been higher yields of about 5% relative to conventional corn. The impact on average costs of production has been +$2/ha to −$10/ha (based on an average cost of the technology of $35–$42/ha and an insecticide cost saving of $32–$37/ha). As a result, the net impact on farm profitability has been +$28/ha to +$79/ha.

At the national level, farm incomes increased by $4.6 million in 2003, rising to $1.1 billion in 2008. Cumulatively since 2003, the total farm income gain from the use of CRW technology in the USA corn crop has been $2 billion.

CRW cultivars were also planted commercially for the first time in 2004 in Canada. In 2008, the area planted to CRW-resistant varieties was 119,380 ha. Based on US costs, insecticide cost savings and yield impacts, this has resulted in additional income at the national level of $8.65 million in 2008 (cumulative total since 2004 of $13 million).

At the global level, the extra farm income derived from biotech CRW maize use since 2003 has been just over $2 billion. In 2008, the additional farm income generated from use of the technology was equal to 0.9% of the value of the global maize crop.

Virus-Resistant Papaya

Ring spot-resistant papaya has been commercially grown in the USA (State of Hawaii) since 1999, and in 2008 (85% of the state's papaya crop was GM virus resistant (700 ha).

The main farm income impact of this biotech crop has been to significantly increase yields relative to conventional varieties. Compared to the average yield in the last year before the first biotech cultivation (1998), the annual average yield increase of biotech papaya relative to conventional crops has been within a range of +15% to +77% (29% in 2008). At a state level, this is equivalent to a 25% increase in total papaya production in 2008.

In terms of profitability (Impact data based on Sankala and Blumenthal [6, 7] and Johnson and Strom [8]), the net annual impact has been an improvement of between $3,000/ha and $29,000/ha, and in 2008 this amounted to a net farm income gain of $5,790/ha and an aggregate benefit across the state of $4 million. Cumulatively, the farm income benefit since 1999 has been $53.4 million.

Virus-resistant papaya is also reported to have been grown in China in 2008, on 4,500 ha. No impact data on this technology has been identified.

Virus-Resistant Squash

Biotech virus-resistant squash has also been grown in some states of the USA since 2004 and is estimated to have been planted on 2,900 ha in 2008 (17% of the total crop in the USA – mostly found in Georgia and Florida).

Based on analysis from Johnson and Strom [8], the primary farm income impact of using biotech virus-resistant squash has been derived from higher yields, which in 2008, added a net gain to users of $26 million. Cumulatively, the farm income benefit since 2004 has been $107 million.

Insect-Resistant Potatoes

GM insect-resistant potatoes were also grown commercially in the USA between 1996 and 2000 (planted on 4% of the total US potato crop in 1999 (30,000 ha). This technology was withdrawn in 2001 when the technology provider (Monsanto) withdrew from the market to concentrate on GM trait development in maize, soybeans, cotton, and canola. This commercial decision was also probably influenced by the decision of some leading potato processors and fast-food outlets to stop using GM potatoes because of perceived concerns about this issue from some of their consumers, even though the GM potato provided the producer and the processor with a lower cost, higher yielding, and more consistent product. It also delivered significant reductions in insecticide use Carpenter and Gianessi (2002).

Indirect (Nonpecuniary) Farm-Level Economic Impacts

Apart from the tangible and quantifiable impacts on farm profitability presented above, there are other important, more intangible (difficult to quantify) impacts of an economic nature.

Many of the studies of the impact of biotech crops have identified the following reasons as being important influences for adoption of the technology:

Herbicide-Tolerant Crops

- Increased management flexibility and convenience that comes from a combination of the ease of use associated with broad-spectrum, post-emergent herbicides like glyphosate and the increased/longer time window for spraying. This not only frees up management time for other farming activities but also allows additional scope for undertaking off-farm, income-earning activities.
- In a conventional crop, post-emergent weed control relies on herbicide applications before the weeds and crop are well established. As a result, the crop may suffer "knock-back" to its growth from the effects of the herbicide. In the GM HT crop, this problem is avoided because the crop is both tolerant to the herbicide and spraying can occur at a later stage when the crop is better able to withstand any possible "knock-back" effects.
- Facilitates the adoption of conservation or no tillage systems. This provides for additional cost savings such as reduced labor and fuel costs associated with plowing, additional moisture retention, and reductions in levels of soil erosion.
- Improved weed control has contributed to reduced harvesting costs – cleaner crops have resulted in reduced times for harvesting. It has also improved harvest quality and led to higher levels of quality price bonuses in some regions and years (e.g., HT soybeans and HT canola in the early years of adoption respectively in Romania and Canada).

- Elimination of potential damage caused by soil-incorporated residual herbicides in follow-on crops and less need to apply herbicides in a follow-on crop because of the improved levels of weed control.
- A contribution to the general improvement in human safety (as manifest in greater peace of mind about own and worker safety) from reduced exposure to herbicides and a switch to more environmentally benign products.

Insect-Resistant Crops

- Production risk management/insurance purposes – the technology takes away much of the worry of significant pest damage occurring and is, therefore, highly valued. Piloted in 2008 and more widely operational from 2009, US farmers using stacked corn traits (containing insect resistance and herbicide-tolerant traits) are being offered discounts on crop insurance premiums equal to $7.41/ha.
- A "convenience" benefit derived from having to devote less time to crop walking and/or applying insecticides.
- Savings in energy use – mainly associated with less use of aerial spraying and less tillage.
- Savings in machinery use (for spraying and possibly reduced harvesting times).
- Higher quality of crop. There is a growing body of research evidence relating to the superior quality of GM IR corn relative to conventional and organic corn from the perspective of having lower levels of mycotoxins. Evidence from Europe (as summarized in Brookes [35] has shown a consistent pattern in which GM IR corn exhibits significantly reduced levels of mycotoxins compared to conventional and organic alternatives. In terms of revenue from sales of corn, however, no premia for delivering product with lower levels of mycotoxins have, to date, been reported although where the adoption of the technology has resulted in reduced frequency of crops failing to meet maximum permissible fumonisin levels in grain maize (e.g., in Spain), this delivers an important economic gain to farmers selling their grain to the food using sector. GM IR corn farmers in the Philippines have also obtained price premia of 10% [37] relative to conventional corn because of better quality, less damage to cobs and lower levels of impurities.
- Improved health and safety for farmers and farm workers (from reduced handling and use of pesticides, especially in developing countries where many apply pesticides with little or no use of protective clothing and equipment).
- Shorter growing season (e.g., for some cotton growers in India), which allows some farmers to plant a second crop (notably maize) in the same season. Also some Indian cotton growers have reported knock on benefits for beekeepers as fewer bees are now lost to insecticide spraying [63].

Some of the economic impact studies have attempted to quantify some of these benefits (e.g., Qaim and Traxler [9] quantified some of these in Argentina (a $3.65/ha saving (−7.8%) in labor costs and a $6.82/ha (−28%) saving in machinery/fuel costs associated with the adoption of GM HT soybeans). Where identified, these cost savings have been included in the analysis presented above. Nevertheless, it is important to recognize that these largely intangible benefits are considered by many farmers as a primary reason for adoption of GM technology, and in some cases farmers have been willing to adopt for these reasons alone, even when the measurable impacts on yield and direct costs of production suggest marginal or no direct economic gain.

Since the early 2000s, a number of farmer-survey based studies in the USA have also attempted to better quantify these nonpecuniary benefits. These studies have usually employed contingent valuation techniques to obtain farmers valuations of nonpecuniary benefits. A summary of these findings is shown in (Table 22).

Aggregating the Impact to US Crops 1996–2008

The approach used to estimate the nonpecuniary benefits derived by US farmers from biotech crops over the period 1996–2008 has been to draw on the values identified by Marra and Piggot ([1, 2]: Table 22) and to apply these to the biotech crop planted areas during this 13-year period. Figure 9 summarizes the values for nonpecuniary benefits derived from biotech crops in the USA (1996–2008) and shows an estimated (nominal value) benefit of $855 million in

2008 and a cumulative total benefit (1996–2008) of $5.99 billion. Relative to the value of direct farm income benefits presented above, the nonpecuniary benefits were equal to 21% of the total direct income benefits in 2008 and 25.6% of the total cumulative (1996–2008) direct farm income. This highlights the important contribution this category of benefit has had on biotech trait adoption levels in the USA, especially where the direct farm income benefits have been identified to be relatively small (e.g., HT cotton).

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 22 Values of nonpecuniary benefits associated with biotech crops in the USA

Survey	Median value ($/ha)
2002 IR (to rootworm) corn growers survey	7.41
2002 soybean (HT) farmers survey	12.35
2003 HT cropping survey (corn, cotton, and soybeans) – North Carolina	24.71
2006 HT (flex) cotton survey	12.35 (relative to first generation HT cotton)

Source: Marra and Piggot 2006 and 2007 [1, 2]

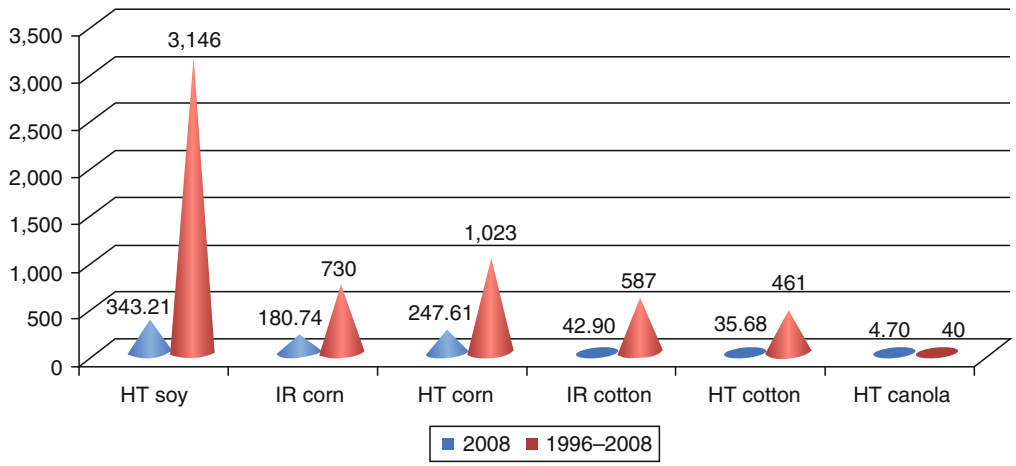
Estimating the Impact in Other Countries

It is evident from the literature review that farmers in other countries, who use GM technology, also value the technology for a variety of nonpecuniary/intangible reasons. The most appropriate methodology for identifying these nonpecuniary benefit valuations in other countries would be to repeat the type of US farmer surveys in other countries. Unfortunately, the authors are not aware of any such studies having been undertaken to date.

Production Effects of the Technology

Based on the yield assumptions used in the direct farm income benefit calculations presented above and taking account of the second soybean crop facilitation in South America, biotech crops have added important volumes to global production of corn, cotton, canola, and soybeans since 1996 (Table 23).

The biotech IR traits, used in the corn and cotton sectors, have accounted for 99% of the additional corn production and almost all of the additional cotton production. Positive yield impacts from the use of this technology have occurred in all user countries (except GM IR cotton in Australia: this reflects the levels of *Heliothis* pest control previously obtained with intensive insecticide use. The main benefit and reason for adoption of this technology in Australia has arisen from significant cost savings (on insecticides) and the associated environmental gains from reduced insecticide use)



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 9
Nonpecuniary benefits derived by US farmers 1996–2008 by trait ($ million)

when compared to average yields derived from crops using conventional technology (such as application of insecticides and seed treatments). Since 1996, the average yield impact across the total area planted to these traits over the 12-year period has been +7.1% for corn traits and +14.8% for cotton traits (Fig. 10).

Although the primary impact of biotech HT technology has been to provide more cost-effective (less expensive) and easier weed control versus improving yields from better weed control (relative to weed control obtained from conventional technology), improved weed control has, nevertheless occurred, delivering higher yields in some countries. Specifically, HT soybeans in Romania improved the average yield by over 30% in early adoption years and biotech HT corn in Argentina and the Philippines delivered yield improvements of +9% and +15% respectively.

Biotech HT soybeans have also facilitated the adoption of no tillage production systems, shortening the production cycle. This advantage enables many farmers in South America to plant a crop of soybeans immediately after a wheat crop in the same growing season. This second crop, additional to traditional soybean production, has added 73.5 million tons to soybean production in Argentina and Paraguay between 1996 and 2008 (accounting for 99% of the total biotech-related additional soybean production).

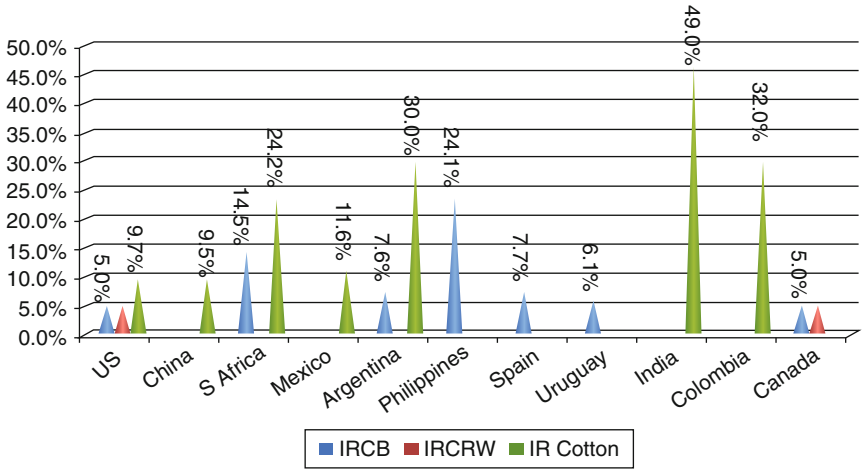
Using the same sensitivity analysis as applied to the farm income estimates presented in the executive summary to the production impacts (one scenario of consistent lower than average pest/weed pressure and one of consistent higher than average pest/weed pressure), Table 24 shows the range of production impacts.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 23 Additional crop production arising from positive yield effects of biotech crops

	1996–2008 additional production (million tons)	2008 additional production (million tons)
Soybeans	74.0	10.1
Corn	79.7	17.1
Cotton	8.6	1.8
Canola	4.8	0.6

Summary of Economic Effects of Transgenic/Biotech Crops

Overall, GM technology has had a significant positive impact on farm income derived from a combination of enhanced productivity and efficiency gains (Table 25). In 2008, the direct global farm income benefit from biotech crops was $9.37 billion. This is equivalent to having added 3.6% to the value of global production of



Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Figure 10
Average yield impact of biotech IR traits 1996–2008 by country and trait Notes: IRCB, resistant to corn-boring pests; IRCRW, resistant to corn rootworm

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 24 Additional crop production arising from positive yield effects of biotech crops 1996–2008 under different pest/weed pressure assumptions and impacts of the technology (million tons)

Crop	Consistent below average pest/weed pressure	Average pest/weed pressure (main study analysis)	Consistent above average pest/weed pressure
Soybeans	73.8	74.0	74.3
Corn	48.0	79.7	140.9
Cotton	6.2	8.6	11.8
Canola	3.3	4.8	5.2

Note: No significant change to soybean production under all three scenarios as 99% of production gain due to second cropping facilitation of the technology

the four main crops of soybeans, maize, canola, and cotton. Since 1996, farm incomes have increased by $52 billion.

The largest gains in farm income have arisen in the soybean sector, largely from cost savings. The $2.93 billion additional income generated by GM herbicide-tolerant (GM HT) soybeans in 2008 has been equivalent to adding 4.3% to the value of the crop in the biotech growing countries, or adding the equivalent of 4.1% to the $71 billion value of the global soybean crop in 2008. These economic benefits should, however be placed within the context of a significant increase in the level of soybean production in the main biotech adopting countries. Since 1996, the soybean area in the leading soybean producing countries of the USA, Brazil, and Argentina increased by 63%.

Substantial gains have also arisen in the cotton sector mainly from the adoption of GM insect-resistant (GM IR) cotton (through a combination of higher yields and lower costs). In 2008, cotton farm income levels in the biotech adopting countries increased by $2.9 billion and since 1996, the sector has benefited from an additional $15.6 billion. The 2008 income gains are equivalent to adding 19.3% to the value of the cotton crop in these countries, or 11.1% to the $26 billion value of total global cotton production. This is a substantial increase in value-added terms for two new cottonseed technologies.

Significant increases to farm incomes have also resulted in the maize and canola sectors. The combination of GM insect resistant (GM IR) and GM HT technology in maize has boosted farm incomes by $10.24 billion since 1996. In the canola sector (largely North American) an additional $1.83 billion has been generated.

Of the total cumulative farm income benefit, $31.2 billion (60%) has been due to yield gains (and second crop facilitation), with the balance arising from reductions in the cost of production. Within this yield gain component, 76% derives from the GM IR technology and the balance to GM HT crops.

Table 26 summarizes farm income impacts in key biotech adopting countries. This highlights the important farm income benefit arising from GM HT soybeans in South America (Argentina, Brazil, Paraguay, and Uruguay), GM IR cotton in China and India, and a range of GM cultivars in the USA. It also illustrates the growing level of farm income benefits obtained in South Africa, the Philippines, and Mexico.

In terms of the division of the economic benefits obtained by farmers in developing countries relative to farmers in developed countries, Table 27 shows that in 2008, 50.5% of the farm income benefits have been earned by developing country farmers. The vast majority of these income gains for developing country farmers have been from GM IR cotton and GM HT soybeans. Over the 13 years, 1996–2008, the cumulative farm income gain derived by developing country farmers was also 50% ($26.2 billion).

Examining the cost farmers pay for accessing GM technology, Table 28 shows that across the four main biotech crops, the total cost in 2008 was equal to 27% of the total technology gains (inclusive of farm income gains plus cost of the technology payable to the seed supply chain: the cost of the technology accrues to the seed supply chain including sellers of seed to farmers, seed multipliers, plant breeders, distributors, and the GM technology providers).

For farmers in developing countries the total cost was equal to 15% of total technology gains, while for farmers in developed countries the cost was 36% of the total technology gains. While circumstances vary between countries, the higher share of total technology gains accounted for by farm income gains in developing countries relative to the farm income share in developed countries reflects factors such as

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 25 Global farm income benefits from growing biotech crops 1996–2008: million US $

Trait	Increase in farm income 2008	Increase in farm income 1996–2008	Farm income benefit in 2008 as % of total value of production of these crops in biotech adopting countries	Farm income benefit in 2008 as % of total value of global production of crop
GM herbicide-tolerant soybeans	2,925.7	23,342.0	4.3	4.1
GM herbicide-tolerant maize	433.5	1,896.0	0.6	0.3
GM herbicide-tolerant cotton	14.6	855.8	0.1	0.06
GM herbicide-tolerant canola	391.8	1,829.2	6.9	1.5
GM insect-resistant maize	2,645.5	8,344.2	3.7	2.0
GM insect-resistant cotton	2,904.5	15,612.7	19.3	11.1
Others	51.5	162.1	Not applicable	Not applicable
Totals	9,367.1	52,042.0	5.71	3.65

Notes: All values are nominal. Others = Virus-resistant papaya and squash and herbicide-tolerant sugar beet. Totals for the value shares exclude "other crops" (i.e., relate to the four main crops of soybeans, maize, canola, and cotton). Farm income calculations are net farm income changes after inclusion of impacts on yield, crop quality, and key variable costs of production (e.g., payment of seed premia, impact on crop protection expenditure)

weaker provision and enforcement of intellectual property rights in developing countries and the higher average level of farm income gain on a per hectare basis obtained by farmers in developing countries relative to that obtained by farmers in developed countries.

Concluding Comments

Biotechnology has, to date delivered several specific agronomic traits that have overcome a number of production constraints for many farmers. This has resulted in improved productivity and profitability for the 13.3 million adopting farmers who have applied the technology to 115 million hectares in 2008.

During the last 13 years, this technology has made important positive socioeconomic and environmental contributions. These have arisen even though only a limited range of biotech agronomic traits have so far been commercialized, in a small range of crops.

The biotechnology has delivered economic and environmental gains through a combination of their inherent technical advances and the role of the

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 26 GM crop farm income benefits 1996–2008 selected countries: million US $

	GM HT soybeans	GM HT maize	GM HT cotton	GM HT canola	GM IR maize	GM IR cotton	Total
The USA	11,028	1,705.6	799	185.0	7,107	2,444.1	*23,268.7*
Argentina	8,764.1	113.8	34.2	N/a	269.8	95.4	*9,277.3*
Brazil	2,745.8	N/a	N/a	N/a	69.8	5.0	*2,820.6*
Paraguay	503.2	N/a	N/a	N/a	N/a	N/a	*503.2*
Canada	116.1	45.8	N/a	1,643.2	265.4	N/a	*2,070.5*
South Africa	4.1	3.8	2.2	N/a	475.8	21.0	*506.9*
China	N/a	N/a	N/a	N/a	N/a	7,599	*7,599*
India	N/a	N/a	N/a	N/a	N/a	5,142	*5,142*
Australia	N/a	N/a	8.3	0.9	N/a	214.9	*224.1*
Mexico	3.3	N/a	11.7	N/a	N/a	76.1	*91.1*
The Philippines	N/a	27.1	N/a	N/a	61.2	N/a	*88.3*
Romania	44.6	N/a	N/a	N/a	N/a	N/a	*44.9*
Uruguay	49.4	N/a	N/a	N/a	3.9	N/a	*53.3*
Spain	N/a	N/a	N/a	N/a	77.9	N/a	*77.9*
Other EU	N/a	N/a	N/a	N/a	11.1	N/a	*11.1*
Columbia	N/a	N/a	N/a	N/a	N/a	13.9	*13.9*
Bolivia	83.4	N/a	N/a	N/a	N/a	N/a	*83.4*

Notes: All values are nominal. Farm income calculations are net farm income changes after inclusion of impacts on yield, crop quality, and key variable costs of production (e.g., payment of seed premia, impact on crop protection expenditure). N/a = not applicable. US total figure excludes $182.3 million for other crops/traits

technology in the facilitation and evolution of more cost-effective and environmentally friendly farming practices. More specifically, it covers the following main issues:

- The gains from the GM IR traits have mostly been delivered directly from the technology (yield improvements, reduced production risk, and decreased the use of insecticides). Thus farmers (mostly in developing countries) have been able to both improve their productivity and economic returns while also practicing more environmentally friendly farming methods.
- The gains from GM HT traits have come from a combination of direct benefits (mostly cost reductions to the farmer) and the facilitation of changes in farming systems. Thus, GM HT technology (especially in soybeans) has played an important role in enabling farmers to capitalize on the availability of a low-cost, broad-spectrum herbicide (glyphosate) and in turn, facilitated the move away from conventional to low/no tillage production systems in both North and South America. This change in production system has made additional positive economic contributions to farmers (and the wider economy) and delivered important environmental benefits, notably reduced levels of GHG emissions (from reduced tractor fuel use and additional soil carbon sequestration).
- Both IR and HT traits have made important contributions to increasing world production levels of soybeans, corn, cotton, and canola.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 27 GM crop farm income benefits 2008: developing versus developed countries: million US $

	Developed	Developing
GM HT soybeans	1,232.1	1,693.6
GM IR maize	2,380.5	265.0
GM HT maize	357.4	76.1
GM IR cotton	213.8	2,690.8
GM HT cotton	5.5	9.1
GM HT canola	391.8	0
GM virus-resistant papaya and squash and GM HT sugar beet	51.5	0
Total	4,632.6	4,734.6

Developing countries = all countries in South America, Mexico, Honduras, Burkino Faso, India, China, the Philippines, and South Africa

The impact of GM HT traits has, however contributed to increased reliance on a limited range of herbicides and this has contributed to some limited development of weed resistance to these herbicides. Some degree of reduced effectiveness of glyphosate (and glufosinate) against certain weeds is to be expected and the extent to which this may develop further, will depend on farming practice and behavior relating to mixing, rotation, and sequencing of herbicides. Where resistance has occurred, this has resulted in low-dose rate applications of other herbicides in weed control programs (commonly used in conventional production systems) occurring and hence, has marginally reduced the level of net environmental and economic gains derived from the current use of the biotechnology. Nevertheless, to date, the overall environmental and economic gains arising from the use of biotech crops have been substantial.

Global Economic Impact of Transgenic/Biotech Crops (1996–2008). Table 28 Cost of accessing GM technology (million $) relative to the total farm income benefits 2008

	Cost of technology: all farmers	Farm income gain: all farmers	Total benefit of technology to farmers and seed supply chain	Cost of technology: developing countries	Farm income gain: developing countries	Total benefit of technology to farmers and seed supply chain: developing countries
GM HT soybeans	1,058.2	2,925.7	3,983.9	334.4	1,693.6	2,028.0
GM IR maize	1,045.9	2,645.5	3,691.4	99.7	265.0	364.7
GM HT maize	547.8	433.5	981.3	32.5	76.1	108.6
GM IR cotton	434.6	2,904.5	3,339.1	353.0	2,690.8	3,043.8
GM HT cotton	167.1	14.6	181.7	10.4	9.1	19.5
GM HT canola	109.0	391.8	500.86	N/a	N/a	N/a
Others	41.5	51.5	93.0	N/a	N/a	N/a
Total	3,404.1	9,367.1	12,771.26	830.0	4,734.6	5,564.6

N/a, not applicable. Cost of accessing technology based on the seed premia paid by farmers for using GM technology relative to its conventional equivalents

Appendix 1: Argentine Second Crop Soybeans

Year	Second crop area (million hectares)	Increase in income linked to GM HT system (million $)	Additional production (million tons)
1996	0.45	Negligible	Negligible
1997	0.65	25.4	0.3
1998	0.8	43.8	0.9
1999	1.4	116.6	2.3
2000	1.6	144.2	2.7
2001	2.4	272.8	5.7
2002	2.7	372.6	6.9
2003	2.8	416.1	7.7
2004	3.0	678.1	6.9
2005	2.3	526.7	6.3
2006	3.2	698.9	11.2
2007	4.9	1,133.6	9.88
2008	3.4	764.6	9.62

Additional gross margin based on data from Grupo CEO

Bibliography

1. Marra M, Piggott N (2006) The value of non pecuniary characteristics of crop biotechnologies: a new look at the evidence, North Carolina State University
2. Marra M, Piggott N (2007) The net gains to cotton farmers of a national refuge plan for Bollgard II cotton. AgBioforum 10(1):1–10 (www.agbioforum.org)
3. Marra M, Pardey P, Alston J (2002) The pay-offs of agricultural biotechnology: an assessment of the evidence. International Food Policy Research Institute, Washington, USA
4. Carpenter J, Gianessi L (1999) Herbicide tolerant soybeans: why growers are adopting roundup ready varieties. AgBioforum 2:65–72
5. Carpenter J (2001) Comparing Roundup ready and conventional soybean yields 1999. National Centre for Food & Agriculture Policy, Washington
6. Sankala S, Blumenthal E (2003) Impacts on US agriculture of biotechnology-derived crops planted in 2003 – an update of eleven case studies. NCFAP, Washington, www.ncfap.org
7. Sankala S, Blumenthal E (2006) Impacts on US agriculture of biotechnology-derived crops planted in 2005 – an update of eleven case studies. NCFAP, Washington, www.ncfap.org
8. Johnson S, Strom S (2008) Quantification of the impacts on US agriculture of biotechnology-derived crops planted in 2006. NCFAP, Washington, www.ncfap.org
9. Qaim M, Traxler G (2002) Roundup ready soybeans in Argentina: farm level, environmental and welfare effects. In: 6th ICABR conference, Ravello, Italy
10. Qaim M, Traxler G (2005) Roundup ready soybeans in Argentina: farm level and aggregate welfare effects. Agric Econ 32(1):73–86
11. Parana Department of Agriculture (2004) Cost of production comparison: biotech and conventional soybeans. In: USDA GAIN report BR4629 of 11 November 2004. www.fas.usad.gov/gainfiles/200411/146118108.pdf
12. Galveo A (2009) Farm survey findings of impact of herbicide tolerant soybeans and insect resistant corn and cotton in Brazil, Celeres, Brazil. www.celeres.co.br
13. George Morris Centre (2004) Economic and environmental impacts of the commercial cultivation of glyphosate tolerant soybeans in Ontario (Unpublished report for Monsanto, Canada)
14. Brookes G (2005) The farm level impact of using roundup ready soybeans in Romania. AgBioforum 8(4) (Also available on www.pgeconomics.co.uk)
15. Monsanto Romania (2007) Roundup ready soybeans: survey growers crops in 2006 and intentions for 2007
16. Monsanto Comercial Mexico (2005) Official report to Mexican Ministry of Agriculture. (Unpublished)
17. Monsanto Comercial Mexico (2007) Official report to Mexican Ministry of Agriculture of the 2006 crop. (Unpublished)
18. Monsanto Comercial Mexico (2008) Official report to Mexican Ministry of Agriculture of the 2008 cotton crop. (Unpublished)
19. Fernandez W et al (2009) GM soybeans in Bolivia. In: Paper presented to the 13th ICABR conference, Ravello, Italy, June 2009
20. Doyle B et al (2003) The Performance of roundup ready cotton 2001–2002 in the Australian cotton sector. University of New England, Armidale, Australia
21. Canola Council of Canada (2001) An agronomic and economic assessment of transgenic canola. Canola Council, Canada, www.canola-council.org
22. Gusta M et al (2009) Economic benefits of GMHT canola for producers, University of Saskatchewan, College of Biotechnology (Working Paper)
23. Monsanto Australia (2009) Survey of herbicide tolerant canola licence holders 2008
24. Fischer J, Tozer P (2009) Evaluation of the environmental and economic impact of roundup ready canola in the Western Australian crop production system. In: Curtin Univeristy of Technology Technical Report 11/2009
25. Kniss A (2009) Farm scale analysis of glyphosate resistant sugar beet in the year of commercial introduction in Wyoming, University of Wyoming
26. Khan M (2008) Roundup ready sugar beet in America. British Sugar Beet Review Winter 2008. 76(4):16–19
27. James C (2003) Global review of commercialized transgenic crops 2002: feature Bt maize, ISAAA No 29

28. Gianessi L, Carpenter J (1999) Agricultural biotechnology insect control benefits. NCFAP, Washington, USA
29. Trigo E, Cap E (2006) Ten years of GM crops in Argentine Agriculture, ArgenBio
30. Gouse M et al (2005) A GM subsistence crop in Africa: the case of Bt white maize in S Africa. Int J Biotechnol 7(1/2/3)
31. Gouse M et al (2006) Three seasons of insect resistant maize in South Africa: have small farmers benefited. AgBioforum 9(1):15–22
32. Gouse M et al (2006) Output and labour effect of GM maize and minimum tillage in a communal area of Kwazulu-Natal. J Dev Perspect 2:2
33. Van der Weld W (2009) Final report on the adoption of GM maize in South Africa for the 2008/09 season. South African Maize Trust
34. Brookes G (2003) The farm level impact of using Bt maize in Spain, ICABR conference paper 2003, Ravello, Italy (Also on www.pgeconomics.co.uk)
35. Brookes G (2008) The benefits of adopting GM insect resistant (Bt) maize in the EU: first results from 1998-2006. Int J Biotechnol 10(2/3):148–166 (www.pgeconomics.co.uk.)
36. Gonsalves D (2005) Harnessing the benefits of biotechnology: the case of Bt corn in the Philippines. ISBN 971-91904-6-9. Strive Foundation, Laguna, Philippines
37. Yorobe J (2004) Economics impact of Bt corn in the Philippines. In: Paper presented to the 45th PAEDA convention, Querzon City, Philippines
38. Ramon G (2005) Acceptability survey on the 80–20 bag in a bag insect resistance management strategy Galveo A (2009) Unpublished (in January 2010) data on first survey findings of impact of insect resistant corn (first crop) in Brazil, Celeres, Brazil. www.celeres.co.br
39. Falck Zepeda J et al (2009) Small 'resource poor' countries taking advantage of the new bio-economy and innovation: the case of insect protected and herbicide tolerant corn in Honduras. In: Paper presented to the 13th ICABR conference, Ravello, Italy
40. Mullins W, Hudson J (2004) Bollgard II versus Bollgard sister line economic comparisons. In: 2004 Beltwide cotton conferences, San Antonio, USA, Jan 2004
41. Pray C et al (2001) Impact of Bt cotton in China. World Dev 29(5):1–34
42. Pray C et al (2002) Five years of Bt cotton in China – the benefits continue. Plant J 31(4): 423–430
43. Doyle B (2005) The performance of Ingard and Bollgard II cotton in Australia during the 2002/2003 and 2003/2004 seasons. University of New England, Armidale, Australia
44. Taylor I (2003) Cotton CRC annual report, UNE, Armidale, Cotton Research Institute, Narrabri, Australia
45. CSIRO (2005) The cotton consultants Australia 2005 Bollgard II comparison report. CSIRO, Australia
46. Qaim M, De Janvry A (2002) Bt cotton in Argentina: analysing adoption and farmers willingness to pay. American Agricultural Economics Association Annual Meeting, California
47. Qaim M, De Janvry A (2005) Bt cotton and pesticide use in Argentina: economic and environmental effects. Environ Dev Econ 10:179–200
48. Elena M (2001) Economic advantages of transgenic cotton in Argentina, INTA. As cited in Trigo and Cap (2006)
49. Traxler G et al (2001) Transgenic cotton in Mexico: economic and environmental impacts. In: ICABR conference, Ravello, Italy
50. Martinez-Carillo J, Diaz-Lopez N (2005) Nine years of transgenic cotton in Mexico: adoption and resistance management. In: Proceedings Beltwide Cotton Conference, Memphis, USA, June 2005
51. Ismael Y et al (2002) A case study of smallholder farmers in the Mahathini flats, South Africa. In: ICABR conference, Ravello Italy 2002
52. Kirsten J et al (2002) Bt cotton in South Africa: adoption and the impact on farm incomes amongst small-scale and large-scale farmers. In: ICABR conference, Ravello, Italy 2002
53. Morse S et al (2004) Why Bt cotton pays for small-scale producers in South Africa. Nat Biotechnol 22(4):379–380
54. Bennett R, Ismael Y, Kambhampati U, Morse S (2004) Economic impact of genetically modified cotton in India. AgBioforum 7(3):96–100
55. IMRB (2006) Socio-economic benefits of Bollgard and product satisfaction (in India). IMRB International, Mumbai, India
56. IMRB (2007) Socio-economic benefits of Bollgard and product satisfaction (in India). IMRB International, Mumbai, India
57. Monsanto Brazil (2008) Farm survey of conventional and Bt cotton growers in Brazil 2007. (Unpublished)
58. Zambrano P et al (2009) Insect resistant cotton in Columbia: impact on farmers. In: Paper presented to the 13th ICABR conference, Ravello, Italy, June 2009
59. Vitale J et al (2006) The Bollgard II field trials in Burkina Faso: measuring how Bt cotton benefits West African farmers. In: Paper presented at the 10th ICABR Conference, Ravello, Italy
60. Vitale J et al (2008) The economic impact of 2nd generation Bt cotton in West Africa: empirical evidence from Burkino Faso. Int J Biotechnol 10(2/3):167–183
61. Rice M (2004) Transgenic rootworm corn: assessing potential agronomic, economic and environmental benefits. Plant Health Progress 10, '094/php-2001-0301-01-RV
62. Alston J et al (2003) An ex-ante analysis of the benefits from adoption of corn rootworm resistant, transgenic corn technology. AgBioforum 5(3), Article 1
63. Manjunath T (2008) Bt cotton in India: remarkable adoption and benefits, Foundation for Biotech Awareness and Education, India. www.fbae.org

GM Crop Risk Debate, Science and Socioeconomics

Klaus Ammann
Botanical Garden, University of Bern, Bern, Switzerland

Article Outline

Introduction

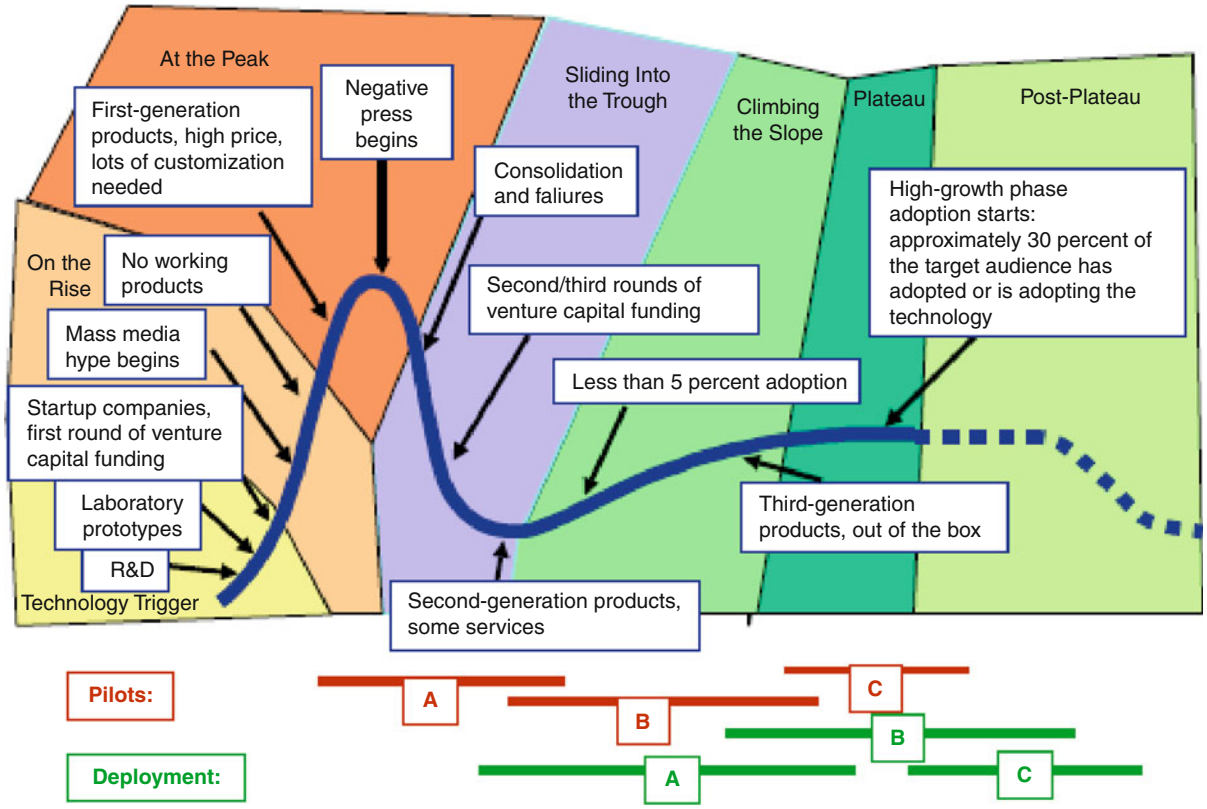
The General Strategic Situation of the Debate About Green Biotechnology Today

The aim of this text is to set the framework for a better communication about science and regulation, and production of GM crops. GM stands for Genetic Modification, basically an unfortunate denomination, because actually *all* crops are genetically modified, but it is a worldwide accepted term for genetically engineered crops, including transgenes, auto- and allotransgenes, cis- and infra-genes, and synthetic genes, for details see Beardmore [1]. By including gene stacking of various kinds, the situation is getting even more complex [2]. With the introduction of in Vivo Mutation (with Zink-Finger Technology and the latest transformation method transcription activator-like family of type III effectors [TALEs]) the situation will change even more, the age of a high precision and targeted change of genomes has only begun and will develop rapidly, see section Innovation in Agriculture on All Levels Will Speed Up and Makes it a Necessity to Rethink Regulation Basically and Radically, Most often in the Direction of Lowering the Regulatory Hurdles with details. The term LMOs (Living Modified Organisms), which is generally used in the United Nations Biosafety Protocol (Cartagena Protocol) is nothing but a "Living Proof" that the scientific basis of the Protocol remains questionable, since firstly the term is creating misunderstandings and secondly it is based on an erroneous assumption that GM crops are basically different from conventional crops, as is discussed with detail in the sections Molecular Processes Similar in Natural Mutation and Transgenesis and Dissent over Differences Between GM- and Non-GM Crops Causes Transatlantic Regulatory Divide. More detailed clarification about the terminology of GMOs is given in a text block of the published Statement of the Pontifical Academy of Sciences: [3].

- There are many different terms used to describe the processes involved in plant breeding. All living organisms are made up of cells in which are contained their genes, which give them their distinctive characteristics. The complete set of genes (the genotype) is encoded in DNA and is referred to as the genome; it is the hereditary information that is passed from parent to offspring. All plant breeding, and indeed all evolution, involves genetic change or modification followed by selection for beneficial characteristics from among the offspring. Most alterations to a plant's phenotype or observable traits (such as its physical structure, development, biochemical and nutritional properties) result from changes to its genotype. Plant breeding traditionally used the random reshuffling of genes among closely-related and sexually compatible species, often with unpredictable consequences and always with the details of the genetic changes unexplored. In the mid-twentieth century this was supplemented by mutagenesis breeding, the equally random treatment of seeds or whole plants with mutagenic chemicals or high-energy radiation in the hope of generating phenotypic improvements; this, too, gave rise to unpredictable and unexplored genetic consequences from which the plant breeder selected the beneficial traits. Most recently, techniques have been developed allowing the transfer of specific, identified and well characterized genes, or small blocks of genes that confer particular traits, accompanied by a precise analysis of the genetic and phenotypic outcomes: this last category is called 'transgenesis' (because genes are transferred from a donor to a recipient) or 'genetic engineering' (abbreviated to GE in this report) but, in truth, this term applies to all breeding procedures.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



GM Crop Risk Debate, Science and Socioeconomics. Figure 1
Gartner Hype cycle, extended view from [6] after Fig. 3. Technologically aggressive ("Type A") enterprises are relatively comfortable adopting the technology, and moderately aggressive ("Type B") enterprises start to investigate and pilot the technology. Conservative ("Type C") enterprises remain wary (From [6])

The strategic situation in the debate on GM crops is difficult, but not desperate, particularly in Europe – this is an evaluation shared by lots of experts of the debate about agricultural biotechnology; in Europe, it is negatively affecting research and researchers [4]. We have reached in Europe the peak of anxiety related to GM-crops since the introduction of the new technologies, and some opponents to transgenic crops have taken advantage of this situation. They have organized themselves in a veritable protest industry, see section The Dispute Between Scientists and Opponents Today. Nevertheless, the next years should lead to reassurance and scientific consolidation on biotechnology views. We encounter the same repeating dynamics as described for previous technology introductions [5]. The Gartner Hype Cycle [6] adds another dimension to technology life cycle models: it characterizes the typical progression of an emerging technology from user and media overenthusiasm through a period of disillusionment to an eventual understanding of the technology's relevance and role in a market or domain (Fig. 1).

In the details of the cycle [6], amended by the author – specified for the technology push in transgenic cropdevelopment – it should be noted that there are differences between the development of the technologies in the mind of Linden and Fenn and agricultural technologies, where life sciences, combined with regional and cultural diversity, results in a much more diversified picture, often not following the below described phases.

- **2.1 Technology Trigger.** The Technology Trigger is a technological breakthrough, public demonstration, press release or other event that generates significant publicity and industry interest in an emerging technology. Typically no usable products exist, only research and laboratory prototypes (from the first transgenic plants in the 80ties [7]. Venture capitalists may provide some early funding just after the Trigger, if they expect the technology to be a fast runner.

- **2.2 On the Rise.** On the rise to the Peak of Inflated Expectations, media articles explain the technology and discuss its potential impact on business and society. First-generation products emerge like the Flavr-Savr-Tomato [8], but they usually are highly specialized products or extremely difficult to use or with other hitches in the introductory phase. Products are high margin because vendors are still trying to recover R&D costs, and the technology is expensive compared to its cost of production. For example, in 2002, Bluetooth products such as headsets cost $200, while the final silicon cost of Bluetooth chips likely will be approximately $5. This is a good stage for venture capitalists to enter the market, before evaluations are at their apex. During this phase, some particularly aggressive enterprises may start to pilot the technology, particularly if it contributes to critical business issues. These enterprises work closely with the vendors to create customized solutions for their requirements

- **2.3 At the Peak of Inflated Expectations.** As the Peak crests, the number of vendors offering the technology increases. These vendors are primarily startup companies and small vendors that try to use the increasing amount of hype for their marketing benefit. A growing number of enterprises start to examine how the technology may fit within their business strategies, although most do not take action at this stage. Venture capitalists may be interested in selling some of the startups that they equipped with early funding. As problems with first-generation products become visible (e.g., emerging pest resistance in the Bt cotton regions [9, 10] and the latest success message of Huang et al. [11], often because the technology is pushed to its limits, negative publicity starts to push the technology into the Trough of Disillusionment, often the pertinent publications are pushed for negative statements beyond the limit of scientific rules (for example, Web services in 2002 and biometrics in 2003 and two example from the debate on non-target insects related to Bt crops: a) the case of the monarch butterfly [12] and b) Lovei et al. [13] giving false alarm for ladybirds and its prompt rebuttal by Antony Shelton et al. [14]).

- **2.4 Sliding into the Trough of Disillusionment.** Because the technology does not live up to enterprises' and the media's overinflated expectations, it is rapidly discredited. Some of the early trials end in highly publicized failures. Media interest wanes, except for a few cautionary tales. A significant amount of vendor consolidation and failure occurs. Later-stage investors may be interested in funding vendors during this phase because equity is fairly inexpensive after the "microbubble" at the Peak of Inflated Expectations has burst. However, amid the disillusionment, trials are ongoing and vendors are improving products based on early feedback regarding problems and issues. Some early adopters find some benefit in adopting the technology. For some slow-moving technologies (for example, biometrics), workable and cost-effective solutions emerge and provide value in niche domains, even while the technology remains in the Trough. The Trough of Disillusionment coincides with the "chasm" in Geoffrey Moore's classic book, "Crossing the Chasm" [15]. During this stage, vendors need to launch their products from a few early adopters to adoption by a majority of enterprises to begin the climb up the Slope of Enlightenment. There is no real parallel in the GM crop history, except that the differences in GM crop regulation and perception between the Americas and Europe caused a deep transatlantic divide [16].

- **2.5 Climbing the Slope of Enlightenment.** Focused experimentation and real-world experience by an increasingly diverse range of enterprises lead to a better understanding of the technology's applicability, risks and benefits. Vendors seek mezzanine or later-round funding for marketing and sales support to pull them-selves up the Slope. Second- and third-generation products are launched by the leading seed companies, and methodologies and tools are added to ease the development process, see the

sections under 1.2. The service component declines as a percentage of the sale. Technologically aggressive ("Type A") enterprises are relatively comfortable adopting the technology, and moderately aggressive ("Type B") enterprises start to investigate and pilot the technology. Conservative ("Type C") enterprises remain wary. At the beginning of the slope, the penetration often is significantly less than 5 percent of the potential market segment. This will grow to approximately 30 percent and more as the technology enters the Plateau of Enlightenment. Examples of more or less unexpected enhancements in science and risk assessment of transgenic crops come from a higher precision of gene transfer methods (see sections under 1.2.), also compare to the latest developments in resistance management with a clear success story this year [11].

- **2.6 Entering the Plateau of Productivity.** The Plateau represents the beginning of mainstream adoption, which began in the Americas much earlier from 2000 onwards, when the real-world benefits of the technology are demonstrated and accepted, see the consecutive reports on the world development of transgenic crops on www.isaaa.org. Technologies become increasingly embedded into solutions that increasingly are "out of the box," with decreasing service elements as the technology matures (example conservation tilling). The majority of Type B, then Type C, enterprises adopt the technology. As a high-profile technology matures, an "ecosystem" often evolves around it. The ecosystem supports multiple providers of products and services, and also a market for related products and services that extend or are based on the technology (for example, virtual private networks in 2003 or the growing market for suppliers of molecular laboratories or the growing market for electronic equipment for precision agriculture).

  The final height of the Plateau varies according to whether the technology is broadly applicable or benefits only a niche market, depending heavily on crop and region.

- **2.7 Post-Plateau.** As a technology achieves full maturity and supports thousands of enterprises and millions of users, producers and consumers, its hype typically disappears, as seen in the Americas. Only a few specialist magazines continue coverage of new aspects of implementing and maintaining the technology. Often there may be innovations around this technology that will follow their own Hype Cycles (new crop varieties on stress resistance, on bio-fortification, pharmaceutical crop lines etc.).

- **3.0 The Time-to-Maturity Assessment.** Technologies do not move at a uniform speed through the Hype Cycle. It often takes years for a technology to traverse the Hype Cycle — some technologies like GM crops may take decades, with considerable regional differences. There are three adoption speeds:

  "Fast-track" technologies go through the Hype Cycle within two to four years. This occurs when the performance curve inflects early in the life cycle of a technology. These technologies find themselves adopted without much fanfare, bypassing the Peak of Inflated Expectations and Trough of Disillusionment. Many enterprises are unaware of their sudden maturity and applicability, such as what has happened with instant messaging and Short Message Service.

It is interesting to note that the Showalter "hystories" on the introduction of most new technologies [5] report no real damage in their subsequent introductory phase, or the benefits were so overwhelming that the debate was soon fading away. This alone demonstrates clearly that it is the sociocultural environment strongly influencing the risk debate [17]. The most recent events seem to hint that Europe finally finds to a more de-contracted way of looking at GM crops: The new report of the Royal Society [18] tries to unite conventional and biotechnology approaches for the sake of making progress on agricultural management in developing countries:

- Past debates about agricultural technology have tended to involve different parties arguing for either advanced biotechnology including GM, improved conventional agricultural practice or low-input methods. We do not consider that these approaches are mutually exclusive: improvements to all systems require high-quality science. Global food insecurity is the product of a set of interrelated local problems of food production and consumption. The diversity of these problems needs to be reflected in the diversity of scientific approaches used to tackle them. Rather than focusing on particular scientific tools and techniques, the approaches should be evaluated in terms of their outcomes.

It might well be that we arrive sooner than expected from a period of disillusionment to an eventual understanding of the technology's relevance and role in a market or domain.

Innovation in Agriculture on all Levels will Speed up and Makes it a Necessity to Rethink Regulation Basically and Radically, most Often in the Direction of Lowering the Regulatory Hurdles

Unfortunately, regulatory legislation is in its nature static, needs a long time to be settled in international negotiations, and then, finally, settled and approved with an important number of signatory states as the Cartagena Protocol; therefore, it is nearly impossible to make the necessary changes based on good science. At the time of the establishment of the Cartagena Biosafety Protocol, the similarities between nontransgenic and transgenic organisms on the molecular level were not widely known, although properly published (see latest review with early publications [19]), and a correction about these grave errors (recently called by the author as "Genomic Misconception," publication in preparation) in concept is now nearly impossible – details in section GM- and Non-GM-Crop Differences Over-Estimated, the "Genomic Misconception". But the situation is not getting better: the accelerating speed of scientific progress and discoveries used for new (agricultural) technologies is breathtaking. A short overview is provided in the following sections.

New Biotechnology Approaches in Plant Breeding, Introduction In an early paper, Britt et al. give an overview on many molecular possibilities which will develop for new breeding successes [20], they address the current status of plant gene targeting and what is known about the associated plant DNA repair mechanisms. One of the greatest hurdle that plant biologists face in assigning gene function and in crop improvement is the lack of efficient and robust technologies to generate gene replacements or targeted gene knockouts. They also face an old problem in plant breeding summarized under the complex term of epigenetics [21, 22], a problem corrected in conventional plant breeding by careful and often tedious selection processes. Unfortunately, opponents abuse epigenetics as a seemingly new problem for genetic engineering [23], avoiding the mention of modern molecular insight and its ease to correct such problems in a more targeted way. It is clear that transgenesis will remain a solid technology for breeding, but new approaches will appear – as science is always open for progress and new breakthroughs. Here, we only mention shortly progress from another more holistic perspective of systems biology: the dynamics of Metabolomics [24], and also the growing speed of discovery in proteomics [25], techniques which will increasingly augment more common types of experimentation, especially as they provide the capacity of generating data sets that can be compared across studies and laboratories [26], and because quantitative proteomics data are generated with unprecedented sensitivity, accuracy, and reproducibility. There are many new biotechnologies enhancing the speed of achieving targeted breeding successes such as the high throughput marker finding technology [27, 28], only a few can be mentioned here:

***Cis*- and Intragenic Approaches** A new technology has now proven to be a successful strategy: As Romments describe it, cisgenetics is a welcome way of combining the benefits of traditional breeding with modern biotechnology. It is an understandable enthusiasm of the first researchers using this technology to emphasize the positive sides by also comparing to transgenesis as an "old-fashioned" method with its problems. But things are certainly not so easy: In sections Molecular Processes Similar in Natural Mutation and Transgenesis and Dissent Over Differences Between GM- and Non-GM Crops Causes Transatlantic Regulatory Divide, it is made clear that on the genomic level, particularly on the level of molecular processes, there is no difference between transgenic and nontransgenic crops (supported by an important body of scientific literature), and this is certainly also true to cisgenic and intragenic varieties. This is why it is questionable and based on false grounds to make claims that those new methods in transformation would be safer, as Giddings has made it clear in his letter [29], and his arguments against the views of [30–32] and later publications [33–35] could have been targeted as well: they try to demonstrate that the new cisgenics and intragenics are safer than transgenics, which is not

based on any facts, rather it is based on accepting without scientific scrutiny the negative public perception on transgenic crops. It is also wrong to use without clarification the term "alien genes" in view of confirmed and widely accepted universality of DNA and genomic structures.

However, there is nothing to say against the application of such new methods per se, as [33, 34] can demonstrate:

- The classical methods of alien gene transfer by traditional breeding yielded fruitful results. However, modern varieties demand a growing number of combined traits, for which pre-breeding methods with wild species are often needed. Introgression and translocation breeding require time consuming backcrosses and simultaneous selection steps to overcome linkage drag. Breeding of crops using the traditional sources of genetic variation by cisgenesis can speed up the whole process dramatically, along with usage of existing promising varieties. This is specifically the case with complex (allo)polyploids and with heterozygous, vegetative propagated crops. Therefore, we believe that cisgenesis is the basis of the second/ever green revolution needed in traditional plant breeding. For this goal to be achieved, exemption of the GM-regulation of cisgenes is needed.

Reverse Screening Methods: Tilling and Eco-Tilling Two rather independent publications [36, 37] with largely incongruent literature lists promote a new technology of finding useful genes within the genome of the crops involved: They both promote powerful reverse genetic strategies that allow the detection of induced point mutations in individuals of the mutagenized populations, can address the major challenge of linking sequence information to the biological function of genes, and can also identify novel variation for plant breeding [37]. Rigola et al. [36] develop reverse genetics approaches which rely on the detection of sequence alterations in target genes to identify allelic variants among mutant or natural populations. Current (pre-) screening methods such as *tilling* and *eco-tilling* are based on the detection of single base mismatches in heteroduplexes using endonucleases such as CEL 1. However, there are drawbacks in the use of endonucleases due to their relatively poor cleavage efficiency and exonuclease activity. Moreover, prescreening methods do not reveal information about the nature of sequence changes and their possible impact on gene function. Rigola et al. [36] present a *KeyPointTM* technology, a high-throughput mutation/polymorphism discovery technique based on massive parallel sequencing of target genes amplified from mutant or natural populations. Thus, *KeyPointTM* combines multidimensional pooling of large numbers of individual DNA samples and the use of sample identification tags ("sample barcoding") with next-generation sequencing technology. Rigola et al. [36] can demonstrate first successes in tomato breeding by identifying two mutants in the tomato eIF4E gene based on screening more than 3,000 M2 families in a single GS FLX sequencing run, and discovery of six haplotypes of tomato eIF4E gene by re-sequencing three amplicons in a subset of 92 tomato lines from the EU-SOL core collection. This technology will prove to be useful and does not need for its own breakthrough to refer to a scientifically unjustified critique of transgenesis. Whether the new technology will replace the transgenic "Amflora potato" has still to be proven by further scrutinizing of the results of the equivalent trait [38].

Zinc Finger Targeted Insertion of Transgenes Plant breeding has gone through dynamic developments, from marker-assisted breeding to transgenesis with steadily improved methods to the latest development of the Zink-finger enzyme-assisted targeted insertion of transgenes in complex organisms [39–42]. Zinc-finger nucleases (ZFNs) allow gene editing in live cells by inducing a targeted DNA double-strand break (DSB) at a specific genomic locus. However, strategies for characterizing the genome-wide specificity of ZFNs remain limited. According to [43], comprehensive mapping of ZFN activity in vivo will facilitate the broad application of these reagents in translational research.

The development toward more insertion precision and less genomic disturbance is so rapid that promoters of organic farming will see dwindling one of their pet arguments even more rapidly: Genomic disturbance of modern breeding is certainly less important and will even be negligible compared to the old breeding methods, still promoted stubbornly by the

organic plant breeding community [44]: It is very likely that the transcriptomic disturbances will be even smaller in future – compared to the clumsy and tedious methods of conventional breeding, see also the latest developments in sections TALEs: Transformation Method Transcription Activator-like Family of Type III Effectors and Precision Engineering Through DNE Meganucleases below.

TALEs: Transformation Method Transcription Activator-like Family of Type III Effectors The generation of double-strand DNA breaks (DSBs) promotes homologous recombination in eukaryotes and can facilitate gene targeting, additions, deletions, and inactivation. Zinc-finger nucleases have been used to generate DSBs and subsequently for genome editing, but with low efficiency and reproducibility. In contrast, the transcription activator-like family of type III effectors (TALEs) contains a central domain of tandem repeats that could be engineered to bind specific DNA targets. The new method is capable of generating site-specific DNS Breaks and has great potential for site-specific genome modification in plants and eukaryotes in general [45]. See also comments on the newswire CNBS [46] on the discovery:

- Dr. Mahfouz has developed a "repair tool" (molecular scissors) made out of protein that does two things: it finds the exact place on the genome where it is to be cut using a genetic "postcode" and then deletes, adds or edits the gene with great accuracy and precision.

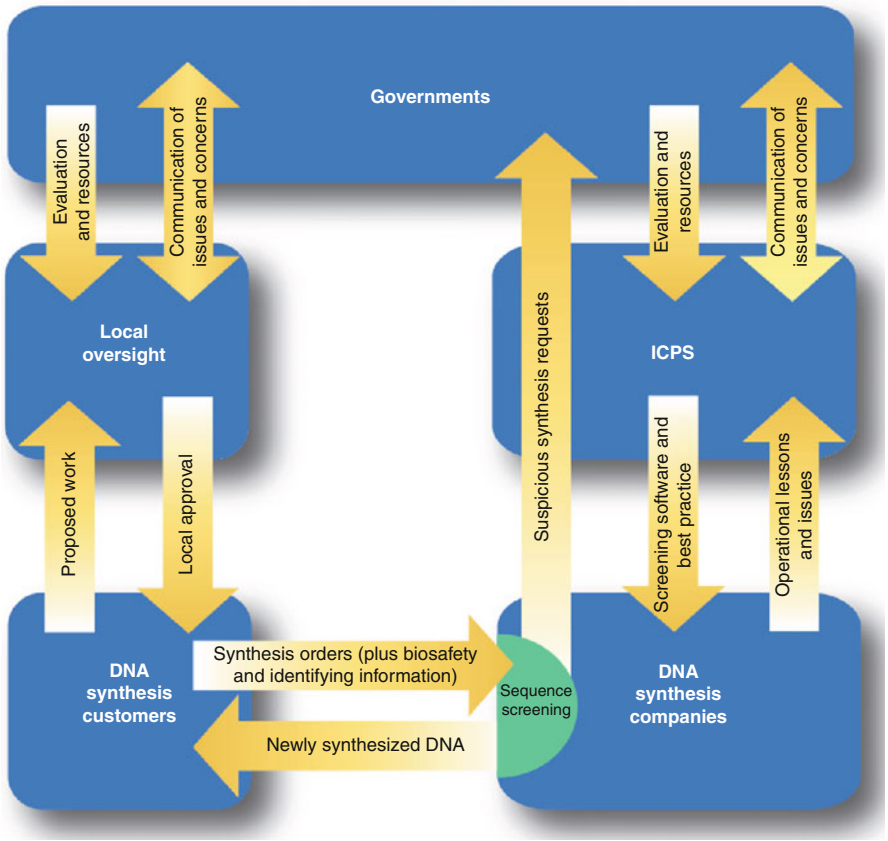
  Dr. Mahfouz's work has the potential for much broader applications including human health. This new technology could enhance the technique that may be used to substitute "good" genes for bad, or to cut out or silence the defective genes that cause disease.

  Commenting on the research, KAUST Provost Stefan Catsicas saw the technology as a scientific breakthrough and, if the patent is eventually successful, having potentially promising revenues. Dr. Nina Fedoroff, Professor of the Life Sciences at Penn State University, said the Mahfouz paper "shows the practicability of creating DNA-cutting enzymes tailored to cut a desired target sequence with very high specificity. This is an excellent step forward toward creating very specific genetic improvements in crop plants, while avoiding the potential risks many are concerned about with more conventional genetic modification strategies. Moreover, the paper gives the first evidence that this particular strategy will work in plants." Professor Fedoroff is "delighted to see such cutting-edge contributions emerging from a university as young as KAUST!".

Precision Engineering Through DNE Meganucleases Engineered DNE meganucleases can be used for cloning and molecular analysis purposes in much the same ways as conventional restriction enzymes. The important difference, of course, is that meganucleases recognize much rarer DNA sequences than restriction enzymes. This makes them particularly well suited to the manipulation of extremely large DNA sequences such as intact genomes. Importantly, DNE meganucleases cleave to leave four base pair 3' overhangs suitable for "sticky-end" cloning. The first application with a new tool called Directed Nuclease Editor™ in plant breeding by Bayer Crop Science http://www.precisionbiosciences.com/ seems promising: The meganucleases have been first used to do precision work in human gene therapy, but an outlook into various other applications was announced as early as 2003 [47–49].

Synthetic Biology In some 150 laboratories, synthetic biology is intensively researched, and it seems clear that the future will bring here some unexpected revolutions: A new field, synthetic biology, is emerging on the basis of these experiments [50], where chemistry mimics biological processes as complicated as Darwinian evolution. According to [51], the emerging field of synthetic biology is generating insatiable demands for synthetic genes, which far exceed existing gene synthesis capabilities. Tian et al. claim that technologies and trends potentially will lead to breakthroughs in the development of accurate, low-cost, and high-throughput gene synthesis technology – the capability of generating unlimited supplies of DNA molecules of any sequence or size will transform biomedical and any biotechnology research in the near future. And, according to [52], already in 1998 the redesigning of nucleic acids has been judged in an optimistic way, this was confirmed in an important Nature review in 2005 [53].

The real breakthrough came with the synthesis of an organism including its reproduction, achieved after



GM Crop Risk Debate, Science and Socioeconomics. Figure 2

Our framework calls for the immediate and systematic implementation of a tiered DNA synthesis order screening process. To promote and establish accountability, individuals who place orders for DNA synthesis would be required to identify themselves, their home organization, and all relevant biosafety information. Next, individual companies would use validated software tools to check synthesis orders against a set of select agents or sequences to help ensure regulatory compliance and flag synthesis orders for further review. Finally, DNA synthesis and synthetic biology companies would work together through the ICPS, and interface with appropriate government agencies (worldwide), to rapidly and continually improve the underlying technologies used to screen orders and identify potentially dangerous sequences, as well as develop a clearly defined process to report behavior that falls outside of the agreed-upon guidelines. ICPS, International Consortium for Polynucleotide Synthesis (From [58])

years of research and a firm belief in success, typical of the senior author of the mega project still continuing, [54–57].

A pragmatic view of a new regulatory scheme answering the new biosafety tasks of synthetic biology is proposed by [58] (Fig. 2):

This kind of new regulatory approach will be necessary in order to avoid unnecessary hindering of research progress in synthetic biology, a demand supported with other innovative suggestions for interactive procedures [59]. Another balanced view [60] demonstrates also the new risks arising from synthetic organisms and the accidental (or purposeful) release in the environment. As always, the ethical awareness and behavior has to be developed further, agreeing with [61] not in a way which gives forfeit power to social sciences. What we really need is a new interfaculty, interdisciplinary or, even better, transdisciplinary discursive scheme as proposed in sections Long Term Discourse and Decision Making

Processes and The Second Generation Systems Approach as a New Decision Making Process.

What happened some 35 years ago in the US National Institute of Health in the words of Henry I. Miller [62] should be a warning.

> Thirty-five years ago, the US National Institutes of Health adopted overly risk-averse guidelines for research using recombinant DNA, or "genetic engineering," techniques. Those guidelines, based on what has proved to be an idiosyncratic and largely invalid set of assumptions, sent a powerful message that scientists and the federal government were taking seriously speculative, exaggerated risk scenarios – a message that has afflicted the technology's development worldwide ever since.

A final remark: In a way, the artificial altering of genes producing Bt toxins can, strictly spoken, also be summarized under synthetic biology since the specifically altered Bt toxins in order to facilitate resistance management of Bt crops: Bruce Tabashnik, who works on problem solving programs for Bt crops with field research and new concepts of resistance management [63]: Relative to native toxins, the potency of modified toxins was >350-fold higher against resistant strains of *Plutella xylostella* and *Ostrinia nubilalis*. Previous results suggested that the modified toxins would be effective only if resistance was linked with mutations in genes encoding toxin-binding cadherin proteins [64]. Tabashnik et al. report evidence from five major crop pests refuting the Soberon hypothesis.

Illusions and Realities on Educational Effects in the Debate, the Dialogue Between Science and the Public

There is no doubt that there is hope and need to simply start and/or maintain an open dialogue between major stakeholders among young scientists, politicians, industry, and society [65], although there are many obstacles such as asymmetric relationships among the partners, which can render the discourse complex and unpredictable. And it is uncontested here that education on all school levels has its justified place; this has again been shown with empirical results from Spain [66, 67]. Gensuisse should also be mentioned here with educational activities in schools and a popular open day of Genetics in major Swiss cities organized by researchers and institutes every year [68]. And education on biotechnology in the developing world is especially important, if done in a participative way, and with proper ramifications in all institutions of communication, science, and regulation: In April 2007, biosafety and biotechnology scientists, regulators, educators, and communicators from Kenya, Tanzania, and Uganda met to examine the status and needs of biosafety training and educational programs in East Africa [69].

Thus, educational efforts on all levels are not in vain, and deplorably there are too few academic institutions active in biotechnology education [70]. The structure of the debate has shifted: Today, the GM crop debate is steered by scientific *and* pseudoscientific arguments. And this also includes an element of hope for the pro-scene: Slowly but surely the pseudoscientific arguments are fading away for the opponents, since there is no serious incident known despite the fact that millions of hectares are grown with GM crops worldwide [71].

There is a widespread mistrust against new technologies where everybody feels it will change their own life, and this often happens in a phase where the benefits are not yet clearly visible, especially for the consumers/users. But it is not correct to reduce those difficulties to an exclusive criticism of the so-called deficit model [72–74] where the people just have to be educated and then they would refrain from negative emotions. A question mark on the exclusive use of the "deficit model" is justified, but surprising conclusions emerge from the above-mentioned critics themselves: They do not discard altogether the traditional deficit model, rather they propose to combine it with the *contextual approach*, thus emphasizing the complex and interacting nature of the knowledge-attitude interface. This highlights the sophistication and value of lay understandings of science that can exist in the absence of formal scientific knowledge [75, 76]. Surprisingly, positive are results of polls which are conducted by Philip Aerni with more closeness to the real life and careful avoiding of polling mistakes [77], the study concludes:

> The results of our discrete choice analysis show that Swiss consumers treat GM foods just like any other type of novel food. We conclude from our findings that consumers tend to appreciate transparency

> and freedom of choice even if one of the offered product types is labeled as containing a genetically modified ingredient. Retailers should allow consumers to make their own choice and accept the fact that not all people appear to be afraid of GM food. [77]

There is growing consensus that scientific knowledge extends beyond the simple learning of "facts" that can be straightforwardly defined and measured [78]. From this perspective, privileging formal scientific knowledge as the sole basis of rational preference formation leads us to overlook other knowledge domains that may be equal or even more important determinants of attitudes toward science.

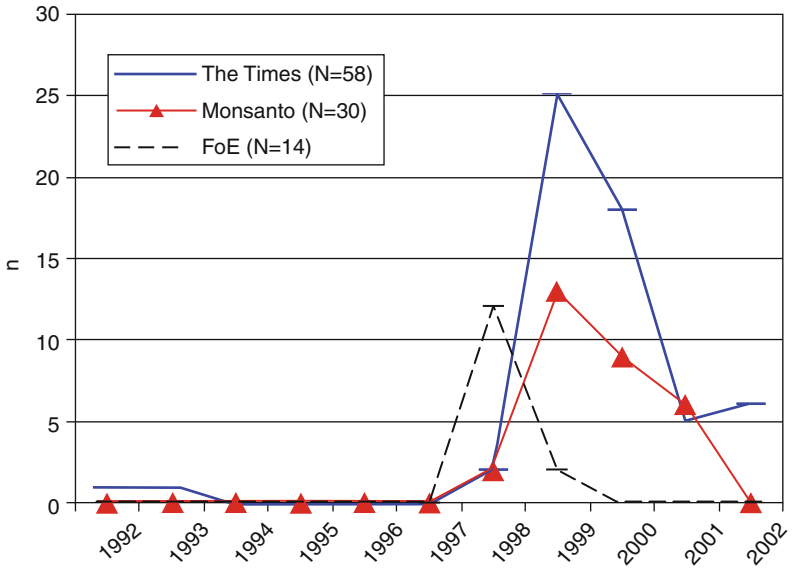
These insights have been condensed into a feasible discursive method of the *Systems Approach* initiated by Churchman [79] and refined by Rittel et al. [80–82]. Details on the methodology are given under sections Long Term Discourse and Decision Making Processes and The Second Generation Systems Approach as a New Decision Making Process, where the *solutions* are discussed.

It is an illusion to solve ill-fated GM-disputes by just adding social and cultural aspects, or that the dispute should, so to say, start from the other end of the controversy ignoring the biosafety science [83] or even worse to primarily appeal to feelings and emotions of the public and indulge in entertaining but ultimately meaningless discussions in order to catch the interest of the public – we should not mimic the strategy of the protest corporations. That said, this does not mean that sociocultural aspects including emotions should be neglected – even the boulevard press sends out strong signals for learning processes. Vaughan's [84] plea is that regulatory officials should engage in an interactive process of information and opinion exchange that is reasonable and effective within vastly different socioeconomic and cultural contexts, This is often a challenge to government employees concentrating on office work routine. Patricia Osseweijer [85, 86] offers an interesting compromise: a mix of science, ethics, and emotions with her "Three E-Model" Entertainment (getting attention), Emotion (identification), and Education (information and skills for [future] decision making). It has been developed on the basis of long-term experience and observation of public communication by individuals in the Department of Biotechnology of the Delft University of Technology [87, 88].

Despite all possible refinements and enhancements in the dialogue with the public, we should not underestimate the negative role of the opponents of genetic engineering in plant breeding organized as professional protest corporations, see section The Costs and Loss Benefits of Overregulation.

How the Internet is Influencing the Debate The Internet as a worldwide literacy practice environment is still underestimated, nevertheless it has created a new situation in communication, providing a new dynamic field for research and knowledge accumulation [89]. It has created an Internet-based debate culture with all its ramifications from classic email over blogs and better organized social media to twitter and this not only in nanotechnology [90], but also in other research realms and E-business [91]. The evolution in this kind of debate is still going on with unprecedented dynamics and is not yet fully understood in all its consequences [92], [93], and [94]. The hope is that easier communication through the Internet will invite a *collaborative* instead of *confronting* modus [95]. Some advice on how to behave in chats and blog debates on the Internet might be useful [96]; compare a list of useful websites and databases on biosafety by DeGrassi et al. [97] and [98]. A list of pertinent websites can be expanded ad libitum, the present state of error of 2011, with all the personal bias in [99].

Informatics and the new ease to access huge amounts of scientific information on the Internet causes a democratization effect on the science debate. But this can only then lead to positive developments if the new flood of information is also well organized and provided people make serious efforts to analyze the available information, so that our understanding of complex scientific knowledge can indeed be improved. As Janetzko (2008) shows, it is not enough to make use of the most common search machines, only professionally organized searches and databases on scientific literature can help and create some limited reliability and sustainability of scientific knowledge. And: clearly, the usual citation clusters among opinion-buddies will not suffice. And it should be emphasized: Electronic ease does not replace the tough job of scholarly reading and



GM Crop Risk Debate, Science and Socioeconomics. Figure 3
Web site pages addressing the "Frankenfood" and "Frankenstein food" issues at Monsanto, the *Times*, and the Friends of the Earth Web sites. jcmc.indiana.edu/vol8/issue4/hellsten.html

understanding. It will be a difficult task for the future to divide up clever knowledge accumulation and genuine thinking work among active scientists. A caveat already signaled by Seneca: Thoughtful Action creates more wisdom than knowledge accumulation, can be interpreted related to social electronic networking in two ways: On one side, the immense intensification of social networking via the Internet creates among other things a new possibility for post-publication reviewing and filtering out the really relevant publications and ideas. On the other hand, it hinders systematically the deepening of your own knowledge in an individual way, and be it only by reading every year a dozen or two really relevant book publications.

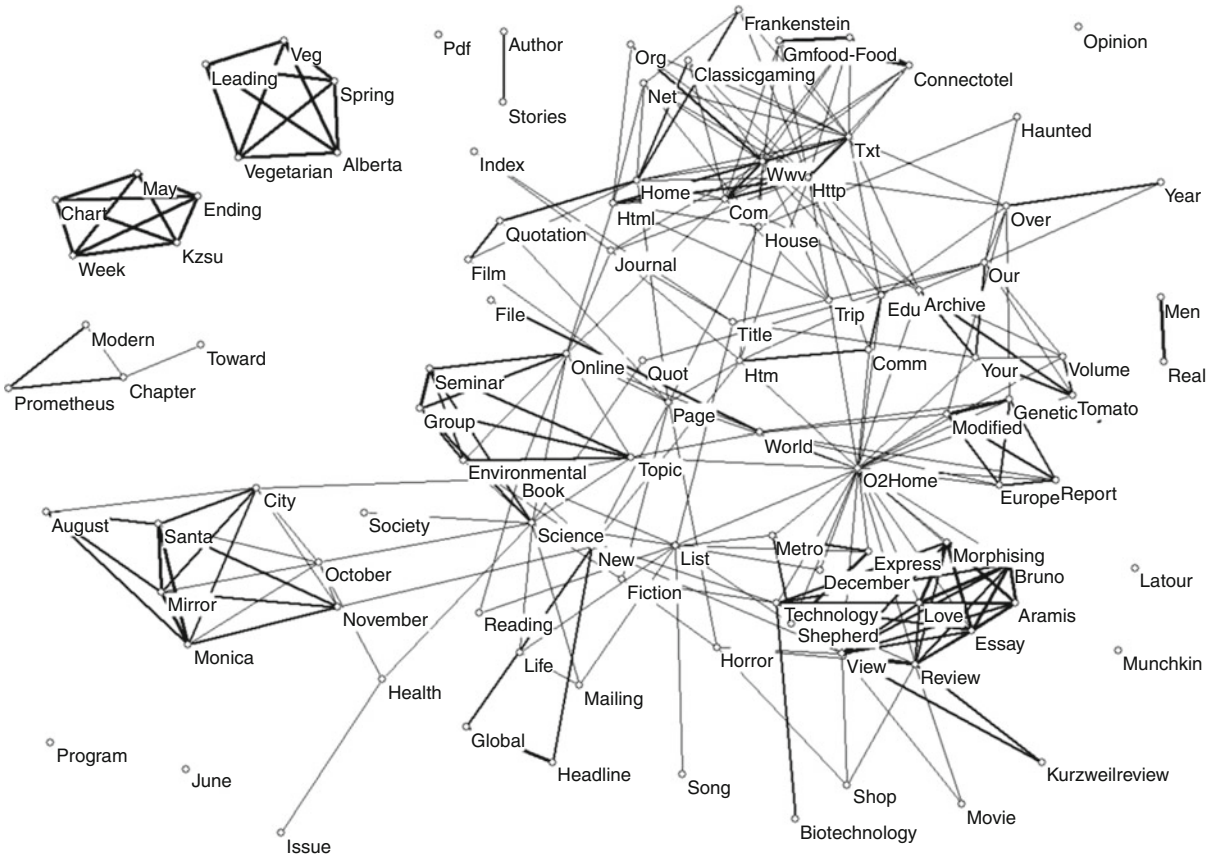
This major shift from paper to electronics is also creating new methods of *quantitative* analysis of scientific work: see the Scientometrics Wikipedia: http://en.wikipedia.org/wiki/Scientometrics. Actually, this newly emerging science can provide with caveats and insights into changes in research priorities, reveal citation habits, evaluate journals with new scales, etc. [100–103]. A typical example is given in the analysis of the coming and going of the Frankenfood myth [104], with a somewhat surprisingly early and sharp peak of appearances of the word Frankenfood in websites for 1998, followed by a sharp decline to virtually zero 2 years later (Fig. 3).

This figure is confirmed in [104] with the following statements and figures (Figs. 4 and 5):

The comments in [104]:

- Our interpretation of these results is as follows: the decline of the organizing power of the metaphor was rapid in 1999 and 2000 when the metaphors of 'Frankenfood' and 'Frankenstein food' began to be outdated. Due to its generalized meaning, the metaphor was used increasingly across domains and therefore lost its domain-specificity and the ability to organize distinctions among domains. This might also explain why the NGOs stopped using the metaphor in 2000 (HELLSTEN, 2003). From [104]

Scientometrics can do much more, [105] have shown the potential of a sophisticated statistical analysis combined with modeling of community interactions in the web: Besides tracking just the description-to-acquisition behavior of users, scientometrics can do much more by longer observation periods which offers the chance to make richer inferences about both group and individual user intentions – trends of intruding into human behavior and making



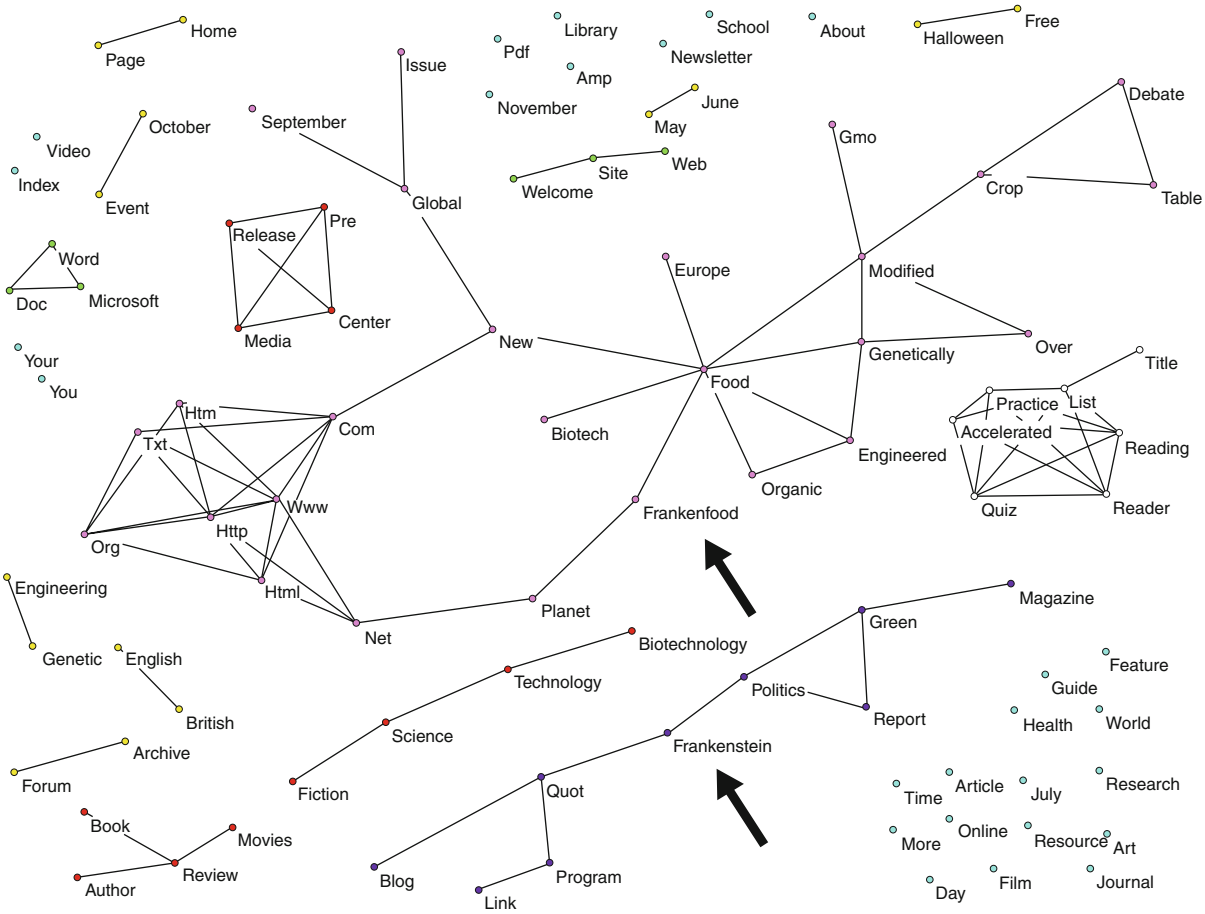
GM Crop Risk Debate, Science and Socioeconomics. Figure 4
The cosine map of 107 words used more than once in the 205 documents on Frankenfoods in 1999 (cosine $\geq$ 0.1) (From [104])

conclusions, which are actually beyond Orwell's imagination. Yet we should have no illusions, since a lot of work and application is already going on in the marketing and advertisement scene, which has also an often manifested interest in knowledge accumulation methods [106, 107]. It is somehow amazing to realize that the academic world in most fields of specialization have not yet reached the realms of professional knowledge accumulation and consolidation – not to speak about an efficient way of reaching out from knowledge accumulation to efficient development of new technology. Scientometrics would have the potential to get instrumentalized in research and development, with some good chance to be used also in new peer review processes.

A *qualitative* evaluation of science should involve additional elements – see below under peer review in the section Developments in Risk Handling of GM Crops on regulation.

Deplorably, important networks are often only known in specific reader clusters, these awareness gaps should be minimized. We need knowledge exchange, jumping over geographical and ideology fences.

Science Education and New Developments on the Internet In a successful initiative, Ron LaPorte and his group "Supercourse" started in 2002 [108] a new educational Internet-based system: In his view, Journals do not have an exclusive "right" to science. A publication and a scientific presentation do virtually the same thing – they share scientific knowledge. Publication and presentation have been separate but could "morph" into a single entity. This metamorphosis is taking place and is driven by a juggernaut called



GM Crop Risk Debate, Science and Socioeconomics. Figure 5
The cosine map of 100 words used more than 31 times in the 6,101 documents on Frankenfoods in 2003 (cosine $\geq$ 0.1) (From [104])

PowerPoint, Microsoft's graphics and slide presentation software, and today enriched with more media from Twitter over YouTube to all the numerous blog systems, networking enhanced with RSS, etc. More on the Supercourse program in [109–113], also in connection with the Bibliotheca Alexandrina in Egypt: [114] Another possibility on a well-organized collection of Powerpoint slides is offered for free by the University of California by Peggy Lemaux and Barbara Alonso, University of California http://ucbiotech.org/resources/slide_archive/index.html. A series of over 100 slideshows is offered by the bibliography of the author; new slide shows are continuously added, they can be downloaded from [115]. An important new development started 2002 at the Bibliotheca Alexandrina, where a new world center of electronic knowledge is emerging, which is based on thoughtful new structures [116].

On Biosafety Education Biosafety is today a permanent topic on local, national, and international level, and basically, it is good to see educational activity. As demonstrated in this contribution, the topic of biosafety is highly controversial, and so are the views on the various educational activities. The most blatant misunderstandings in biosafety education stem again from the "Genomic Misconception," which forces authors seemingly to focus on transgenic crops alone, which is scientifically unacceptable as we will see in section GM- and Non-GM-crop Differences

Over-estimated, the "Genomic Misconception". A symptomatic example on the enumeration of risks related to transgenic crops is given by Craig et al. [117]: All risks duly mentioned can be attributed just as well to conventional crops. The only difference between modern and conventional breeds can be found in risk mitigation, which is much easier in the case of the transgenic crops. Here, just two recent examples related to the successful prevention of upcoming resistant pest insects (a problem arising in all kinds of agricultural management systems): [63] and [11]. It is deplorable, that most biosafety education is still based on the erroneous "Genomic Misconception," which results automatically into a biosafety risk view focusing on the process of transgenesis instead of working on a product-oriented basis. More about the "Genomic Misconception" is discussed in section GM- and Non-GM-crop Differences Over-estimated, the "Genomic Misconception".

Proposal for a Website of Websites There are simply too many websites (see ASK-FORCE Organization and Related Websites) and not enough coordination, so there is a need for networking structures among the most important websites, a *network of networks* with all the fancy new buttons available like RRS, etc. There should be a place where people see with one glance on the first page what news they can expect on various important sites. It should also not be difficult to add possibilities for an individual choice.

Those website connection activities need professional support with some secretarial/managerial help. We must work out ways in which the broad public can easily reach rebuttals on all the myths, facts, and benefits in the debate on green biotechnology. It will not be difficult to establish a platform for a better communication among the most important websites – in the field of agricultural biotechnology, there are a few very successful ones, but this is not the whole task. We need to look deeper into the theory of networks in order to be really successful; comprehensive reviews demonstrate how complex the networking task really is [118, 119].

As of now, this is just an idea and needs to be discussed with Internet and website specialists. One of the main difficulties will be to establish permanent existence, this is why it would be best to use structures having proofed long years' activities and assured permanence, such as ISAAA, the International Service for the acquisition of Agri-Biotech Applications, www.isaaa.org. After all, the leading webmasters and coordinators agree that it is time to *enhance collaboration through better communication*, see section ASK-FORCE Organization and Related Websites. ASK-FORCE. The task on uniting the most relevant websites and blogs should not be underestimated, see the list already given above [99].

Developments in Risk Handling of GM Crops

General Views on the Dialogue Related to Regulation of GM Crops

The dialogue between scientists and regulators is very complex, as accurately described by Saner [120]. This should be a reminder that it is not about facts alone:

- It should be clear without explanation that each and every rational decision is a combination of facts and values – a decision requires judgment. The agents of judgment are, of course, people, and this leads us to an entirely different interface – that between scientists and policy-makers.

We should keep this in mind when we concentrate here on the *science* of GM crop regulation. See also the analysis of the debate in The General Strategic Situation of the Debate About Green Biotechnology Today. These philosophical thoughts of Saner are at the basis of the discursive methodology for complex decision-making processes, [121–123]. For details, see below in this contribution in sections Long Term Discourse and Decision Making Processes and The Second Generation Systems Approach as a New Decision Making Process.

A valid overview on the regulatory science and traceability related to GM crops has been published by Gasson and Burke [124, 125], there is no intention to repeat these reviews.

Biotechnology and Economics

How Economics Are Influencing the GM Crop Debate The example of the Flavr Savr Tomato demonstrates that in earlier times, even in Europe, GM food was well received, but several factors just made it clear

that economic success was missing [8, 126–128]. And regulation of this pioneer work needs to get a new look; with modern screening methods, the gene silencing on the molecular level revealed some surprises [129].

Economics play a very important role in the process of technology acceptance: This can be illustrated with the present day feed import situation in Europe. First it should be mentioned, that it is the trade policy of Europe still going the wrong way, which causes a lot of difficulties in the transatlantic dialogue: As Graff et al. [130] explain:

> ▶ European policies blocking genetically engineered crops are conventionally attributed to the concerns of European consumers, but they can be attributed to the self-interests of European industry and farmers as well. Biotech policies maintained in the name of consumer interests are helping European chemical firms to slow their losses in the global crop protection market and are helping European farmers differentiate their conventional crops on environmental and safety grounds, maintain their agricultural subsidies and win new non-tariff trade protections.

The recent development in feed supplies, see Lawrence in *The Guardian* [131], in the EU provides argument, and the reports and letters below give excellent examples:

- Food Chain Dossier 2009: http://www.botanischergarten.ch/Feed/Food-Feed-Chain-Dossier-20090616.pdf
- DG AGRI feed report: http://www.botanischergarten.ch/Feed/EC-DG-AGRI-Rep-feed-situation-UnapprovedGMOs-200709.pdf
- EU Report on Pipeline: http://www.botanischergarten.ch/Feed/Stein-EU-Report-GMO pipeline-LLP-2009.pdf
- Letter to the President of the EU Commission Barroso: http://www.botanischergarten.ch/Feed/Letter-big-Producers-Tolerance-Value-Barroso-20090624.pdf

Strict labeling and thus a discrimination of European meat from animals fed with GM crops will soon be impossible as a political goal due to *economic* reasons – as it is also scientifically not justifiable [132, 133].

An interesting thesis with economic arguments is promoted by Paarlberg [134]: Today, Africa's production of GM crops is exported mainly to other African countries, and this might go on this way in the coming years, so the reasoning that Africans would destroy export opportunities to Europe by developing their own GM crops is not really convincing. But in reality, there is growing concern: Commercial fear over potential loss of export sales to Europe and East Asia is also a reason for mounting pressure on biosafety approvals in developing countries. Consumer misgivings toward GM food in rich countries combined with restrictive import and labeling policies are prompting GM-free agricultural production in developing countries. The long-term costs of these negative trends could be enormous [135]. Good arguments for this view are produced with lots of facts on economics and negative labeling effects of European developed countries, published by Gruère et al. [136–138]:

> ▶ In this context, the marketing decision of avoiding GM ingredients in food items rapidly became a quality attribute employed in the competition among the retails chains of Europe, Japan and South Korea. A report by the international NGO, Greenpeace, which has encouraged companies to adopt GMfree policies, provides evidence of the widespread adoption of such practices in Europe [139] as follows:
>
> - Fourteen of these retailers have a policy of not selling GM-branded products under their company name for all European countries. These include Carrefour, Auchan, Sainsbury's, Safeway, Marks & Spencer, Coop Switzerland, Coop Italia, Migros, Big Food Group, Somerfield, Morrison's, Kesko, Boots, and Co-op UK.
> - Seven of these retailers have a non-GM policy for their own branded products for their main markets (mainly in their home countries). These include Tesco, Rewe, Metro Group, Casino, Edeka, Schwarz group, Tengelmann).
> - Out of the top 30 European food and drink producers, 22 have a non-GM commitment in Europe, including Nestle, Unilever, Coca Cola, Diageo, Kraft Foods (Altria), Masterfoods (Mars), Heineken, Barilla, Carlsberg, Dr. Oetker, Arla Foods, InBev (Interbrew), Heinz, Chiquita, Cirio del Monte, Orkla, Ferrero, Northern Foods, Eckes Granini, Bonduelle, Kellogg and McCain.

- Thirteen of these 22 multinationals have a company-wide non-GM policy beyond Europe. These include Diageo, Heineken, Barilla, Carlsberg, Arla Foods, Dr. Oetker, Chiquita, Cirio del Monte, Orkla, Ferrero, Northern Foods, Eckes Granini, and Bonduelle [138].

Some companies even go beyond banning processed products derived from GM ingredients to include requirements on GM-free animal feed in animal products. Virtually all supermarkets sell only poultry fed with non-GM feeds, whereas the policies for dairy products, beef, and pork vary. The usual crude Greenpeace mix of facts and interpretation helped efficiently to push the companies for the European market to go GM crop free [139, 140]. The simple fact of labeling allows opponent NGOs to drive a polemic campaign of pompous "contamination" reports, thus delivering junk science "evidence" that there is some risk involved in the numerous events of minute admixtures of transgenes traces.

In India, there is a clear positive trend visible since some years after some difficulties in the beginning because local traits had to be created for the many Indian regions and also because there was right from the beginning a black market with illegal cotton traits developing (which often did better commercially than the legal ones. Presently, there are 38 traits of GM cotton in India [141].

The whole complex story has been recently summarized by [142]:

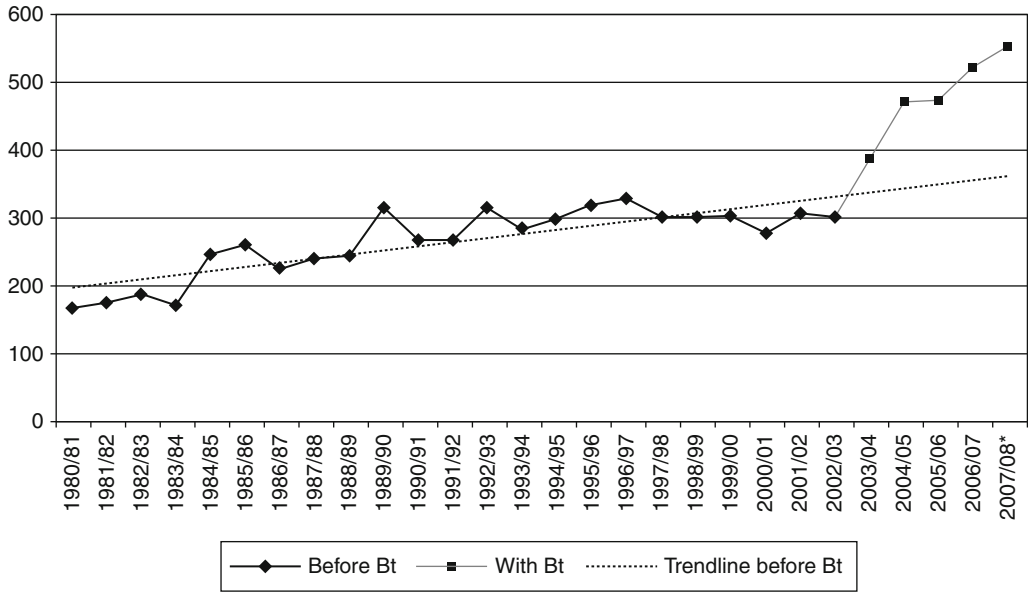
- On average, Bt-adopting farmers **realize pesticide reductions of roughly 40%, and yield advantages of 30-40%. Profit gains are at a magnitude of US $60 per acre**. These benefits have been sustainable over time. Farmers' satisfaction is reflected in a high willingness to pay for Bt seeds. Nonetheless, in 2006 Indian state governments decided to establish price caps at levels much lower than what companies had charged before. This intervention has further increased farmers' profits, but the impact on aggregate Bt adoption was relatively small. Price controls might have negative long-term implications, as they can severely hamper private sector incentives to invest in new technology. [142]

At the end of the day the profitability of Bt cotton is now uncontested, see comments of Müller-Jung Frankfurter Allgemeine: [143]

Also the old wrong connection between suicides of Indian farmers and the introduction of GM cotton in India has been thoroughly falsified [144, 145]. This does not hinder activists like Vandana Shiva from continuing with cheap propaganda linking GM crops with the sad tradition of farmers' suicides in India, which started decades before the introduction of GM crops and beginning activities of multinational seed companies. Here are two of the many graphs from [145] (Figs. 6 and 7):

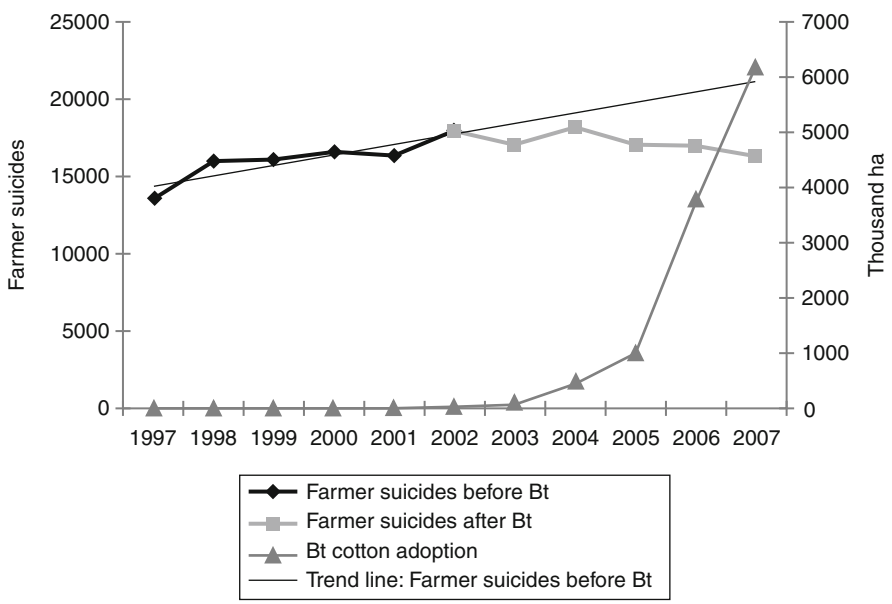
- **Abstract.** Bt cotton is accused of being responsible for an increase of farmer suicides in India. In this article, we provide a comprehensive review of evidence on Bt cotton and farmer suicides. Available data show no evidence of a 'resurgence' of farmer suicides. Moreover, Bt cotton technology has been very effective overall in India. Nevertheless, in specific districts and years, Bt cotton may have indirectly contributed to farmer indebtedness, leading to suicides, but its failure was mainly the result of the context or environment in which it was planted [145].

- **From the discussions.** The absence of irrigation systems in drought-prone areas (especially in Maharashtra), combined with specialisation in high-cost crops, low market and support prices, and the absence or failure of the credit system, is a clear recipe for failure. It is possible, therefore, that under the conditions in which it was introduced, Bt cotton, an expensive technology that has been poorly explained, often misused and initially available in only a few varieties, might have played a role in the overall indebtedness of certain farmers in some of the suicide-prone areas of these two states, particularly in its initial years. But none of these possible links has been explicitly demonstrated with a sufficiently robust analysis. One implication of this study is the critical need to distinguish the effect of Bt cotton as a technology from the context in which it was introduced. Revealed preferences based on farmer adoption rates and official or unofficial data all point toward the overall success it has had in controlling pest damage and therefore raising average yields in India. In particular, the increasing adoption rate in two suicide-prone states, Andhra Pradesh and Maharashtra, indicates that farmers in these states found this technology economically beneficial.



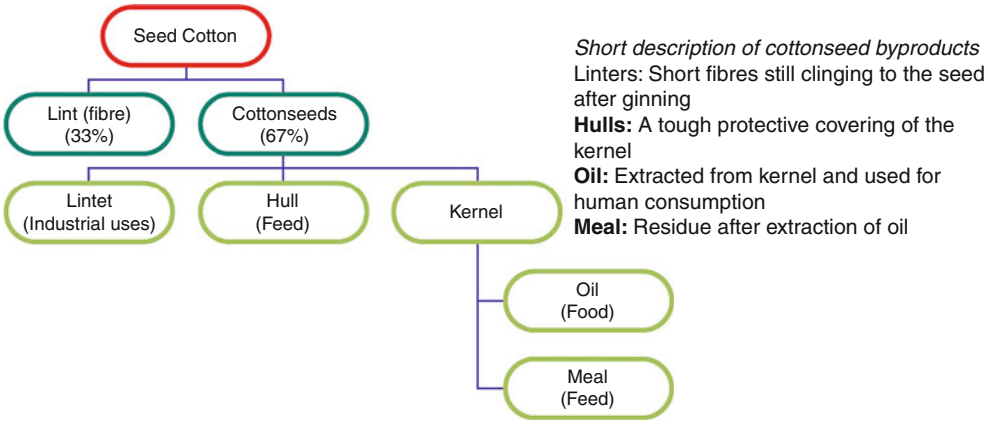
GM Crop Risk Debate, Science and Socioeconomics. Figure 6

Average cotton yields in India (kg/ha), 1980–2007 (Source: International Cotton Advisory Committee (2008). Note: Data for 2007/2008 is an estimate. From [145])



GM Crop Risk Debate, Science and Socioeconomics. Figure 7

Farmer suicides and Bt cotton area in India, 1997–2007 (Source: Combined data from Table 1 and Table 2. From [145])



GM Crop Risk Debate, Science and Socioeconomics. Figure 8
Cotton seed byproducts (From [148])

In contrast, marketing constraints and institutional issues may have played a significant role. Our analysis suggests the need for a better extension system, more controlled seed marketing system, anti-fraud enforcement and better information dissemination among farmers in all regions, before the introduction of any costly new technologies like Bt cotton. Farmers should also be encouraged to diversify their farming and non-farming activities to spread the risks they may incur.

The second implication is that, as farmer suicides are not new or specific to recent cases or to the introduction of Bt cotton, they point toward the failure of the socioeconomic environment and institutional settings in rural dry areas of India. This has nothing to do with cotton or the use of new technology and would suggest many potential policy changes. In several states, such as Karnataka and Andhra Pradesh, some policy changes have already been proposed. Lastly, much more and better federal and state investment could help prevent the 80 percent or more other cases of suicides.

This does not hinder activists like Vandana Shiva from proclaiming Indian farmers' suicides to be the fault of international corporations: [146] and lately also at a Barilla webinar July 20, 2011 in Milano: http://www.barillacfn.com/en/biotecnologie, she also does not shy away from connecting the sad tradition of farmers suicides in India with the emergence of GM crops, despite hard facts as demonstrated above. In the same picture you can see her pompous literature list she gives in her curriculum of "over 300 scientific publications in important journals" – a quick test in the comprehensive database of the Web of Knowledge http://apps.isiknowledge.com/ reveals some 47 papers, most of them in less important journals and magazines – so much about her scientific achievements.

A new perspective is open since 2006 for the production of cotton seed (oil for human consumption), seed meal for feed, made possible thanks to the detoxification (gossypol) successfully done by modern breeding including genetic engineering [147], see the latest summary on the matter (Fig. 8) [148]:

This latest development will open new doors for the cotton production and marketing.

The Political Economy of Biosafety Regulation in Agriculture An in-depth analysis of how politics is influenced by multiple factors of discursive processes, influenced by economics, has been developed by Graff et al. [149]. They are giving highly differentiated insights in the network of self-interests with some interesting examples of units influencing in their own interest the debate on GM crops: opponents of genetically engineered crops and also industrial units fearing losses in pesticide sales. Often these important socioeconomic elements in the regulatory debate are neglected and it seems to be difficult for all the regulatory analysis to bring together socioeconomic *and* molecular plant breeding aspects.

- This article develops a political-economy framework to analyze the formation of agricultural biotechnology policies. Going beyond accounts, that largely attribute differences between US and European regulatory environments to consumer attitudes, we consider the impact of what amounts to a Schumpeterian process of "creative destruction" across the entire range of relevant economic sectors and interests. **The analysis suggests that in Europe and in some developing countries a "strange bedfellows" constellation of concentrated economic interests (including incumbent agrochemical manufacturers, certain farm groups, and environmental protest activists)** act in rational self-interest to negatively characterize GM technology in the public arena and to seek regulations that block or slow its introduction. In contrast, those interests most likely to experience welfare gains from biotechnology are the more diffused and less informed – including consumers and small farmers. The most profound implications of overregulation of agricultural biotechnology are (1) delays in the global diffusion of proven technologies, resulting in a lower rate of growth in the global food supply and higher food prices, and (2) disincentives for investing in further R&D, resulting in a slowdown in innovation of second generation technologies anticipated to introduce broad consumer and environmental benefits." [149]

Ayal and Hochman [150], started in some intricate experimental setups working on the cognitive processes underlying choice behavior. With a mix of behavioral actions combined with opinion polls they found that people *do not rely on limited arguments only, but tend to integrate all acquired information into their choice processes*. This could explain the delay in such opinion finding and decision-making processes influencing politics over years, described in the Gartner hype cycles, see The General Strategic Situation of the Debate About Green Biotechnology Today.

Although this would be an epic theme, we shall concentrate here more on the debate of the *Science* of regulation and some discursive elements.

Brazil, A Case Where Politics Positively Influences the Development and Adoption of GM Crops

Studying the biosafety law of Brazil, the similarities with the European legislation cannot be overlooked: Both legislations are process-oriented and obey strict rules on biosafety assessment, including field experimentation:

A closer look at the Brazilian legislation [151] shows the similarities to the European legislation.

- Article 3. Under this Law, it shall be considered:
  V – genetically modified organism – GMOs: an organism the genetic material of which – DNA/RNA has been modified by any genetic engineering technique;

And compare some exclusion rules, typically reducing the safety assessments strictly to the process of genetic engineering.

- Article 4. This Law is not applicable when a genetic modification results from the following techniques, provided they do not imply in using a GMO as the receiver or donator:
  I – mutagenesis;
  I – the formation and use of animal hybridome somatic cells;
  III – cellular fusion, including plant cells protoplasm, which can be produced from traditional culture methods;
  IV – the self-cloning of naturally processed non-pathogenic organisms.

The same is the case in the European law:

[152], in the introduction the definition of GMOs is given:

- In order to protect human and animal health, food and feed consisting of, containing or produced from genetically modified organisms (hereinafter referred to as genetically modified food and feed) should undergo a safety assessment through a Community procedure before being placed on the market within the Community.

The intention of this "exclusive" definition is clear in this European Law: it should be restricted to GMOs which are wrongly defined as "genetically modified crops," a scientifically questionable denomination, since in the strict sense of modern genomic science this means to include all crops and horticultural traits having been modified also by conventional breeding. This kind of now false but routine denomination is a symbol for the disregard of proper science in regulation.

A further comparison demonstrates that legislations in Europe and Brazil are both rather strict, the decisive difference is that in Brazil there are clear (political) decision-making rules, whereas these are lacking in Europe. Until lately, the decisions were depending on majority voting rules of the European states, and this caused a lot of confusion and an almost complete stall in decision-making. This is why Commissioner Dalli [153], in July 2010 opened a debate on delegating some important decisions to the national level: Comments from http://www.gmo-compass.org/eng/news/523.docu.html

- (13 July 2010) As expected, the EU Commission decided on 13.07.2010 changes in the legal regulation of green biotechnology. Accordingly, Member States should be able to prohibit the cultivation of genetically modified (GM) crops that have been approved EU-wide. As the next step, the EU Parliament and Council of Ministers must agree.

The outcome will again depend on complex negotiations and it is not sure whether Commissioner Dalli and the EU will come to concrete legislative results. And, except for some modest GMO corn cultivation in Spain, the present day acreage of GM cultivars remains disappointingly low [154].

In contrast to the complex and stalled situation on European GMOs, the case in Brazil documents in the last few years successful regulation of GMOs: Recent reports document steadily growing acres on GMO crops in Brazil: [155, 156]

- The 1st survey on agribiotechnology in Brazi l for the 2010/11 growing season showed there was a substantial growth in the adoption rate of biotech soybeans, corn, and cot ton. The Brazilian farmers are expected to plant 17.2 million hectares with GM soybean cultivars, or 75.6% of the total harvested surface, in 2010/11.

For a general survey of the Brazilian situation, see the recent publication of Mendonca-Hagler et al. [157], where a clearly optimistic picture is developed. The abstract reads:

- Biotechnology is a Brazilian priority, and has been recognized for its potential to promote sustainable development. The Government recently announced an ambitious program for Science and Technology, which includes strategies to develop modern biotechnology, continuing three decades of public investments on capacity building and infrastructure, aimed principally at the development of technologies applied to health, agriculture and the environment (MCT 2008 http://www.mct.gov.br/). Research initiatives have focused on genomics, proteomics, genetically modified organisms (GMOs), gene therapy, stem cells, bio-fuels and nanotechnology, among other biotechnological topics. Research projects in Brazil have been mainly developed in public universities and institutions funded by federal and state agencies, with a minor participation from the private sector [158]. Genomics, an area of considerable success in the country, was launched a decade ago by S. Paulo State Research Foundation (FAPESP), with the organization of a virtual institute, called ONSA, comprising several laboratories with the main task of sequencing the genome of the citrus pathogenic bacterium Xylella fastidiosa [159, 160].

The success of this genomic network stimulated biotechnology startup companies and projects with the focus on other genomes, such as sugarcane and coffee, including functional genomics and proteomics. Following in the footsteps of the ONSA network, the Ministry of Science and Technology created a National Genome Project Consortium involving institutions located in the major regions of the country, with the task of sequencing eight microbial and two plant genomes. Recently, they concluded the sequence of Chromobacterium violaceum, a bacterium with exploitable properties, such as the ability to produce a bactericidal purple pigment (violacein) and bioplastics [161]. Later on, several states launched their own genome programs. A group from Rio de Janeiro, part of the Riogene network, recently sequenced the genome of the nitrogen-fixing bacterium Gluconoacetobacter diazotrophicus, a sugarcane endophyte involved in enhancing growth of large crops without the addition of nitrogen fertilizer [162, 163], see also the websites of EMBRAPA http://www.embrapa.br/english and the Ministerio Biotecnologia e Tecnologia http://www.mct.gov.br/.

Agriculture plays an important role in the Brazilian economy, being responsible for ca. 40% of the exports and employing 20% of the active work force. About

> one third of the Brazilian GDP comes from agribusiness. Traditionally, this country has been competitive in tropical agriculture, supported by strong research programs on conventional and modern technologies. Intense capacity building initiatives resulted in the formation of a critical mass of scientists working in molecular biology and agricultural sciences [158]. Despite these favorable factors, the adoption of GM crops has been delayed due to intense opposition organized by environmental groups and additional difficulties resulting from a conflicting regulatory framework. In this overview, we address the current status of Brazilian biosafety legislation, and discuss the perspectives for the development of molecular biotechnology in Brazil.

This view is confirmed in a recent editorial in *Nature*, [164], interestingly enough with the same emphasis as above on gene sequencing projects which are the basis of independent biotechnological research and development in Brazil.

Also, the latest success of approving regulatory decisions is symptomatic of the positive biotech climate in Brazil: The first fully developed transgenic crop in Brazil has been approved for commercialization, published in 2007: [165]. The press release of the president of AnBio (National Biosafety Association) Leila Oda emphasizes also the socioeconomic importance of this approval: [166].

Without going into a survey on the Brazilian opponent's activities and reports in detail, here just a typical example published by a medical group (not linked in any way with environmental toxicology) [167] on how science is distorted in order to make a negative and totally unfounded point against glyphosate is given. This paper produces negative toxicological effects on clearly doubtful experimental scenarios: experimental Xenopus frog embryos were *injected* with glyphosate, as mentioned in the introduction.

- We show here that sublethal doses are sufficient to induce reproducible malformations in Xenopus and chicken embryos treated with a 1/5000 dilution of a GBH formulation (equivalent to 430 μM of glyphosate) or in frog embryos **injected with glyphosate alone (between 8 and 12 μM per injected cell).** GBH treated or glyphosate injected frog embryos showed very similar phenotypes, including shortening of the trunk, cephalic reduction, microphthalmy, cyclopia, reduction of the neural crest territory at neurula stages, and craniofacial malformations at tadpole stages.

This absurd experiment methodology contradicts all internationally agreed rules on environmental toxicology testing, as described and cited in detail in [168].

But opponents are well organized on an international level, and promptly, the Paganelli paper is cited in many of those reports, here is just one example: [169]. In this extensive report, dozens of papers are cited which do not match the high quality standards of biosafety science; they are cited because they produce negative results related to modern soybean agriculture. The following is an example on how the authors do not even shy away from distorted reporting of published results.

- Very few studies directly examine the effects of GM foods on humans. However, two studies examining possible impacts of GM RR soy on human health found potential problems.

  Simulated digestion trials show that GM DNA in GM RR soy can survive passage through the small intestine and would therefore be available for uptake by the intestinal bacteria or cells [170]. Another study showed that GM DNA from RR soy had transferred to intestinal bacteria before the experiment began and continued to be biologically active [171]. These studies were not followed up. GM proponents often claim that GM DNA in food is broken down and inactivated in the digestive tract. These studies show that this is false.

Actually, if you read the above Newcastle study properly, you notice that the GM DNA is completely decomposed in the colon, the only traces measurable were found in fresh, undigested stomach probes of human ileostomy patients. Reading the summary alone shows the blatant incorrectness of the comments. Two previous studies, after careful reading, reveal the same results [170, 172]. The conclusion therefore is that the interpretation of [169] is false, as confirmed in the latest publication of the Newcastle research team:

- The transgene did not survive the gastro-intestinal tract of human subjects fed GM soya.

A recently published paper of Zhang is seen as a breakthrough in our knowledge on interkingdom relations between plant and animal genomics: [173].

First data, obtained with modern genomic analysis, demonstrate the surprising finding that exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake. MIR168a is abundant in rice and is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. In addition, these findings demonstrate that exogenous plant miRNAs in food can regulate the expression of specific target genes in mammals.

This could lead to erroneous conclusions that horizontal gene transfer is possible also for the antibiotic resistance genes and even for genes expressing Bt toxins into mammals and humans, and one can see already that opponents to genetic engineering take advantage of the news by clear misinterpretation of the results: They use it as an argument for the unforeseen risks of the technology. See the comments of anonymous scientists in GMwatch [174]:

- The study is yet another nail in the coffin of the already discredited 'safety assessment' process for GM foods in the EU and elsewhere. These assessments do not consider the effects described.

This rather naive statement is typical of the thinking of GM crop opponents: Firstly, they mix up in an unscientific way various categories of transgenes; secondly, they mix up scientific progress and the inevitable adaptation of risk assessment methodology with the present day regulatory rules in place in the laboratories. It is a matter of simple scientific consensus that biosafety assessment has to adapt in methodology with the progress of genetic engineering: on one side, Zink-Finger and TALES methods (details see Zinc Finger Targeted Insertion of Transgenes and TALEs: Transformation Method Transcription Activator-like Family of Type III Effectors.) with all their precision and elegance are prone to simplified risk assessments after detailed studies. On the other hand, technologies using small RNA molecules will undoubtedly force risk assessment researchers to adapt to appropriate methods of analysis, as already proposed by [175]:

- In the future, the predictive ERA process will need to be flexible and adaptable for analysis of the next generation of crops engineered using RNAi and HD-RNAi. As a first step, regulatory agencies and risk analysts need to become familiar with the science of RNAi and its application to plant biotechnology. A concerted effort is needed to develop a pool of expertise to ask the right questions about potential hazards and exposures, to ensure that relevant data are collected and to characterize uncertainty in risk assessments.

  Regulators will have to evaluate the design and implementation of research protocols for laboratory experiments and confined experimental field trials. Scientific questions will need to be answered about off-target effects, non-target effects and the impact of genetic mutations and polymorphisms. Understanding the stability, persistence and half-life of small RNAs in various aquatic and terrestrial ecosystems will be essential for the characterization of exposure pathways. New diagnostic tools will probably be required for the identification and quantification of small RNAs for a range of purposes, including crop identity preservation, monitoring and segregation. Ideally, these tools should have a low detection limit and a high degree of specificity for each RNAi crop, while being relatively inexpensive, functional under field conditions and operable by individuals with diverse backgrounds and training. With all this in mind, it should be possible for stakeholders, regulators and citizens to develop policies and ERA frameworks for RNAi and HD-RNAi crops. [175]

It is correct that small RNA molecules are considered and used for GM plant improvements, as suggested by [175]. And it is also correct that the risk assessment of GM crops up to now does not specifically include the effects described by Zhang et al., that is, that small miRNAs are obviously passing mammal stomach environments and can be integrated in the organism and even be active genetically. This seems to be routine in the evolution of life (and undoubtedly calls for verification and further studies). And the question arises whether we should *automatically* include in the risk assessment small miRNAs, the answer should be *no*: rather it should be another reason to switch European and UN-Risk assessment to product-oriented mood, following the conclusions drawn in the section on the GM- and Non-GM-crop Differences Overestimated, the "Genomic Misconception".

The above examples of misleading statements and publications of the opponents lead in a logical way to the following section on the quality of scientific papers:

Peer Review in the Biosafety Science Debate on Regulation

Before we start talking about regulation, a word on the science debate shall precede, which depends on the process of peer review, but it may be flawed in many ways, although there is no real good alternative in sight, despite some attempts to change this situation like the proposal to involve respected science journalists. But there are objections: journalists might become part of the system [176] and give up indirectly their strict impartiality and neutrality – which is, maybe, anyway an illusion. Or it might be that they may simply not have the scientific expertise as demonstrated recently in a contribution of a science journalist in Nature [177], extensive critical comments in ASK-FORCE contribution on the Rosi-Marshall publication on aquatic insects, see [178] (more comments about this study are given below). It should also be admitted, that a fresh look of a "greenhorn" might reveal new aspects of the GMO battle.

The quality of biotechnological research is also influenced by the research environment offered to students and is evaluated in a differentiated way for Europe by Reiss et al. [179]. Peer review is a very fragile instrument and needs constant inquiry, as demonstrated also on the Wikipedia website on the subject of peer review http://en.wikipedia.org/wiki/Peer_review. It should also be seriously considered that the present day peer review system is basically "faith based," as described with convincing details by [180].

A trend toward a magazine style is documented for some important journals as *Nature* and others. The facts show that the percentage of externally peer-reviewed articles has dropped dramatically. Facts will be given in a forthcoming publication of R. Laporte, F. Linkov, and K. Ammann.

We should also include a new element in the reviews and evaluation of science as proposed by Lubchenco [181]: the scientific community should formulate a new *Social Contract for science.*

> This contract would more adequately address the problems of the coming century than does our current scientific enterprise. The contract should be predicated upon the assumptions that scientists will (1) address the most urgent needs of society, in proportion to their importance; (2) communicate their knowledge and understanding widely in order to inform decisions of individuals and institutions; and (3) exercise good judgment, wisdom, and humility. The paper concentrates, according to the zeitgeist of the publication date, too much on environmental issues alone, today we should put into the center of our science strategy debates **humanity as a whole – and this means to take care of the most urgent needs, namely to work on the eradication of hunger**.

However, this process should not be mollified on the costs of hard science. The line between science and pseudoscience is often difficult to draw.

An interesting new aspect has been introduced by the Supercourse Group with Faina Linkov and Ron LaPorte: [182]. It is true that quality control of Internet texts need rethinking, and it is also important to analyze in a critical way peer review of print material: Their comments can be summarized as follows: High-quality, Internet-distributed lectures are not basically different from written science publications, they also must be documented and references properly. A further element could be a method of quality management introduced originally for the industry by Edwards Deming Wikipedia of Edward Deming http://en.wikipedia.org/wiki/W._Edwards_Deming, who very successfully taught management and quality control also in Japan in the 1950s.

Two more initiatives should be mentioned here, they can be summarized under a kind of *post-publication peer review.*

Faculty of 1,000 System With a total of nearly 84,000 articles reviewed by May 2011, the system has accumulated an important body of comments, see http://f1000.com/, the comments, although really critical sentences are not foreseen, the system is now linked to The Scientist and provides helpful orientation about important publications. Some examples have been evaluated by the author [183].

Frontiers of Science Frontiers of Science has been developed over 2 years in consultation with scientists and other faculty, as well as with students and postdoctoral fellows, to address manifest intellectual, logistical, and pedagogical issues, see http://www.sciencecore.columbia.edu/s2.html and http://www.fos-online.org/

Declaration of a New Global Business Ethos as a Barrier Against Undue Influence on the Publication Policy of Scientific Journals On October 6, 2009, Hans Küng, Josef Wieland and Klaus Leisinger presented the Declaration of a NEW GLOBAL BUSINESS ETHOS at the United Nations in New York http://www.novartisstiftung.org/platform/content/element/3177/Newsletter_3-09_2.pdf.

Although coming from a pharmaceutical company like Novartis, multinational seed companies will (or should) most likely join. Such efforts are important, because there is a constant pressure of undue influence on scientific papers, although resisted successfully by most researchers, but the influence of multinational (in this case pharmaceutical) companies can be hidden but nevertheless powerful:

An example of such influence by units sponsoring scientific journals has popped up in Australia: See the debate around the withdrawal of six Australia-based Elsevier "fake" journals sponsored by the pharmaceutical industry, see the statement of Elsevier's CEO Michael Hansen [184] and [185–187]. This kind of influence might still be under control, and peer review is usually functioning in an unbiased way – but the difficulties are deep-rooted, and it is a constant fight for quality, as is summarized comprehensively by Scott [188].

It is a cheap and intellectually intolerable slogan of opponents of genetic engineering in agriculture when they discredit researchers for their relationships with industry, since the great majority of researchers all over the world act as independent persons, although sometimes also funded by industry. The sole quality criteria on science are transparency in applied methods agreed upon by the science community and the reproducibility of the data. For more details see section More on the Quality of Scientific Publications.

In the "dangerous" waters of corporate influence, we need renewed efforts of scientometric analysis, as given earlier in a report of bio-era: [189]. The top part of table 5 reveals the few really successful seed companies in relation to the top universities with agricultural research regarding R&D (Fig. 9):

The top 35 R&D orgnizations in agricultural biotechnology

RANK	PARENT ORGANIZATION	SHARE OF INDUSTRY R&D OUTPUT
1.	Monsanto	29.82%
2.	Du Pont / Pioneer	10.98%
3.	Bayer / Aventis	10.14%
4.	Dow	5.81%
5.	Syngenta	5.80%
6.	Savia / Seminis	2.57%
7.	USDA	2.38%
8.	BASF	1.71%
9.	Cornell University	1.25%
10.	Stine Seed Farm Inc	1.15%
11.	Florigene	1.08%
12.	University of California	1.05%
13.	Exelixis	0.98%
14.	Iowa State University	0.91%
15.	Rutgers University	0.83%
16.	University of Guelph	0.79%

GM Crop Risk Debate, Science and Socioeconomics. Figure 9

Table 5, upper part, with a ranking of biotech companies and universities in the USA, from [189], calculation rules above

The calculation rules for the table below:

- The four R&D measures are weighted equally. For example, having 10% of industry patents is just as significant as having 10% of commercialized products. Share of industry R&D output = (share of industry patents + share of industry patent citations + share of industry field trials + share of industry commercialized products)/4 [189].

More on the Quality of Scientific Publications Coming back to the peer review on the quality of scientific papers, all the above statements do not mean to say goodbye to the factual and methodological scrutiny per se – even after a paper is already published. With a focus on the GM food safety research Chassy and Parrott [168] summarize the criterions on how to

judge whether a food study is believable or not: (a) Making sure the samples tested are comparable samples. (b) Testing composition to make sure the tests and controls are comparable. (c) The need for an acceptable balanced and nutritious diet. (d) Why the dose is important. (e) What statistics do and do not tell us. (f) The importance of peer review and scientific publication. (g) Guidelines for dealing with conflicting information. (h) Ethical considerations. A very important additional point is emphasized by Kostoff [190]: "Multiple technical experts should average out individual bias and subjectivity." Two blatant examples of lack of peer review properly done are, among others, discussed in ASK-FORCE (with some additions related to recent publications, all cited in the renewed blog:

- The case of Bt endotoxins supposedly affecting aquatic organisms by Rosi-Marshall et al. [191]

  See comments in ASK-FORCE blog No. 3 on Rosi-Marshall et al. 2007b: [178] (including also the latest publications of [192]. The study has been criticized heavily by [193] and [194], the main points of critique, summarized in a letter to the editor of PNAS [195]: No indication about the nature of Bt toxin, nor any data about its origin. Unscientific extrapolation from lab to field experiments, suppression of an important result of Fig. 3: low toxicity of normal Bt toxin levels for aquatic organisms etc. It is good to know that the authors of the original study admitted some mistakes and tuned down their alarmist interpretation in the first study:
- The case of the Austrian mice experiments supposedly affecting fertility after some generations [196]. After lots of public and scientific debate, which caused serious and unfounded damage to the image of Bt crops, the study results were distributed on hundreds of websites of GM crop opponents. But critique came up, and since there was no publication in a peer reviewed journal available, the rebuttals were not published in journals either. The whole bitter debate is summarized extensively in two ASK-FORCE blogs: [197].

  The subsequent official retraction done by the Austrian Government itself is hidden in an European Commission Health and Consumers Directorate-General Summary Record of the Standing Committee on the Food Chain and Animal Health from October 19, 2008: European Commission Health and Consumer Directorate-General, Summary Record of the Standing Committee on the Food Chain and Animal Health Held in Brussels October 19, 2008: http://ec.europa.eu/food/committees/regulatory/scfcah/modif_genet/sum_19102009_en.pdf

  See also the published comments of Ammann in [198]:

> Studies that look at non-obvious risks are a welcome addition to the literature, say critics, but poorly conducted studies do more harm than good. "It's just bad science," says Ammann. "There are a lot of scientists producing these studies in a very sloppy way. They bolster public fear yet do nothing to resolve conflicts or move the field forward". And:
>
> But the authors aren't to blame, says Klaus Ammann, emeritus professor at the University of Bern in Switzerland. They are merely the latest victims of what has become the political gerrymandering of science to bolster and support anti-GM sentiment in Europe. "The Austrian government had exhausted all legal avenues to ban cultivation of GM crops," Ammann says. "The Ministry of Health decided to avoid the peer-review process and announce study results at a conference, hide the data from scientists, and let the activists run amok with the help of uncritical media." Indeed, in the ensuing months the Austrian government has backpedaled. The Ministry of Health responded to a request to interview Zentek or other authors with the following: "We asked the scientists to reevaluate their statistical analysis. Additionally the external evaluation will soon be started. I kindly ask you to wait with your proposal until the reevaluation is completed." [198]

- The case of a review by Dona and Arvanitoyannis [199]. This review would never pass tests designed by Tang et al. [200], which can detect biased filtering of citations and words: According to Tang et al., it is important to distinguish between *subjectivity classification* retrieved from opinionated and factual statements, and combine it with a multiclass *sentiment classification*, and to get a better scale by using neutral training examples. An extensive scientific analysis on [199] has been placed in ASK-FORCE with critical comments: [201]

A caveat at the end of this paragraph on peer review is appropriate. Although it is in principle necessary to ask ethical questions, we should first concentrate on the scientific assessment of a professional peer review strictly following a factual agenda such as [168, 202] are demanding. Only then when this filter has been passed successfully, it is important to go into ethical and socioeconomic questions. But as often, it is the farmers and the market regulating efficiently, and – no surprise – they follow quite naturally socioeconomic principles. It is wrong to mix scientific and ethical questions as de Melo et al. and Interman et al. are asking for [203, 204], the result is then to accept for discussion a paper like the one of [205], which has been seriously and repetitiously criticized on a factual basis by EFSA [206–208]. Such papers should not be seen as a publication which takes also into account a "balanced view," because they are flawed in the first place. Papers from the laboratory of Séralini are then often cited as done by *independent* scientists, which is not very convincing, since digging into the financial support of Séralini and his CRIIGEN lab it is highly interesting to realize that they also receive funds which come from opponents of GMO technology, such as Sevene Pharma, commercializing homeopathic products which claim to detoxify various toxic products [209] and more. CRIIGEN has been created with the financial support of the retailer Carrefour, which has also contributed financially to certain studies of Séralini and his group. Interestingly enough, Carrefour, the second largest food distributor in the world, sells its own brand of "GMO-free" products. . .Source: [210].

- The result of this discussion: it will be necessary to call for new, Internet-based methods to create a more efficient peer review system. A nucleus of such a system is given in Ron LaPorte's supercourse system http://www.pitt.edu/□super1/.

GM- and Non-GM Crop Differences Overestimated, the "Genomic Misconception"

Early Phase of Risk Assessment In the wake of molecular breeding, in particular with the first successes of "gene splicing," the safety debates started soon after the discovery of the DNA structure by Watson & Crick [211–213], followed by the Asilomar Conference [214, 215] – see also some historical accounts [7, 216, 217]. The fascination about the novelty of transgenesis was justified, but also overwhelming, and the many unforeseen scientific breakthroughs following were unprecedented in the history of molecular biology. Unfortunately, the enthusiasm also lashed back in an overacting in risk assessment, when the first GM crops went into production. The debate on how GM crops should be regulated started very early with an emerging divide between regulation in the USA and Great Britain, including later the whole of Europe [218, 219]. Some more traces of early disputes about regulatory decisions in the USA and in Great Britain can be seen in letters to Nature in 1992: [220, 221]. Some support tighter regulation including field biosafety assessments, others fear strangulation of biotechnology research. During the wake of the Cartagena Biosafety Protocol most countries adopted (around 2003) the European way of risk analysis of genetic engineering, emphasizing process-oriented regulation and rejecting product-oriented regulation.

The seemingly absolute novelty of genetic engineering on the molecular level has been contested already in the early days of molecular biology in the 1930s and 1950s with the discovery of cellular systems for genome restructuring discovered with the classic papers of McClintock [222, 223] and with later commentaries of Fedoroff [224, 225], also summarized under "natural genetic engineering" [226, 227].

Molecular Processes Similar in Natural Mutation and Transgenesis Genetic engineering has been brought into evolutionary perspective of natural mutation by authorities such as Werner Arber: his view remains scientifically uncontested that molecular processes in transgenesis and natural mutation are basically similar [228–232]. In a recent paper, Werner Arber [19] reemphasized those similarities on a broader organismal and evolutionary basis; the abstract reads:

- By comparing strategies of genetic alterations introduced in genetic engineering with spontaneously occurring genetic variation, we have come to conclude that both processes depend on several distinct and specific molecular mechanisms. These mechanisms can

be attributed, with regard to their evolutionary impact, to three different strategies of genetic variation. These are local nucleotide sequence changes, intragenomic rearrangement of DNA segments and the acquisition of a foreign DNA segment by horizontal gene transfer. Both the strategies followed in genetic engineering and the amounts of DNA sequences thereby involved are identical to, or at least very comparable with, those involved in natural genetic variation.

Therefore, conjectural risks of genetic engineering must be of the same order as those for natural biological evolution and for conventional breeding methods. These risks are known to be quite low. There is no scientific reason to assume special long-term risks for GM crops.

For future agricultural developments, a road map is designed that can be expected to lead, by a combination of genetic engineering and conventional plant breeding, to crops that can insure food security and eliminate malnutrition and hunger for the entire human population on our planet. Public-private partnerships should be formed with the mission to reach the set goals in the coming decades. "from [19].

The same claim is made with a more organismic view by Hackett [233].

It is therefore no surprise that a natural transgene species has been discovered in a widespread grass genus [234]. An extensive overview on "natural transgenic organisms" is given in the excellent blog of David Tribe GMO pundit on natural transgenics: http://gmopundit2.blogspot.com/2005/12/collected-links-to-scientific.html.

Recent publications demonstrate that transgenesis, for example, has less impact on the transcriptome of the wheat grain than traditional breeding [235–237] (more details see [44, 238]).

One should also take into account that many of the conventional breeding methods such as colchicination [239, 240] and radiation mutation breeding [241] can be obviously more damaging to the genome, and it is, in addition, not possible to clearly define what impact the untargeted process could have caused. Or, on the other hand, as [242] have demonstrated, that irradiation-induced wheat – Aegilops biuncialis intergenomic translocations will facilitate the successful introgression of drought tolerance and other alien traits into bread wheat. In their review, [243] criticized the biased statements of [244, 245] who focus in an unjustified manner on transgenesis alone when describing unwelcome mutations. Still, it has to be admitted that repair mechanisms on the DNA level are powerful [246–248]. It is thus not logical that opposition within organic farming toward genetic engineering is now expanding also to some of those conventional breeding methods, some go even so far as to reject marker-assisted breeding – symptomatic for the organic agriculture scene, this trend is based on the myth of "intrinsic integrity of the genome" [249, 250], for which term it is not possible to find a proper scientific definition, which inevitably should be based on comparisons [44]. The addition of rejected breeding methods would ultimately lead to an absurd situation where most of the modern time traits would have to be rejected and breeding would be forced to virtually start from scratch.

Basically, many of the first-generation GM crops should be today subject to a professional debate on *deregulation*, and there is good and sturdy reason to state that many of these GM crops should not have been treated in such a special way in the first place, they can be compared in their risk potential to many crops created with traditional methods.

- This should not be misunderstood as a plea for general deregulation of GM crops, rather for a strictly science-based, risk-based regulation and clearly for a shift from process-based regulation toward product-based regulation.

Dissent over Differences Between GM- and non-GM Crops Causes Transatlantic Regulatory Divide This actually includes a critical questioning about some basic rules of the United Nations Convention on Biological Diversity (CBD). Transgenic crops of the first-generation should not have been *generally* subjected to regulation purely based on the *process* of transgenesis alone; rather it would have been wiser to have a close look at the *products* in each case, as John Maddox already proposed in 1992 in an editorial in Nature [251]. This is also the view of Canadian regulators [252–254], where the *novelty* of the crop is the primary trigger for regulation. This transatlantic contrast has been

commented by many [16, 218, 255–258], and although for many years a solution and mediation seemed to be too difficult, contrasts can be overcome:

In a letter to the executives of the Convention on Biological Diversity (CBD), the Public Research and Regulation Initiative (PRRI) http://www.pubresreg.org/index.php?option=com_docman&task=doc_download&gid=490 is asking for a scientific discussion in order to exempt a list of GM crops from the expensive regulatory process for approval, here is only the final statement:

- Bearing in mind that the **method of transformation itself is neutral**, *i.e.,* that there are no risks related to process of transformation, PRRI believes that there are several types of LMOs and traits for which - on the basis of the characteristics of the host plant, the functioning of the inserted genes and experience with the resulting GMO - **it can be concluded that they are as safe as its conventional counterpart with respect to potential effects on the environment, taking also into account human health**.

Unfortunately, there was no substantial reaction from the leading Cartagena organizers.

To be quite explicit once more, this does not mean to exempt transgenesis from biosafety assessment as a whole, but it should say that "several types of LMOs and traits, where the inserted genes demonstrate in large scale commercialization (of course after risk assessment done in due course) can be deemed as safe as conventional counterparts according to several years of beneficial agricultural practice, should be exempt under article 7.4 of the Cartagena Protocol for further expensive and time-consuming risk assessment and regulatory procedures. This motion has now officially been repeated by PRRI (Public Research and Regulation Initiative at the occasion of the COP10-MOP5 negotiations in Nagoya, Japan, see the interventions on the website www.pubresreg.org with recent additions.

In a recent paper, an indiscriminate continuation of food biosafety research is questioned on the basis of all the above arguments by Herman et al. [259] with good reason:

- Compositional studies comparing transgenic crops with non-transgenic crops are almost universally required by governmental regulatory bodies to support the safety assessment of new transgenic crops. Here we discuss the assumptions that led to this requirement and lay out **the theoretical and empirical evidence suggesting that such studies are no more necessary for evaluating the safety of transgenic crops than they are for traditionally bred crops.**

Perspectives for Solutions, a Synthesis of Divergent Views in 2.4

These new perspectives create hope that solutions can be found. Even within the difficult and for GMOs totally negative legal environment of the Cartagena Protocol, there are some slim possibilities:

In a first phase some of the widespread transgenic crops like transgenic maize with the Cry1Ab endotoxin could be exempt from regulation. This is indeed possible according to art. 7.4 in the Cartagena Protocol. In COP-MOP5 2010, in Japan (Fifth meeting of the Conference of the Parties serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety (COP-MOP 5), 11–15. 10. 2010 Nagoya, Japan http://bch.cbd.int/protocol/meetings/) it should be possible, to amend the protocol with the introduction of a dynamics which allows to start the regulatory process with an initial phase focusing on the process of transgenesis, first following procedures proposed for nontarget insects by [260, 261].

Indeed, in COP10-MOP5 in Nagoya October 2010, PRRI www.pubresreg.org has made a request for the exemption of widely adopted Bt maize crops of the endotoxin type of Cry1Ab, see the press release for the context (PRRI press release: http://www.pubresreg.org/index.php?option=com_docman&task=doc_download&gid=586), here the original text as read at the plenary meeting in Nagoya: PRRI Statement on exemptions MOP5: http://www.ask-force.org/web//PRRI-MOP5/PRRI-MOP5-statement-Strategic-Plan-delivered.pdf:

- Third, there is an underlying misperception that there are demonstrated cases of adverse effects. This is incorrect. Over the last 15 years GM crops have been planted over a billion hectares by tens of millions of farmers in the developing and developed world. These crops

have been grown in numerous different environments, and they have been consumed in billions of meals. The substantial scientific evidence accumulated shows that there are **no** verifiable reports of any adverse effect to environment or human health.

The Strategic plan includes an indicator "Number of reports to the BCH on the identification of LMOs or specific traits that may have adverse effects". Such an indicator makes little sense, because it is never possible to rule out that any organisms, LMO or non LMO, may have adverse effects. What is crucial is the question whether they are likely or unlikely to have adverse effects, and PRRI proposes that the strategic plan includes these two questions. PRRI is ready to submit examples of categories of LMOs of which the risk assessments and accumulated evidence indicate that they are unlikely to have more adverse effects on biodiversity or human health than their non modified counterparts, and that consequently those LMOs can be exempted from the AIA procedure on basis of article 7.4 of the Protocol.

In future, it should also be possible to shift eventually the focus on the product, making it possible to abbreviate the regulatory process wherever possible and feasible. The ultimate goal of new regulatory concepts should be to minimize obstacles for new and urgent necessities in crop development, such as Swaminathan and Raven are proposing [262, 263]. The author remains pessimistic, since the whole cumbersome process of legal changes in the Cartagena Protocol is also systematically hindered by a strong anti-GMO lobby, having made its way through the institutions to higher and powerful positions within the Cartagena administration quite successfully, starting from MOP1 all the way up through MOP5, thus influencing negatively all change of regulatory appeasement and lowering regulatory costs. Unfortunately, the recent overview of the European legislation on GM crops does not generate much optimism either: [264].

A second negative trend is triggered by a growing community of risk assessment researchers, who have a vested interest to keep the pot cooking, examples can be downloaded at the website of GENOK www.genok.com and also from the website of the Third World Network http://www.twnside.org.sg/ with its intricate mixture of activist statements and questionable and peer-reviewed scientific contributions. Other similar examples supporting this view can be downloaded over the Freiburger Oekoinstitut http://www.oeko.de/ and on the website of ENSSER, European Network of Scientists for Social and Environmental Responsibility http://www.ensser.org/

A conceptual framework is proposed by IFPRI/ISNAR in 2002, the International Service for National Agricultural Research [265]; a careful evaluation of process-based versus product-based triggers in regulatory action can also lead to a merger of both seemingly so contrasting concepts into a legalized decision-making process on which trigger should be chosen in a case-by-case strategy:

- Process-based triggers are the rule in almost all countries that have developed national biosafety regulatory systems; there are exceptions, however, where the novelty of the trait determines the extent of regulatory oversight and not the process by which the trait was introduced. While such a product-based approach to defining the object of regulation is truest to the scientific principle that biotechnology is not inherently more risky than other technologies that have a long and accepted history of application in agriculture and food production, it is less prescriptive than process-based regulatory systems.

Many of the debates on those two concepts suffer from a lack of clear-cut definitions, it will be important to have a close look at the Canadian regulatory system and the definition of PNTs (Plants with Novel Traits). In Canada, the trigger for risk assessment is the *novelty* of the plant rather than the *methods* used to produce it. The difficulties start there, where a clear definition of PNTs is needed to come to a decision. It means that plants produced using recombinant DNA techniques, chemical mutagenesis, cell fusion, cisgenics, or any other in vitro technique leading to a novel trait need to undergo risk assessment in the Canadian system. No wonder the Canadian definition of novel traits is rather wordy, but remains broad minded:

- A plant variety/genotype possessing characteristics that demonstrate neither familiarity nor substantial equivalence to those present in a distinct, stable population of a cultivated seed in Canada and that have

been intentionally selected, created or introduced into a population of that species through a specific genetic change.

Conclusions: There can be no doubt that product-based regulatory approaches are truest to the scientific principle that biotechnology is not inherently more risky than other technologies that have a long and accepted history of application in agriculture and food production, it is also less prescriptive than process-based systems, see for more details McLean et al. [265].

The Costs and Lost Benefits of Overregulation

The Issue

The Cartagena Protocol on Biosafety (CPB) has now been adopted by 157 parties http://www.cbd.int/biosafety/signinglist.shtml. It still builds on the principle that GM crop plants might bare risks in contrast to the conventional crops, objective of CPB: http://www.cbd.int/biosafety/articles.shtml?a=cpb-01. The huge apparatus on risk assessment based on this protocol is building on the principle that the mechanism of transgenicity is totally artificial and is not found in nature. Modern molecular science insights have proven the contrary, as shown in ASK-FORCE AF-9 [201] on the molecular basis of transgenesis. This results in maintaining the concept of an asymmetric risk assessment of innovation of GM crops. The possible exemption of widespread GM crops in Art. 7.4 (Cartagena Protocol on Biosafety, Article 7: http://www.cbd.int/biosafety/articles.shtml?a=cpb-07) is not even considered officially up to now.

Summary

An excellent summary graph is given in [266] in Fig. 10b: innovations active in the R&D pipeline were growing at an increasing rate during the period before 1998, but declined after 1998. Apart from competition of reasonably close nontransgenic substitutes, the authors consider one regulatory reason to be the main culprit: The halting of regulatory approvals in 1998 in Europe. Although the authors consider the full extent of reasons still to be conjectural, their data suggest that changes in regulatory environment may have been a cause. In a combination of high costs for lost implementation and high costs for regulatory approvals, the present state and operational experience has grown into a major obstacle of modern crop breeding (Fig. 11).

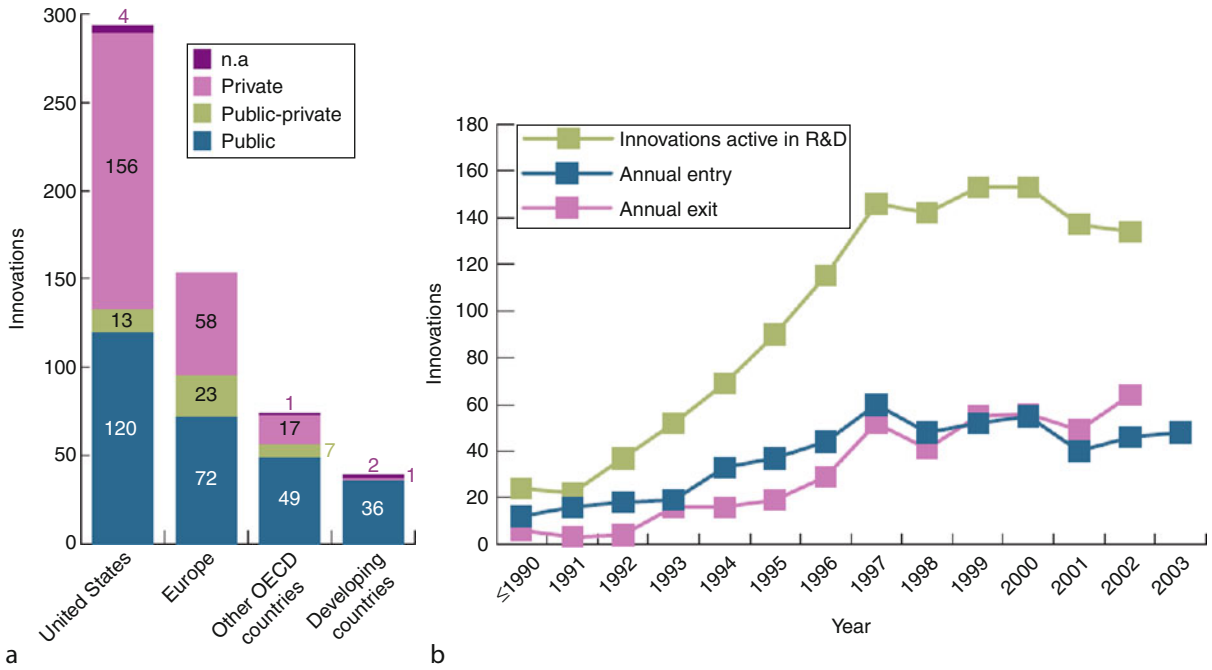
- Commentary from Table 1 in [266]: The primary survey combined records from scientific publications, field trial records, and regulatory filings to identify 558 transgenic plants with quality improvements and determine how far they had progressed through stages of R&D by 2004, including those that had only been published in the scientific literature; those that had reached initial field trials (defined as having completed 1–3 field trials), mid-stage field trials (4–9 field trials) or advanced field trials (>10); those that had entered regulatory filings; and those that were commercialized. The secondary survey canvassed expectations of firms and analysts about the likelihood and time frame for future commercialization of transgenic product quality innovations. Complete one-to-one correspondence between individual observations of the two surveys was not possible.

In a recent publication [267] document the same dramatic negative trend for specialty GM crops is demonstrated:

Costs and Lost Benefits Worldwide and Europe

An excellent summary graph is given in [266] in Fig. 6 above: innovations active in the R&D pipeline were growing at an increasing rate during the period before 1998, but declined after 1998. Apart from competition of reasonably close nontransgenic substitutes, the authors consider one regulatory reason to be the main culprit: The halting of regulatory approvals in 1998 in Europe. Although the authors consider the full extent of reasons still to be conjectural, their data suggest that changes in regulatory environment may have been a cause.

The full extent of the GM crop development pipeline can be evaluated in websites like the Information Systems for Biotechnology alone from the USA, there are (October 23, 2009) 14,204 notifications with 1,586 full field release permits registered in this Database, ISB: Information Systems of Biotechnology: Field Test Releases in the US: http://www.isb.vt.edu/cfdocs/fieldtests1.cfm



GM Crop Risk Debate, Science and Socioeconomics. Figure 10

Innovation in Ag-Biotech. (**a**) Location and sector of organizations conducting R&D for the 558 transgenic product quality innovations identified. Private sector consists of corporate and privately held firms. Public sector consists of government research laboratories, universities, and nonprofit research institutes. (**b**) Annual entry, exit, and the numbers of innovations active in the R&D pipeline were calculated from observations of the 558 innovations tracked in the primary survey. The number of active innovations stopped growing in 1998, after which those new innovations that entered were more likely to be published and less likely to move toward commercialization. Figure 1 from [266]

Overall, the present day regulatory regime detains public research in molecular breeding considerably due to enormously high regulation costs. More information about this effect on the development of GM trees is in Strauss and McLean [268, 269]; the abstract reads:

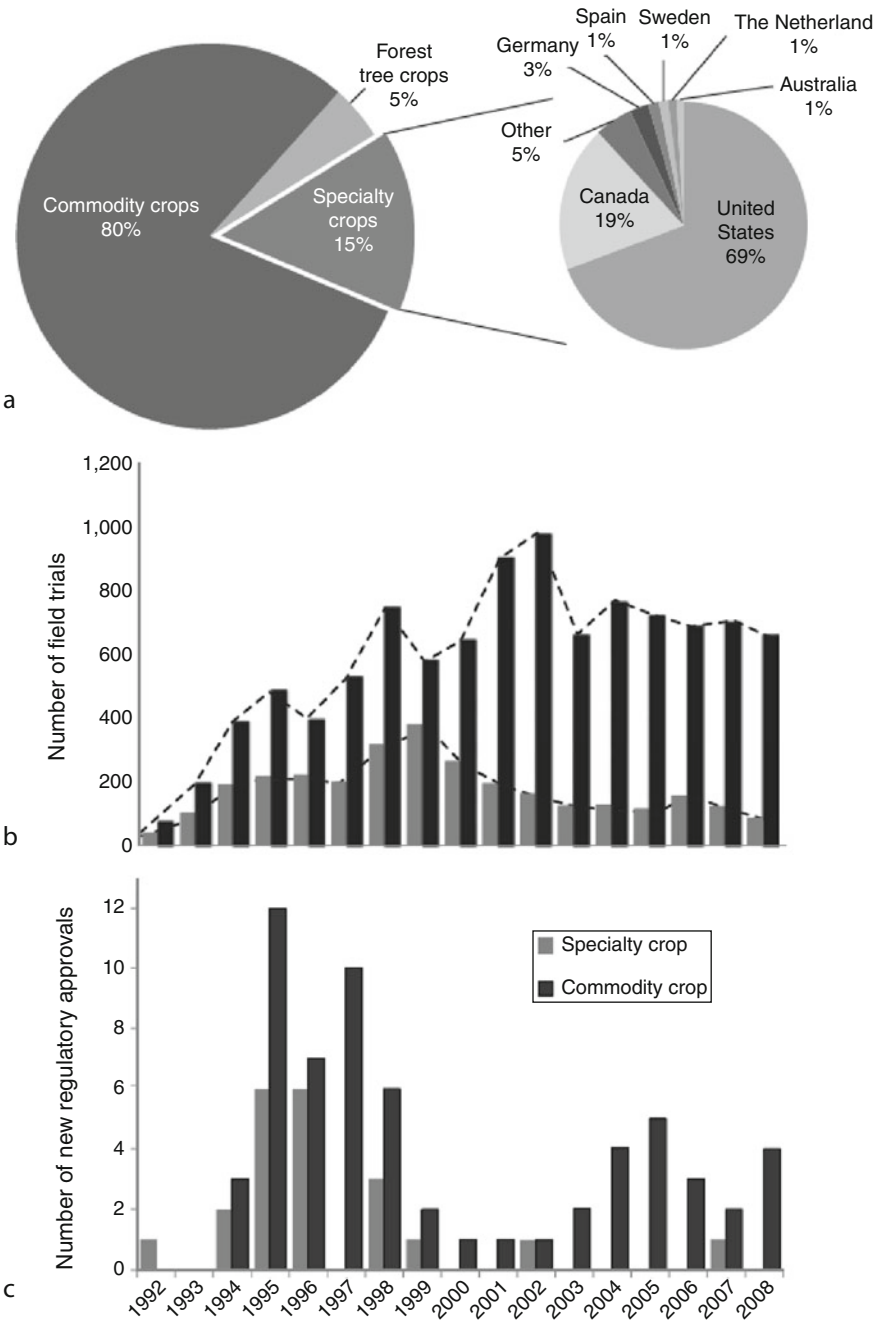
- **Against** the Cartagena Protocol and widespread scientific support for a case-by-case approach to regulation, the Convention on Biological Diversity has become a platform for imposing broad restrictions on research and development of all types of transgenic trees.

Some comprehensive tables on the massive costs of regulation of the major commodity crops are given by Kalaitzandonakes [270]. The compliance costs for herbicide tolerant maize alone have been calculated based on the events in 2006 for the USA. They amount to US $6,180,000–14,510,000 – a sum most likely to be prohibitive for any trait developed by a public institution.

Another case is reported by Piero Morandini from Italy. A scientific assessment on a field trial on Bt maize is delayed in publication by the Italian Government, although (or because?) it yields very positive results [271, 272].

- The grain yield data (tons/ha, GM crop *vs*. their conventional counterparts) were rather spectacular: 15.9 *vs*. 11.1 and 14.1 *vs*. 11.0, translating into a 43 and 28% yield increases for the P67 and Elgina, respectively. These data have already been released by the INRAN (National Institute for Research on Food and Nutrition, a research institution funded and run by the government) in 2006, albeit without the emphasis they deserved.

  The delay in properly communicating these data can be considered as a very costly omission. In fact, taking into account the total area of maize cultivation in Italy together with yield differences, maize prices



GM Crop Risk Debate, Science and Socioeconomics. Figure 11

Field trials and regulatory approvals. (**a**) Using the UNU-MERIT database, field trials conducted in 24 developed countries between 2003 and 2008 were separated on the basis of commodity, forest tree, or specialty crop. From this, the specialty crops were further subdivided based on the country in which the field trial was conducted. (**b**) The numbers of field trial permits acknowledged or issued in the USA are plotted by year for commodity crops and specialty crops. (**c**) The 84 unique transgenic events that have been granted regulatory approval by one or more countries are plotted by year of approval. If the year of approval varied among countries, the first year of regulatory approval granted by any agency for a given event was used. From [267]

and pest pressure, **these data translate into a forfeited value of between roughly € 300 million and € 1 billion a year because Italian farmers are not allowed to plant Bt maize.**

A summary of the Lombardia maize case has also been published in Nature Biotechnology [273]. Unfortunately, the original research report is still not published, it is "resting" in an Italian government drawer. . .

The present day regulatory "cropping apartheid" of high tech farming versus organic farming, large-scale farming against smallholders seriously hampers the development of GM crops, which could foster a more ecological production [44, 274] [275] and [276] – in short, Gene Peace instead of Greenpeace.

Costs and Lost Benefits in Developing Countries

Even more drastically, in the developing world, there is regulatory legislation in place hindering the development of transgenic crop breeding for the benefit of the poor, Driessen, Herring, Paarlberg [277–280].

Doubling agricultural research investment per se (no regulatory costs included in the calculation), would reduce poverty in Sub-Saharan Africa by 9% according to Alene & Coulibaly [281]. But these prospects are seriously hindered and as a result are practically nullified by the exorbitantly high regulatory costs during the implementation phase. Moreover, GM-free private standards set up by food companies and distributors in developed countries have influenced biosafety policymaking in developing countries: Gruère and Sengupta [282] found 29 cases where private importers have affected policy decisions in numerous countries due to irrational fear of export losses. This is based on two generally misleading premises: (1) Europe or Japan represents the only market for exports, and (2) non-GM segregation is too costly. It is amazing to realize, that many of the cases rely on unpublicized lobbying activities, and because of the lack of comprehensive evidence, many cases do not provide straightforward evidence of causality links between importers or traders and policy decisions. There is evidence that development of GM crops in Africa is mainly based on public research, and that the private sector only reluctantly invests in projects for developing countries, although the situation is getting better in the last few years [283, 284].

A blatant case of eco-imperialism is reported from Zambia by Andrew Apel in GMobelus: http://www.gmobelus.com/news.php?viewStory=234, where the Norwegian Government has partly sponsored a $400,000 laboratory, for which GENOK has contributed equipment and training, thus guaranteeing a research policy hostile to GM crops, in accordance with the official policy of the Zambian government, that characterizes GM crops as poisonous. The Norwegian GENOK is a well known anti-biotech NGO, with a very negative attitude toward GM crops, not shying away from spreading myths on allergy caused by pollen of transgenic maize in the Philippines; This is documented in the controversy between GENOK and Rick Roush: http://www.botanischergarten.ch/Allergy/Traavik-Roush-Philippines-controversy-2004.pdf, also supported in favor of Genok without a shred of evidence by John Vidal from the Guardian: http://www.guardian.co.uk/science/2004/feb/27/gm.science. Typically enough, the laboratory's priority will be to detect and search for genetically modified seeds and crops. Former Zambian researcher Ed. Rybicki, now working in Cape Town, said "that the lab would better serve Zambia and the whole region by looking at genuine threats, studying local biodiversity and even making transgenic crops themselves", as reported by SciDev Net http://www.scidev.net/en/news/zambia-s-molecular-biology-lab-fully-functioning-a.html?utm_source=link&utm_medium=rss&utm_campaign=en_news. Indeed, it is rather ironical that many of the biosafety educational efforts undertaken by organizations, highly critical to transgenesis, are turned into the "contrary": the biotechnological methods introduced in those countries are now also used for research and development of GM crops. A comprehensive report on agricultural biotechnology by Alhassan [285] demonstrates that high regulatory hurdles would hinder a reasonable development of modern agriculture in Africa.

Gruère and Smale [286, 287] report in a carefully calculated assessment that if rice cultures in India, Bangladesh, Indonesia, and the Philippines would be based on present day GM traits, the benefits amount to US$4,331 million. For the USA, an earlier assessment calculates similar sums of benefits related to the introduction of biotechnology in agriculture [288].

There has been much more written about regulatory costs and their negative follow-ups. Here only a small selection of important papers [130, 261, 289–294] is given.

The Golden Rice Development Hampered Through Overregulation. Biofortification as an Ideal Sustainable Way of Foreign Aid in Agriculture

In the case of the Golden Rice this tedious and costly regulation forced upon the regulatory authorities by the CBD solely based on the process of transgenesis has serious ethical consequences as documented in http://www.agbioworld.org/biotech-info/topics/goldenrice/index.html and in [270, 295]. A delay of the introduction of the biofortified rice is directly causing each year hundreds of thousands of children to die or to go blind due to severe vitamin A deficiency. Unreasonable and unscientific regulatory obstacles cause massive delay in approvals, especially in developing countries of S.E. Asia [296–311]. The initiator of the Golden Rice Ingo Potrykus project complains bitterly about the unjustified delays due to overregulation in a Nature article: [312].

Specifically related to the developing world, we should refrain from the old myths that international corporate companies are dominating the field – on the contrary Public Research is responsible for 85% of crop developments, 7% private local companies, and only 1% multinational companies according to figures from Cohen [284], supported by FAO statistics [313]. The myth that patenting rules are seriously hampering the spread of helpful biotech crops in poor countries has been seriously contested [314–316].

As an example, the Golden Rice project will result in biofortified rice traits, which will be distributed to the farmers free of royalties. The Asian farmers will also be able to multiply seeds without paying royalties. The homepage of the project is the main information source http://goldenrice.org/. More about the subject can be found in the important and comprehensive Handbook of Intellectual Property Rights of Krattiger et al. 2007 [317], and more: [318–321].

Biofortification programs are prone to get the highest index numbers in the evaluation system for foreign aid programs of Lempert [322]. Biofortification of indigenous landraces by systematically crossing-in the valuable and royalty free traits to enhance the nutritional value is certainly one of the best ways to sustainably help indigenous people suffering from any kind of malnutrition. In all cases known, the technology transfer is royalty free, secured by contracts.

Use of an indicator to assess the quality and success of developing aid projects defined by [322] reveals that most of the major NGO and UN actors in the field of development are actually providing relief rather than development and are creating dependency by treating symptoms rather than long-term solutions. The indicator points to the specific areas where they need to improve in order to fulfill sustainability criteria including tests of whether aid distorts financial markets and business competition, erodes appropriate government functions, and reverses colonial institutions and ideologies that interfere with sustainable consumption within a resource base.

Estimates in costs for vitamin A capsules are clearly incompatible with the living standard in developing countries; a major distribution campaign would result in millions of dollars. Neidecker-Gonzales [323] produced in their study the following figures:

- Total costs are lowest (roughly US$0.50 per capsule) in Africa, where wages and incomes are lowest, US$1 in developing countries in Asia, and US$1.50 in Latin America. Overall, this study derives a much higher global estimate of costs of around US$1 per capsule.

A bibliography of publications of the Golden Rice and Biofortification demonstrates the importance of this field of research; out of a general bibliography of 1,640 references a list of over 200 important papers is assembled: http://www.botanischergarten.ch/Golden-Rice/Bibliography-Golden-Rice-WOS-KA-20091008-links-abstracts.pdf.

It should be mentioned that biofortification strategies are also proposed for feed [324]. Straw from harvested crops can be adapted to higher feeding straw quality for cattle.

Conclusions drawn by Ingo Potrykus [325], the creator of the Golden Rice:

- The huge potential of plant biotechnology to produce more, and more nutritive, food for the poor will be lost, if GMO-regulation is not changed from

being driven by "extreme precaution" principles to being driven by "science-based" principles.

Changing societal attitudes, including the regulatory processes involved, is extremely important if we are to save biotechnology, in its broadest applications, for the poor, so that public institutions in developing as well as industrialized countries, can *harness its power for good.*

As a whole, the new, well-documented review paper of Adenle [326] delivers overwhelming evidence that GM crops are urgently needed in the developing world:

- The world needs fast and reliable solutions to fast growing population and the problems of hunger, malnutrition, ravaging diseases, poverty and global warming crisis. One of ideal technological innovations such as GM technology can be part of solutions to these problems. It is imperative to understand that GM technology cannot establish its ground if continuously faced with the baggage of constraints as discussed above. Moreover, it is not surprising to gather from a variety of literatures that most developing countries lack capacity building and still struggling with the establishment of biosafety system that can facilitate GM field trials and commercial release of GM products. Some of the challenges associated with the development of modern biotechnology still boil down to the fact that individual country government and international organisations have not clearly identified a coherent strategy and enabling policy instrument to deal with the problems. While some progress have been made on GM technology in terms of research and development, capacity building, and biosafety regulation in developed countries and a few developing countries, concerted effort is still needed to make it an accessible technology for every country. [326]

The Dispute Between Scientists and Opponents Today

The Role of Some Activist NGOs in the Debate

There is a continuous need for dialogue with regulators, the public, and specifically consumers, since the new technology emerging from modern life science is affecting all aspects of human life, including food, reproduction, etc. We do have an unfortunate trend toward irrational and antiscience argumentation in the GM crop dispute as clearly diagnosed by [327] in his book "The March of Unreason"), see also [328, 329].

This said, we should not create misunderstandings. There is no room for appeasement politics today when it comes to the activist NGOs like Greenpeace and Friends of the Earth, or websites like the Institute of Science in Society (I-SIS) and GM-Watch. Those professional organizations have proven repetitiously not to be interested in peer-reviewed science in a debate on the science and the sociocultural issues. They rather rely on unconfirmed reports in order to follow their own ideological and commercial interests. Any rational discourse with such organizations would be very welcome, but needs to be based on the latest peer-reviewed science. Their usual tactics is to appeal on fear. A good example from Greenpeace has been described on the EFB forum website http://www.efb-central.org/index.php/forums/viewthread/13/ about baseless accusations that 1,600 sheep have died from feeding Bt cotton leaves. A critique on the distorted picture on Indian cotton cultivation by NGOs is given by Herring [330] with lots of figures, facts, and extensive documentation.

Another blatant example of junk science has been launched recently by Greenpeace on You Tube "Genetic engineering: The world's greatest scam?" http://www.youtube.com/watch?v=1H9WZGKQeYg full of misinformation and hatred against multinational seed companies.

We are also confronted with violence – activities clearly documented and justifiably named and pursued as terrorism [331]. Also, in Europe, there are regularly occurring field destructions [332], which hamper seriously biosafety research – what an irony! Eco-terrorism is not confined to Europe, problems of such kind are very real also in the USA [333]:

According to the Federal Bureau of Investigation (FBI), the Earth Liberation Front, together with its sister organization, the Animal Liberation Front (ALF) has committed from 1997 to 2003 more than 600 criminal acts that have resulted in more than $43 million in damages. Moreover, attacks have been perpetrated in virtually every region of the USA against a wide variety of targets.

Recently, Greenpeace destroyed government field research in Australia [334] and defended the act of eco-terrorism with very thin arguments – and promptly lost lots of supporters and sympathy: Even

some old friends and supporters of Greenpeace (but not all) distanced themselves from the action: [335]. A list of field destruction actions in Europe has been compiled by Marcel Kuntz [336]. This list, far from being complete, demonstrates that activists have lost their moral compass in recent years: [337, 338].

One of the best rebuttals of cheap anti-GMO propaganda coming in attractive book editions, widely distributed in international events by the author Jeffrey Smith [339, 340] has been published on the Internet by Bruce Chassy http://academicsreview.org/reviewed-content/genetic-roulette/. It is actually a scientific comment, section-by-section, based on the best available peer-reviewed literature.

More chagrin emerges from the mounting pressure from within the academia, where, for instance, German university leaders in Giessen ordered to cease field research on GM crops which is unwelcome in the eyes of the extremists, [341] and there are serious complaints about the difficult atmosphere for biotech researchers in Germany [4].

Another symptomatic row is presently taking place in India, related to the approval of Bt brinjal, where activists are in a desperate attempt to stop the regulatory approval of Bt brinjal with outrageous and completely unfounded rumors like "GM brinjal will render the soil sterile," But contradictions have been posted as well: the most recent and comprehensive summary report published by Kameswara Rao [342], which is a review of massive evidence for the safety of Bt Brinjal and the detrimental heavy use of pesticides for the production of conventional Brinjal. It is ironic that one of the main arguments for proponents of the Bt Brinjal moratorium in India is now seriously questioned. There was the seemingly clear evidence on a crop biodiversity center for Brinjal in India, which called for extra protection of indigenous genomes. But recent extensive genomic analysis has clearly demonstrated that Brinjal is originating in Africa [343].

As an exemplary dispute, you can also follow the exchange of letters between the Public Research and Regulation Initiative (PRRI) and Friends of the Earth (FoE) [344]. Some of those anti-GMO activist groups get hefty funding from governments in the EU, as documented accurately by Andrew Apel and his GMobelus website: Europe's massive funding of world-wide activism. Compare also his recent article on the same subject, focusing on global aspects: [345].

The current set of arguments of GM crop opponents is often a mix of anti-American, antiglobal, postmodern, and even antiscience notions, [346], a strategy which has now been taken over very successfully by NGOs like Greenpeace and Friends of the Earth as global actors. These leading protest forces have helped, particularly in Europe, to build up a postmodern negative picture of biotechnology as a whole [347]. In this light, it is easy to act as "opinion leaders" with pseudoscientific arguments. The feedback mechanisms through the media and a network of citations of all the flawed stories make it possible for the global opponents to maintain confirmation of negation mechanisms. We are in a situation where the opponents already try to claim victory, penetrate highest political levels in governments and international organizations like the United Nations, some produce strikingly flawed reports on GM crops.

An analytical article about media and NGO activities in New Zealand has been published by Motion and Weaver [348]: by attracting media attention through dramatic protests, Greenpeace risks to jeopardize its reputation. The abstract reads:

> The challenges of attracting positive media attention are likened to a contest in which various organizations attempt to promote and circulate their version of events; however, this is particularly difficult when attempting to circulate less established, unpopular or critical knowledge. Although complying with, and managing, news values is an important starting point, the need to move beyond news values to consider the commercial values and realities of media organizations is highlighted. In this paper, a case study is undertaken of the Greenpeace media relations in New Zealand when a proposed controversial expiry of a moratorium to release genetically modified organisms into the environment. The predicament for Greenpeace is that in attracting media attention through dramatic protests it risks jeopardizing its reputation as a credible news source that can influence the framing of news stories. Insights are offered into the need for organizations to understand and manage the story or knowledge to be circulated and comply with contradictory news values.

Related to this paragraph on NGOs, it is necessary to write a word on the press: Newspapers and other media usually are mirroring what is important in the public debate, and the NGOs are clever in manipulating both the public and the press, after all, it is easy to provoke with fear and scaremongering, and the majority of journalists of all calibers are also committed to their own product, position, and its commercial situation.

A classic example is the coming and going of the Frankenfood Myth, see Fig. 3 and http://en.wikipedia.org/wiki/The_Frankenfood_Myth. Interestingly enough, this myth had its sharp peak in the press statistics around 1998 (see Fig. 3) and since then it has vanished from the headlines [104] as a major buzz word.

Those mechanisms have been precisely described by Burke for the situation in Great Britain some years ago [349]. But it is also clear that in the last 5 years more balanced voices appeared in the press, although there is no room to extend this topic here, just one recent example from the London Financial Times may suffice [350].

The GM Crop Battle, the Dispute Among Scientists, the Use of Strong Language

First, let us not forget some words of Antony Shelton [291], the most important words can translate into a slogan: "Quality of science must back up personal opinions," the abstract reads:

> In agricultural biotechnology there are roles and responsibilities of scientists, scientific journals, the public media, public agencies, and those who oppose or advocate a specific technology and serious consequences for science in general when those roles and responsibilities go awry. Scientists may feel the pressure of competition, especially in an academic setting. Personal views may continue to decide which issues one will work on, but the quality of science must back up those personal opinions. Common sense tells us that scientific inquiry and the publication and reporting of results to the scientific community and general population should be performed with high standards of ethical behavior, regardless of one's personal perspective on agricultural biotechnology.

One of the arising problems is that there has been recently a tendency to mollify peer review for the sake of politically correct so-called critical views of genetic modification of crops, with some blatant examples of flawed pseudocritical papers having passed for publication in highly respected scientific journals – a few examples have been commented by [351]. Some of those papers just passed due to flawed peer review, others passed despite rejection by some peer scientists, obviously for the sake of public debate (and for the promotion of the journal), see as an example the rather thin justifications of the editor in chief of Lancet Richard Horton to go ahead with the publication of Pusztai's rat experiments [352–356]. For more details about this controversy, see in ASK-FORCE on Pusztai [357], it is an anatomy of the case in 46 pages on the Pusztai affair, which had a big influence on the regulatory climate on GM crops in Great Britain and the world.

It is only between 2005 and 2011 that a certain fatigue of new negative arguments against GM crops is developing, and it is interesting to note that opponents, lacking real negative health and environmental effects, now shift their emphasis on negative arguments in socioeconomics. There are hardly any new issues in food safety and environmental impact to be dealt with in the last few years. This might also be the reason why in a desperate routine of repetitious "negative," GM crop stories get into journals, often also on rehashed events which have been clearly rebutted scientifically many years before. Those "news stories" often pass uncontested and get printed in "news" media due to a mix of short memory effects of uninformed editors and readers of all kind, or worse, they are purposefully repeated by activists counting on short memory of press and public.

A strange effect should also be mentioned that scientists, who defend good science in biosafety research, sometimes get blamed because they use straightforward language when criticizing flawed papers. A paper on such debates has been published by Nature [177], see the comments in a contribution of ASK-FORCE [178] on a paper on aquatic organisms supposedly harmed by Bt toxins of GM maize by Rosi-Marshall [191] and [192]. There are several controversial hints in this Nature story put forward by science journalist Emily Waltz, who is neither specialized nor experienced in the hot scientific regulatory debate on GM crops, suggesting that to criticize flawed papers

with "strong language" is detrimental to the progress of scientific research. This statement was supported by interviewed writers such as Ignazio Chapela (famous for starting the controversy of the Mexican gene flow of transgenic maize with a letter to Nature [358], which later turned out containing insufficient evidence for publication [359], see the latest summary in [360]. Another interview Waltz conducted in the cited *Nature* piece with David Schubert, who tries as a pharmacist to give advice in biosafety rules of GM food, and with his strong anti-corporate mood publishes fraud accusations against pro-GMO scientists [361]. Both interviewees Chapela and Schubert defend independent scientific whistle blowing, but themselves they have a proven negative agenda about GM crops, see more controversy papers: [295, 362, 363]. In the meanwhile, several letters to the editor of Nature have been written commenting the feature of Emily Waltz in Nature, they are all cited in [178], the majority is not supporting her thesis.

Incidentally: Strong language has been used before in the history of science, remember some really bitter and hefty disputes about the history of discovery of the double helix structure of DNA between Watson and Crick [216], who later made their peace again.

Other numerous examples of a fight out in the open are documented about evolution when Darwin proposed his revolutionary ideas. Two citations of strong language may suffice: in a debate on natural selection [364] writes on a dispute with William Bateson:

- By these admission almost the last shred of that teleological fustian with which Victorian philosophy loved to clothe the theory of evolution is destroyed. Those who would proclaim that whatever is right will be wise henceforth to base this faith frankly on the impregnable rock of superstition and to abstain from direct appeals to natural fact.

Another clear example of sharp and relentless scientific controversy on evolutionary biology with strong language has been described in detail by Strick [365], among the numerous juicy examples:

- His [Bastian's] tone was sharp in response to Huxley's public accusations that his technique was sloppy (a much more high-powered attack than Huxley ever adopted in private when attempting to correct young scientists). Huxley replied with an equally sharp tone, now saying sweepingly that "what Bastian got out of his tubes was exactly what he put into them," *i.e.* contaminants.

And one last word about strong language: The word "abuse" has been printed by Nature in the Battlefield paper [177] very prominently in the subtitle, when attacking a group of authors including me who criticize flawed papers in the GM crop debate with blunt, but still polite words – what an irony! – And to be quite clear, no complaints from my side. . . .

Negative Effects of Modern Breeding Methods in Food and Environmental Safety do (or Should) not Pass Strict Scientific Procedure Rules and Peer Review or They Are Based on an Unscientific Focusing on Transgenesis Instead on Management Mistakes

If researchers would follow strict procedural rules, the world of scientific biosafety debate would be far less complex, here are a few papers standing for such in fact uncontestable rules: [168, 260, 261, 267, 312, 366–369]. It is a fact that for some years basically no new arguments against agricultural biotechnology (in particular clearly related to transgenesis) on an agronomic base can be put forward for the most widespread crops, which have run through multiple regulatory processes in many countries.

This does not mean that transgenic crops are completely free of problems, but, in fact, it is that in comparison with conventional crop problems these are minor and manageable in a more efficient way. One of the basic mistakes of GM crop criticism is the unilateral focus on the risks of transgenes inserted, instead of comparing, in a fair and scientific (holistic!) way, with conventional cropping [370].

Still, a growing number of herbicide tolerant weeds are emerging: [371–374]. Powels [375] rightly points to the monotonous fields of glyphosate-resistant soybean landscapes, where the herbicide-tolerant weeds emerge more rapidly:

- Indeed, in spite of longterm use, the evolution of glyphosate-resistant weed populations in non-GRC, burndown systems has been very limited. Thus, functionally competent gene traits endowing glyphosate resistance are relatively rare and not easily enriched in

> plant populations [376], [377]. This is why glyphosate is a remarkably robust herbicide from a resistance avoidance viewpoint. However, as reviewed above, it is clear that, where there is very intense glyphosate selection without diversity, glyphosate resistant weed populations will evolve. In particular, the evolution of glyphosate-resistant weed populations is a looming threat in areas where transgenic glyphosate-resistant crops dominate the landscape and in which glyphosate selection is intense and without diversity. [375]

But it is also a fact that the emergence of glyphosate-resistant weeds is happening on a much slower pace than that of conventional herbicides [378].

Some critical science journalists question the strategies and behavior of the global opposition players. In a kind of last bid, questionable reviews are published, either containing lots of negative *assumptions* [379] or wrong toxico-analytical concepts resulting in an exaggerated risk assessment for nontarget insects as the lacewings as promoted by Hilbeck et al. [380–382] and contradicted clearly in Romeis [383]. Other examples of questionable eco-toxicological conclusions have been drawn by producing or reviewing flawed data or statistics, or drawing questionable conclusions, see the debate on Ermakova's flawed rat experiments: [384], more details in a contribution to the ASK-FORCE [385]. Typical other examples recognizable on filtered citation lists are Dona et al. and Séralini et al. [199, 386]. Séralini conducted his experiments in disrespect of the internationally approved rules of biosafety experiments established by the OECD [387, 388] and also avoided the citation of certain contradicting peer-reviewed references. Many of those papers have been or will be treated in ASK-FORCE [389], where you can read about new or recently updated ASK-FORCE contributions, for more details see section ASK-FORCE Organization and Related Websites.

It also must be said (remember Saner's statements at the beginning of this section) that vested interests can be spotted with some biosafety researchers, who are in need of research grants and thus paint a negative picture on biosafety; they symptomatically have difficulties to distinguish between the "nice-to knows" and the "need-to knows." Example: see the ASK-FORCE contribution [178] on the publication of [13], a paper which is flawed in several ways. It has been completely rebutted by Shelton et al. [14], the questions asked in the Lovei paper are irrelevant for Bt maize cultivation, since the Bt-toxin-technology is overwhelmingly beneficial for majority of nontarget insects [390–394]. One of the major flaws of the Lovei paper is that they used low quality prey for their laboratory feeding studies. A thorough analysis of risk assessment research has been recently published by Raybould [261]: We need to carefully distinguish between basic ecological research and purposeful and targeted risk assessment research which concentrates on the real agronomic risks and needs [395, 396].

The question and negative answer given in the letter of the Public Research and Regulation Initiative (PRRI) to the Secretariat of CBD [397] is fully justified, *and PRRI stands ready to expand on the points made in this letter.*

- 1. Are there LMOs or traits that have caused adverse effects?

  No. Since the first application of genetic modification in the 80s, many thousands of field trials have been conducted with GM organisms (to date mostly plants), and since 1996 many hundreds of millions of hectares have been planted with GM crops by many millions of farmers and consumed by hundreds of millions of consumers in developed and developing countries, without any verifiable reports of adverse effects on the environment or human or animal health.

  In fact, taking a broader look, experience with those GM crops has shown environmental and socio-economic benefits in terms of increases in yield, significant reductions in use of pesticides, fossil fuels and soil erosion, less mycotoxins in grains, as well as increased farmers health and income.

Final remarks: Coming back to the first statement of Saner [120] given under General Views on the Dialogue Related to Regulation of GM Crops, value-laden scientific activity cannot be avoided, but minimized – if you refrain to work with flawed data, with filtered citation lists, and with reviews pontificating on negative assumptions. The only remedy is to work with high-quality data produced in a methodologically transparent way following international agreement.

It is appropriate to end this rather pessimistic section with a positive note, not free of irony:

As Gupta [398] recently stated, there is hope that the introduction of strict biosafety rules in the Cartagena Protocol, originally aiming at a slowing down or even at stopping the transboundary movement (and indirectly development) of GM crops, now seems to turn into its contrary:

- Through analyzing the dynamics of GMO-related information disclosure to the global Biosafety Clearing House (BCH), I argue that the originally intended normative and procedural aims of disclosure in this case to facilitate a GMO-importing country's right to know and right to choose prior to trade in GMOs are not yet being realized, partly because the burden of BCH disclosure currently rests, ironically, on importing countries. As a result, BCH disclosure may even have market-facilitating rather than originally intended market-regulating effects with regard to GMO trade, turning on its head the intended aims of governance by disclosure.

Debate Improvements: What can we do to Enhance the Situation?

Foremost, it is important to *shift from pro-reactive to proactive mode*. This does not automatically mean to filter away negative views on GM crops and to organize a eulogy on the benefits, the pro-active mode should actually engage a new mode of debate, which is more discursive, more structured and definitely concentrates on a solution-oriented decision-making process. It is time for action – as far as a strict scientific view is allowing this. There are several websites working hard on sorting out the strictly science-oriented messages in biotechnology, as mentioned below. We should not, as it often happens, in our struggle against the negative pseudo facts focus on the risk alone and thus trap ourselves in a negativistic perspective.

Rather we should address in a balanced way the obvious (or lost) benefits as well. But this alone will not provoke a turnaround. This shift must be embedded in a discourse with concerned people and organizations and it must clearly oppose untruthful strategies of the global protest corporations and thus also refrain from using the same countertactics. One of the appropriate organizations for this activity will be the two platforms: (1) Public Research and Regulation Initiative PRRI www.pubresreg.org run by public researchers and (2) also the European Federation of Biotechnology http://www.efb-central.org/, so that public science will get a more important place in the international regulatory debate (but also where private seed companies are not fundamentally battled in a naïve neo-Marxist scheme). In many meetings strictly based on science and organized by PRRI, both platforms are well received. The project outline can be described as follows:

ASK-FORCE Organization and Related Websites

There is a flood of papers which cast doubt on the GM crops already regulated in many countries. Most (if not all) of these papers are written in a bad quality, either with flawed methodologies not internationally agreed upon, or with conclusions which are not supported by the data [13], rebutted by [14], details see in [178]. There are also many reviews published in a scientific style, but unfortunately either with a strongly biased set of references or with unsupported assumptions and doubtful conclusions – contradicted by peer-reviewed publications often not cited. In some cases, the flaws are more hidden: Experimental data are achieved on clearly theoretical schemes, working with outdated Bt maize and nontarget butterflies which have in their biology, in nature, no connection to maize fields: [399]. It is therefore important to set the record straight and to try to rebut at least the most important and blatant cases.

Within an EU project with Marc van Montagu and Piet van der Meer, which has been granted to PRRI, a blog was launched with the name ASK-FORCE on the PRRI website www.pubresreg.org with the secretarial help of Kim Meulenbroeks (until 2008) and presently Zuzana Kulikova. A list of about 130 items [400] has been compiled with international help and will be entered step-by-step in the grid of the following six sections. (1) General (2) Human and Animal Health (3) Environmental Safety (4) Agriculture (5) Public Perception (6) Developing Countries.

Up to now, 11 contributions have been published on the Internet; for links and contributions see [389]. These were reviewed by the experts of the steering committees of Public Research and Regulation Initiative and the European Federation of Biotechnology,

some also by the experts united in the blog community of AgBioWorld http://www.agbioworld.org/. All three lists contain some of the best specialists on green biotechnology from all around the world for reviewing and commenting.

In order to become more proactive, we need to develop forward-looking strategies. It is up to the scientists to ask questions to the opposition, and in particular to the professional distorters of the scientific facts. This must escalate into public campaigns if (what is to be expected) those specific questions are ignored. Carefully built contacts with science writers are important here, as a help for networkers a selected list is given here http://www.ask-force.org/web/ASK-FORCE-Summary/Contacts-ASK-FORCE-2011.pdf

Long-Term Discourse and Decision-Making Processes

Let me first be quite clear that I think a dialogue with the professional protest corporations is, as a rule, a waste of time (specifically Greenpeace and Friends of the Earth, not to mention some other organizations). Their only interest is to keep the pot cooking and make sure that the population remains in a state of fear. They should be addressed with a confrontational strategy, which is included in ASK-FORCE. Often such NGOs get the willful help of the press, which acts according to the old proverb (Macbeth, Shakespeare) "evil always fascinates – goodness rarely entertains" [401], see also the arguments produced by Andrew Moore [402]. While some press products concentrate on mirroring public concerns, a press more or less close to boulevard strives to foster its marketing with the help of sensational headlines, creating stories which sell better, but indirectly they are exacerbating the problems. We are also not going to talk about a special discourse, as described by Erjavec [403], related to the politics of the EU commission.

Nevertheless we have to address all segments of the public with its concerns, feelings, and interests. And the discourse we are going to concentrate on is solution oriented. This should be done according to the discursive rules of the management strategies of the second generation, the *Systems Approach* (see under The Second Generation Systems Approach as a New Decision Making Process). As a basic reference with description and citations, see the classic book of Churchman [79]. If we follow some ground rules, this should not be too complicated.

The Second-Generation Systems Approach as a New Decision-Making Process

Instead of making questionable concessions (example: "let's not talk about transgenic crops" as often done by Nestlé and Unilever, with notable exceptions [404] within these two companies!), the dialogue should be organized in an atmosphere of "Active Listening" [405] and understanding in which, apart from the strict rules of scientific argumentation we should send signals that the new technologies also trigger socioeconomic and cultural feedbacks. This will be the key to solve *Wicked Problems* [406], which contain also sociocultural elements besides a set of hard, often contradictory facts [122]. In his usual cynic precision, George Bernard Shaw defined the ultimate problem in the dialogue between scientists and lay people: "Every profession is a conspiracy against the laity."

The new discourse is not about the usual stakeholder meetings; rather it is about instigating modern planning processes of the second generation in evidence based but open ended decision-making processes. This *Systems Approach of the second generation* contrasts to linear planning with predetermined targets and dominating deontic thinking (e.g., of the industrial corporations and government agencies), it contrasts also to *the Systems Approach of the first generation* (e.g., Apollo moon landing with clear target).

The Rationale of New Management and Decision-Making Processes

> ▶ Some problems are so complex that you have to be highly intelligent and well informed just to be undecided about them. Laurence J. Peter [407]

These new strategies should dissolve the traditional stakeholder concept in favor of a much more efficient system respecting *different kinds of knowledge* and other rules (such knowledge differentiation is also known from learning processes, which are related to our decision-making dynamics [408].

There are more practical reasons to employ into the Systems Approach and its concept of different kinds of

knowledge, as Zwart [409] rightly emphasizes: Ever since we have realized that the low number of human genes (approximately, 22,500) cannot be interpreted as a narcissistic offence, since organisms are so highly complex, including the emerging consciousness of our human brain, genomics takes us now beyond a genetic deterministic understanding of life, this must have consequences on societal research and debate as well. Policies for self-improvement will increasingly rely on the use of complex interpretation. *Therefore, the emphasis in our discourse must shift from issues such as genetic manipulation and human enhancement to issues involved in governance of novel forms of information.* The same can be said on the side of agriculture. Ikerd [410] develops with the means of the systems approach a more holistic picture of agricultural management.

Fairclough [411] as a linguist gives an in-depth and critical analysis on discourse related to globalization with lots of facets, and again with a totally different set of terminology, he also presents negative examples of discourse. Objectivism treats globalization as simply objective fact, which discourse may either illuminate or obscure, represent or misrepresent. In the Churchman systems approach, there is no such thing as an objective approach, rather it is objectivation. Ideologism focuses upon how particular discourses of globalization systematically contribute to the legitimation of a particular global order which incorporates asymmetrical relations of power such as those between and within countries.

Scoones et al. [412] come to similar conclusions as the Churchman school, but this time related to agricultural policy, the paper explores the national and transnational character of mobilization against GM crops in India, South Africa, and Brazil in the 10-year period up to 2005. The paper argues for a better understanding of national political and economic contexts which must be taken into account, alongside on how the GM debates articulate with other foci for activism and the complex and often fragile nature of alliances that make up activist networks. It is important to understand that the debate about GM crops has become a much wider one: about the future of agriculture and small-scale farmers, about corporate control and property rights, and about the rules of global trade, see also the new report of the Royal Society [18]. In sum, a debate should not just focus on the pros and cons of a particular set of technologies – after all, they have proven safe – it is more about politics and values and the future of agrarian society. Again we see the plea for the complexity of "*wicked problems*" to be solved.

The downside is that those planning processes of the second-generation are time consuming and need a careful and tedious procedure in developing the most important and difficult *zero-step* – before such decision making can be started. It also implies an exchange of knowledge between the parties beforehand, in order to minimize *hidden agendas*. It also must be emphasized that those decision-making processes do not lead necessarily to a predefined goal, they are often *open-ended* and demand flexibility among the discourse participants, who need to remain open-minded.

The more questions we ask the more answers are possible and vice versa. Limitations of technological solutions are always hidden in the open ecological and social systems: Just compare the (in)famous case of DDT sprayings in the past [413–415]. Today, it is clear that with linear planning, DDT has been banned for ecological and health reasons, not considering the wider argument field of malaria prophylaxes. This inconsiderate DDT ban has caused millions of malaria deaths in Africa. Today, reasonable domestic use of DDT has again lowered the malaria threat measurably. Constraints in possible secondary effects in ecology should be examined carefully. This is well demonstrated in the case of the Monarch larvae being killed by Bt-Maize-Pollen, the result of a laboratory study published in Nature [416] where the subsequent press interpretation got way out of proportion – even though the author Losey himself warned about the limitations of this small lab study. Would researchers have asked the farmers, they would have been able to say that feeding time of the young larvae do rarely overlap with the time of pollen shed of maize, and that the plants the Monarchs are feeding upon are fiercely fought as a weed. Subsequent field studies revealed that there is no problem arising from extensive Bt maize planting for the Monarch larvae [12].

In order to tackle wicked problems, you need to go through *an extensive process of argumentation*, also called objectification, not to be mixed up with an "objective approach" to the problem.

There is rational planning, but there is no way to start to be rational: One should always start a step

earlier, since there are important trends and facts which will make straightforward rational thinking and acting in solving wicked problems useless. It is not the theory component, but rather the political component of the knowledge, which determines the vector of the action. This is the *zero-step* so important in the publications of Horst Rittel [121, 122].

As an example: The fact, that experts can be wrong and farmers know better in certain situations in agriculture because they are better observers out in the field and because they are very experienced in traditional knowledge [417].

The knowledge needed in solving wicked planning problems is not concentrated in a single head. It is absolutely essential to let all partners be involved in the problem solution process, which includes part of the population (mainly farmers' organizations and consumer organizations), the Governmental Regulators, the Non-Governmental Organisations, the Life Science Companies, and the Scientists. There is no monopoly of knowledge. Having illustrated the difficulties in solving wicked problems, we need a new approach in problem solving, in order to avoid the pitfalls of ignoring bottom up feedbacks.

You only can keep to this rule if you are also following another important rule. All partners in the planning process have to avoid hidden agendas, which is certainly eased by a minimum amount of respect paid to each other partner. Nobody should be criticized for speaking up in his own interest.

A caveat: It would be naive to just believe in the discursive capacities of the civil society, contrary to what Gerhards [418] has shown – that Habermas' support for the discursive model is based on the assumption that actors of the civil society argue much more discursively and on a higher level of rationality than other collective actors do. But empirical results show that actors of the civil society are, maybe, even less discursive than other actors.

It is primarily the paradox of rationality which has been severely underestimated in the systems approach of the first generation when tackling *wicked problems*.

How to Solve Wicked Problems in Biotechnology and the Environment What we need in such cases is an action-oriented approach. Risk Assessment and Management must be seen as a planning strategy of the second generation in developing a professional framework for *decision making*.

Strategies have to be developed to recognize the consequences of our doing on one side, and to specify our knowledge on the other side. This knowledge has to be gained step by step and case by case. If we want to clearly distinguish our present state knowledge from appropriate decisions to be made *not* based on our views and opinions, we need to go through the following steps:

- What is the problem?
- What do we want?
- What are the alternatives?
- How do we compare them?
- How can we reach the solution?

All participants need to keep in mind that there are *various types of planning knowledge* (arranged according to the five questions asked above).

Examples given here are lumped together as simple keyword illustrations, taken out of their context in real planning examples, and they cannot be regarded as an example of a realistic situation; this would be exactly the task of a planning process of the second generation.

Factual knowledge is the knowledge of what actually happens (quantitative data or empirical, observational data). Gene flow species by species/region by region/facts about insect resistance in agriculture.

- *Deontic Knowledge*, the very important knowledge of what ought to be. The knowledge about new crops which enhance agricultural production/new agricultural techniques to avoid erosion/new biological approaches to fight insect pests etc.
- *Explanatory Knowledge* explains why things are so or why certain effects will happen. Here, you already start to determine the direction of the solution. The way Bt proteins are acting on specific pest and beneficial insects/what are the main reasons of unwelcome erosion effects/mechanisms of vertical gene flow/mechanisms of resistance development.
- *Instrumental knowledge* on how to steer certain processes, on how to achieve certain goals, knowledge which needs to be balanced against regulation and safety. The way how to build Bt and other genes into crops and how to stabilize them/how to avoid

vertical gene flow/how to avoid unwelcome soil erosion/how to avoid early upcoming pest resistance.
- *Conceptual knowledge* which would allow avoiding conflicts before they pop up. This is the knowledge about complex situations, taking into account all previous kinds of knowledge and also weighing them against arguments coming from open ecological and societal systems. Concepts about transgenic crops compatible to the ideas of a sustainable agriculture. Lawyers and judges also may work with this kind of procedural knowledge.

You need to go through an *extensive, time-consuming process of argumentation*, also called objectification, not to be mixed up with an "objective approach" to the problem. The hopes of this process are:

- To forget less, to raise the right issue
- To look at the planning process as a sequence of events
- To stimulate doubt by raising questions, to avoid short-sighted explicitness
- To control the delegation of judgment. Experts have no absolute power; scientific knowledge is important, but always limited.

There is no such thing as "scientific planning."

- Solving practical problems as to develop sustainable transgenic crops cannot be dealt with by "scientification of planning." Dealing with wicked problems is always political because of its deontic premises (means that you have to involve knowledge what ought to be) and because we deal with traditional knowledge. Science only generates factual, instrumental, and in the best case explanatory knowledge.
- The planner (here the manager of an action plan) is not primarily an expert, but a *mid-wife of problem solving,* a teacher more than a doctor. Moderate optimism and careful seasoned disrespect, casting doubt is a virtue, not a disadvantage of an action plan manager.
- The planning process of wicked problems has to be understood as an *argumentative process*, it should be seen as a venture (or even *ad*venture) within a conspiracy framework, where one cannot anticipate all the consequences of plans.
- Systems methods of the *second generation* are trying to make this deliberation explicit, to support it and to find means in order to make this process more powerful and to get it under better control *for all participants.* Methods like the computer-based argument mapping systems of can be helpful [419].
- It helps making such processes more successful if they are conducted in the spirit of the *Symmetry of Ignorance* [420] – this is the secret of the active listening which often leads to acceptable outcomes and trust.

This seems to be a rather theoretical approach with lots of restrictive rules, but actually it is, on the contrary, an opening for much more freedom in dialogue. Also, it is more practical and efficient in creating results and contrasts with the traditional stakeholder concept where hidden agendas prevail in often disguised authoritarian structures. Such discursive processes are described in detail [80, 121–123, 421–425]. A comprehensive and voluminous monograph on risk-related debate methods has been published by Ortwin Renn [426], see especially the texts related to risk communication with essays 7 and 8 and section 8 on risk participation with numerous references, but notably lacking completely the papers on the "Systems Approach" of the Churchman/Rittel/Webber school.

In a French paper, the origin of negatively connoted words in the debate on GM crops like "contamination," "pollution," "Frankenfood," etc., Moirand [427] clearly reveals the links to negative events like BSE, dioxin scandals, and of course Tchernobyl, etc., thus explaining new words like "mad soya" and "mad colza" in the media. Moirand concludes that a new type of discourse is needed, but also Renn [426] does not refer to the very pragmatic and promising systems approach of Churchman and Rittel.

There are many more schools promoting discourse and new decision-making processes, also in specialized journals, only a few can be summarized here for space reason: [75, 76, 78, 84, 119, 120, 411, 427–441].

See Patrick Moore's practical examples of decision-making processes solving environmental and sustainability problems in forestry, consult his own website Green Spirit http://www.greenspirit.com/index.cfm. These processes need time. Patrick Moore [442–444] has gone successfully through such processes in the

difficult task of reconciliation between the needs of timber production and environmental constraint; he needed months of debate to come to reasonable decisions.

Another good example on how group discourses have good learning effects, has been described by Snyder et al. [258]: Although the US government has assured stakeholders of their safety, the EU continues to be an outspoken opponent. This can largely be attributed to a lack of trust in the regulatory process, and especially a cynical perspective on the underlying science and institutions that govern approval. Such disparities were illustrated in 2003 when the USA donated GM maize to aid African countries stricken by famine. Under purported EU threats, negative propaganda by NGOs, and stressing retaliatory trade sanctions, African officials refused the aid. An examination of this episode contrasts the potential discord between those affected and those who formulate government policy. Using resources from both sides of the debate, this scenario summarizes the pertinent issues regarding EU's refusal to the import of transgenic crops. A group discussion and debate protocol was developed for facilitating small group and entire class consideration of the scenario while strengthening student critical thinking skills.

It helps, if you prepare carefully scenarios before people start the process, a method which has been successfully applied to the reconciliation processes in South Africa after abolishing apartheid by Adam Kahane, one of the principal mediators [445]. He also followed another wise rule: Should only people participate in such processes who are part of the problem. Another excellent example of long-term discourse is described in many aspects by von Grebmer et al. [437]:

- By working collectively the process will be more open, transparent, inclusive and accountable, and sensitive to the normative dimensions of the issues critical to the participants. The themes and processes outlined in this article set the stage for the discussions, internally and between countries, that will shape the policies of agricultural biotechnology in the region. If the dialogue can frame the discussion and be enriched by the information generated from actions taken, it can sustain the interest and commitment of the stakeholders, and more successfully direct biotechnology toward reducing hunger and poverty in the region.

There are too many scientists remaining in the ivory tower, shying away from public debates. They fear losing their independence, a fear which is not just unfounded, but actually it is the contrary: remaining in the academic ivory tower means having lost your independence, since science is not an art per se, it is full of importance for society and humanity. A strong plea in this direction is coming from [446]. Although science should remain at the heart of invention and the drive to make our lives better, scientists should, instead of always having "the answers" ready, should not be afraid to engage in a contradictory evidence-based mode.

In one of the most successful examples of long-term discourse, the author participated as an invited expert in a public hearing in 2000. Strikingly, it was done without the theoretical load described above, but with lots of financial and logistic help from the New Zealand Government, in particular from the Royal Commission on Genetic Modification. A report was finalized after a 14-month inquiry into the risks and benefits of genetic modification. It heard from over 400 experts, including scientists, environmentalists, and ethical specialists. It considered more than 10,000 public submissions and heard the view of many others during a series of public meetings, hui, and workshops around New Zealand.

The Royal Commission's major conclusion was that New Zealand should proceed cautiously with genetic modification (GM) but not close the door to the opportunities offered by the new technology http://www.mfe.govt.nz/issues/organisms/index.html. The discourse is still continuing. Again, it is visible that the discourse is less confrontational and may lead to innovative solutions in the future [447]:

- The debate about genetic modification (GM) can be seen as characteristic of our time. Environmental groups, in challenging GM, are also challenging modernist faith in progress, and science and technology. In this paper we use the case of New Zealand's Royal Commission on Genetic Modification to explore the application of science discourses as used by environmental groups. We do this by situating the debate in the framework of modernity, discussing the use of science by environmental groups, and deconstructing the science discourses evident within environmental groups' submissions to the Commission. We find

science being called into question by the very movement that has relied on it to fight environmental issues for many years. The environmental groups are challenging the traditional boundaries of science, for although they use science they also present it as a culturally embedded activity with no greater epistemological authority than other knowledge systems. Their discourses, like that of the other main actors in the GM debate, are thus part of the constant renegotiation of the cultural construct of 'science'.

However, this process should not be mollified on the costs of hard science. The line between science and pseudoscience is often difficult to draw.

A Remark About the Psychology of the GMO Debate To be written in the next coming days.

It should also be possible to think and act in relation to the reconciliation of science and spirituality, since it will be an important element besides the ratio of science, the ethics of our societal activities, and the emotional elements in human life. But it will be difficult to separate the cheap esoteric chaff from the precious seeds of true spirituality, as Helmut Reich's writings demonstrate [448]. We must endeavor new fields of thought, as done by Papazova Ammann [449], a Bulgarian-born Swiss philosopher with roots in the schools of Muntjan and Rittel.

- What do we need as visionaries: Progress or Development? This is my question today, as I deal with the topic of Biovisionaries here in the Library of Alexandria. I ask this question because I am convinced that we need to build a new culture of questioning. We need a culture orienting itself by authentic questions. How can we develop taste and the ability to distinguish between those questions which are cognitive, statement-oriented and those which are authentic, close to life and to people? What is more important: cognizance or decision for action? How can we move between Statements and Questions? Statements reflect the need to understand the world. But they are the result of past experience and are often contained in frameworks which are coined by society. They may even protect old routines which hinder innovation. Questions, in contrast to statements, can transform our judgements and prejudices. Questions give birth to energy for new orientation, for a more conscious future. This orientation towards the future, towards vision provokes those choice-questions, and they alone will open the way for an urge to change the world. Visions need people who are free! The quality of freedom is inherent in the question. We must strive for this quality through choice-questions. If we cannot befriend these choice-questions with science, it will disengage from the questioners and will not be human science anymore. Thus we need a new humility of thinking – as it has been wonderfully defined by the German philosopher Heidegger: "The question is the devoutness of thinking".

Conclusions Only a multifaceted dialogue over a considerable time span will lead to success. The Internet scene is developing fast and new communication software tools are available now, so careful scrutiny for such a network of networks need to be done first, and the big players like Google and competing networks should be consulted as well.

Personal experience in dialogue with many networkers reveals that sometimes important networks are only known in specific clusters, these lacunas should be closed for many reasons – see section Illusions and Realities on Educational Effects in the Debate, the Dialogue Between Science and the Public. Knowledge exchange, jumping over national fences, and coordination will be a follow-up effect, without even declaring it to be the goal of such activity. As for now, this is just an idea and needs to be discussed with Internet and website specialists. After all, the leading webmasters and coordinators agree that it is time to *enhance collaboration through better communication.*

ASK-FORCE can contribute to this process in making sure that professional peer-reviewed risk assessment papers are fed into the dialogue processes and are ideally fed into a life decision–making process with relevant participants.

Bibliography

1. Beardmore JA (1997) Transgenics: autotransgenics and allotransgenics. Trans Res 6(1):107–108
2. Taverniers I et al (2008) Gene stacking in transgenic plants: towards compliance between definitions, terminology, and detection within the EU regulatory framework. Environ Biosaf Res 7(4). doi:10.1051/ebr:2008018

3. Potrykus I et al (2010) Transgenic plants for food security in the context of development, statement of the pontifical academy of sciences. New Biotechnol 27(5):443–717
4. Rauschen S (2009) German GM research – a personal account. Nat Biotech 27(4):318–319
5. Showalter E (1997) Hystories. Columbia University Press, New York, 244 pp
6. Linden A, Fenn J (2003) Understanding Gartner's hype cycles. In: Strategic Analysis Report. Gartner Research, p 12
7. Chassy BM (2007) The history and future of GMOs in food and agriculture. Cereal Foods World 52(4):169–172
8. Martineau B (2002) First fruit: the creation of the Flavr savr tomato and the birth of biotech food. McGraw-Hill, New York, 224 pp
9. Carrière Y, Crowder DW, Tabashnik BE (2010) Evolutionary ecology of insect adaptation to Bt crops. Blackwell, Oxford, pp 561–573
10. Ellstrand NC et al (2010) Crops gone wild: evolution of weeds and invasives from domesticated ancestors. Blackwell, Oxford, pp 494–504
11. Huang F, Andow DA, Buschman LL (2011) Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomol Experiment Et Appl 140(1):1–16
12. Gatehouse AMR, Ferry N, Raemaekers RJM (2002) The case of the monarch butterfly: a verdict is returned. Trends Genet 18(5):249–251
13. Lovei GL, Andow DA, Arpaia S (2009) Transgenic insecticidal crops and natural enemies: a detailed review of laboratory studies. Environ Entomol 38:293–306
14. Shelton A et al (2009) Setting the record straight: a rebuttal to an erroneous analysis on transgenic insecticidal crops and natural enemies. Trans Res 18(3):317–322
15. Moore GA (2002) Crossing the chasm. Harper Paperbacks, New York, Revised edition 20 Aug 2002, 256 pp
16. Thro AM (2004) Europe on transgenic crops: how public plant breeding and eco-transgenics can help in the transatlantic debate. Commentary. AgBioForum 7:142–148
17. Adams J (1995) Risk. Taylor & Francis, Bristol, 228 pp
18. Royal-Society (2009) Reaping the benefits: science and the sustainable intensification of global agriculture. In: RS Policy document 11/09. Royal Society, London, p 89
19. Arber W (2010) Genetic engineering compared to natural genetic variations. New Biotechnol 27(5):517–521
20. Britt AB, May GD (2003) Re-engineering plant gene targeting. Trends Plant Sci 8(2):90–95
21. Henderson IR, Jacobsen SE (2007) Epigenetic inheritance in plants. Nature 447(7143):418–424
22. Johnson L (2007) The genome strikes back: the evolutionary importance of defence against mobile elements. Evolut Biol 34(3):121–129
23. Moch K, Brauner R, Ott B (2005) Epigenetics, transgenic plants & risk assessment. In: Epigenetics, transgenic plants & risk assessment. 1st Dec 2005, Literaturhaus, Frankfurt am Main, Germany © 2006, Öko-Institut e.V., Box 50 02 40, D-791028 Freiburg, Die Deutsche Bibliothek – CIP Cataloguing-in-Publication-Data, A catalogue record for this publication is available from Die Deutsche Bibliothek
24. Smilde AK et al (2010) Dynamic metabolomic data analysis: a tutorial review. Metabolomics 6(1):3–17
25. Domon B, Aebersold R (2010) Options and considerations when selecting a quantitative proteomics strategy. Nat Biotechnol 28(7):710–721
26. Addona TA et al (2009) Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 27(7):633–U85
27. Wittenberg AHJ et al (2005) Validation of the high-throughput marker technology DArT using the model plant Arabidopsis thaliana. Molec Genet Genomics 274(1):30–39
28. Colbert T et al (2001) High-throughput screening for induced point mutations. Plant Physiol 126(2):480–484
29. Giddings VL (2006) "Cisgenic" as a product designation. Nat Biotech 24(11):1329–1329
30. Schouten HJ, Jacobsen E (2007) Are mutations in genetically modified plants dangerous?. J Biomed Biotechnol: 8261, p 2
31. Schouten HJ, Krens FA, Jacobsen E (2006) Do cisgenic plants warrant less stringent oversight? Nat Biotechnol 24(7): 753–753
32. Schouten HJ et al (2006) Cisgenic plants are similar to traditionally bred plants – International regulations for genetically modified organisms should be altered to exempt cisgenesis. Embo Reports 7(8):750–753
33. Jacobsen E, Nataraja KN (2008) Cisgenics – Facilitating the second green revolution in India by improved traditional plant breeding. Curr Sci 94(11):1365–1366
34. Jacobsen E, Schouten HJ (2007) Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends Biotechnol 25(5):219–223
35. Conner AJ et al (2007) Intragenic vectors for gene transfer without foreign DNA. Euphytica 154(3):341–353
36. Rigola D et al (2009) High-throughput detection of induced mutations and natural variation using keypoint (TM) technology. PLoS One 4(3):e4761
37. Parry MAJ et al (2009) Mutation discovery for crop improvement. J Exper Botany 60(10):2817–2825
38. Davies H, Bryan G, Taylor M (2008) Advances in functional genomics and genetic modification of potato. Potato Res 51(3):283–299
39. Townsend JA et al (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459(7245):442–445, advanced online publication
40. Shukla VK et al (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature 459(7245):437–441, advanced online publication
41. Cai C et al (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Molec Biol 69(6):699–709
42. Osakabe K, Osakabe Y, Toki S (2010) Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc Nat Acad Sci 107(26):12034–12039

43. Gabriel R et al (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotech 29(9):816–823
44. Ammann K (2008) Feature: integrated farming: why organic farmers should use transgenic crops. New Biotechnol 25(2):101–107
45. Mahfouz MM et al (2011) De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc Nat Acad Sci 108(6):2623–2628
46. CNBS (2011) Plant genomics "Molecular scissors" developed at KAUST. CNBS, PR newswire. DOI: http://www.cnbc.com/id/41731207/Plant_Genomics_Molecular_Scissors_Developed_at_KAUST and http://www.ask-force.org/web/Genomics/CNBC-Kaust-Genomic-Scissors-2011.PDF
47. Epinat JC et al (2003) A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. Nucl Acid Res 31(11):2952–2962
48. Paques F, Duchateau P (2007) Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. Curr Gene Ther 7(1):49–66
49. Silva G et al (2011) Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. Curr Gene Ther 11(1):11–27
50. Benner SA (2004) Understanding nucleic acids using synthetic chemistry. Acc Chem Res 37(10):784–797
51. Tian JD, Ma KS, Saaem I (2009) Advancing high-throughput gene synthesis technology. Molec Biosyst 5(7):714–722
52. Benner SA et al (1998) Redesigning nucleic acids. Pure Appl Chem 70(2):263–266
53. Benner SA, Sismour AM (2005) Synthetic biology. Nat Rev Genet 6(7):533–543
54. Rusch DB et al (2007) The sorcerer ii global ocean sampling expedition: Northwest Atlantic through Eastern tropical pacific. Plos Biol 5(3):398–431
55. Gibson DG et al (2008) Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. Science 319(5867):1215–1220
56. Gibson DG et al (2008) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic Mycoplasma genitalium genome. Proc Nat Acad Sci USA 105(51):20404–20409
57. Gibson DG et al (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. Science 329(5987):52–56. doi:10.1126/science.1190719
58. Bugl H et al (2007) DNA synthesis and biological security. Nat Biotechnol 25:627–629
59. Maurer SM, Lucas KV, Terrell S (2006) From understanding to action: community-based options for improving safety and security in synthetic biology, Draft 1.1, 15 April 2006. University of California, Berkeley, Californa, p 93
60. Serrano L (2007) Synthetic biology: promises and challenges. Mol Syst Biol 3:158
61. Edmond G, Mercer D (2009) Norms and irony in the biosciences: ameliorating critique in synthetic biology. Law Literat 21(3):445–470
62. Miller HI (2010) Understanding the frankenstein myth. Project Syndicate, a World of Idas. http://www.ask-force.org/web/Genomics/Miller-Understanding-Frankenstein-Tradition-2010.pdf
63. Tabashnik BE et al (2011) Efficacy of genetically modified Bt toxins against insects with different genetic mechanisms of resistance. Nat Biotech, advance online publication
64. Soberon M et al (2007) Engineering modified Bt toxins to counter insect resistance. Science 318(5856):1640–1642
65. Keller D (2009) Start talking to each other!' – dialogue as key to biotechnology's future in Europe. New Biotechnol 25:185, Corrected proof
66. Ramon D, Diamante A, Calvo MD (2008) Food biotechnology and education. Electron J Biotechnol 11(5)
67. Harms U (2002) Biotechnology education in schools. Electron J Biotechnol 5(3):205–211
68. Gensuisse (2011) Themenfocus. Gensuisse Website Forum 2011. Available from: http://www.gensuisse.ch/focus/index.html
69. Sengooba T et al (2009) Biosafety education relevant to genetically engineered crops for academic and non-academic stakeholders in East Africa. Electron J Biotechnol 12(1)
70. McHughen A (2007) Public perceptions of biotechnology. Biotechnol J 2(9):1105–1111
71. James C (2009) Global status of commercialized biotech/GM crops. Brief 39, Executive Summary. ISAAA, p 20
72. Sturgis P, Allum N (2004) Science in society: re-evaluating the deficit model of public attitudes. Public Understand Sci 13(1):55–74
73. Sturgis P, Cooper H, Fife-Schaw C (2005) Attitudes to biotechnology: estimating the opinions of a better-informed public. New Genet Soc 24(1):31–56
74. Sturgis P, Roberts C, Allum N (2005) A different take on the deliberative poll – Information, deliberation, and attitude constraint. Public Opin Quart 69(1):30–65
75. Gaskell G et al (2000) Biotechnology and the European public. Nat Biotechnol 18(9):935–938
76. Schuman H, Presser S (1980) Public-opinion and public ignorance – the fine line between attitudes and non-attitudes. Am J Sociol 85(5):1214–1225
77. Aerni P, Scholderer J, Ermen D (2011) How would Swiss consumers decide if they had freedom of choice? Evidence from a field study with organic, conventional and GM corn bread. Food Policy
78. Irwin A (2006) The politics of talk: coming to terms with the "new" scientific governance. Soc Stud Sci 36(2):299–320
79. Churchman CW (1979) The systems approach and its ennemies and (Commented German transl.: Der Systemansatz und seine "Feinde," with an Introduction by the editor and translator, Werner Ulrich, ed. and transl., Paul Haupt, Bern, 1981). Basic Books, New York
80. Rittel HWJ, Webber MR (2005) Dilemmas in a general theory of planning. Policy Sci 4(2):155–169
81. Rittel H (1992) Planen, entwerfen, design, ausgewählte schriften zu theorie und methodik. In: Reuter Wolf D (ed) Planen, entwerfen, design. Verlag W. Kohlhammer, Berlin, p 432

82. Protzen JP, Harris DW (2010) The universe of design: Horst Rittel's theories of design and planning, 1st edn. Routledge, London/New York, p 264, 19 June 2010
83. Magnan A (2003) Refeudalizing the public sphere: "Manipulated publicity" in the Canadian debate on GM foods. In: Annual meeting of the canadian-sociology-and-anthropology-association (CSAA). University of Alberta, Halifax, Canada
84. Vaughan E (1995) The significance of socioeconomic and ethnic diversity for the risk communication process. Risk Anal 15(2):169–180
85. Osseweijer P (2006) A new model for science communication that takes ethical considerations into account – The three-E model: entertainment, emotion and education. Sci Eng Ethics 12(4):591–593
86. Osseweijer P (2006) Imagine projects with a strong emotional appeal. Nature 444(7118):422–422
87. Osseweijer P (2006) A short history of talking biotech, fifteen years of iterative action research in institutionalising scientists' engagement in public communication. Vrije Universiteit, Amsterdam
88. Osseweijer P, Ammann K, Kinderlerer J (2010) Societal issues in industrial biotechnology. In: Soethaert W, Vandamme EJ (eds) Industrial biotechnology, sustainable growth and economic success, handbook. Wiley, VCH Verlag, Weinheim, pp 457–481, Chapter 14, 522 pp
89. Koutsogiannis D, Mitsikopoulou B (2004) The Internet as a glocal discourse environment – A commentary on "second language socialization in a bilingual chat room" by Wan Shun Eva Lam and "second language cyberhetoric: A study of Chinese L2 writers in an online usenet group" by Joel Bloch. Lang Learn Technol 8(3):83–89
90. Kostoff RN et al (2006) The structure and infrastructure of the global nanotechnology literature. J Nanoparticle Res 8(3–4):301–321
91. Kanter RM (2000) Are you ready to lead the e-cultural revolution? Inc 22(2):43–44
92. Bruns A (2008) Blogs. Wikipedia. Second life and beyond (digital formations). Peter Lang, Bern
93. Reifer D (2002) Ten deadly risks in internet and intranet software development. IEEE Software 19(2):12–14
94. Kalman ME et al (2002) Motivations to resolve communication dilemmas in database-mediated collaboration. Commun Res 29(2):125–154
95. Borland N, Wallace D (1999) Environmentally conscious product design: a collaborative internet-based modeling approach. J Indust Ecol 3(2–3):33–46
96. Dall'Olio GM et al (2011) Ten simple rules for getting help from online scientific communities. PLoS Comput Biol 7(9): e1002202
97. Degrassi G, Alexandrova N, Ripandelli D (2003) Databases on biotechnology and biosafety of GMOs. Environ Biosaf Res 2(3):145–160
98. Burns CG (2011) Biosafety resources on the Internet. J Int Wildlife Law & Policy http://www.jiwlp.com/ 20110801. Available from: http://www.jiwlp.com/contents/biosafety_resources_net.html
99. Ammann K (2011) List of websites related to GM crops and biotechnology. DOI: http://www.ask-force.org/web/Sustainability/Websites-List-Publ.def.pdf
100. Leydesdorff L (2002) Indicators of structural change in the dynamics of science: entropy statistics of the SCI journal citation reports. Scientometrics 53(1):131–159
101. Leydesdorff L (2008) Caveats for the use of citation indicators in research and journal evaluations. J Am Soc Inform Sci Technol 59(2):278–287
102. Leydesdorff L (2009) How are new citation-based journal indicators adding to the bibliometric toolbox? J Am Soc Inform Sci Technol 60(7):1327–1336
103. Leydesdorff L, Wagner C (2009) Macro-level indicators of the relations between research funding and research output. J Informet 3(4):353–362
104. Leydesdorff L, Hellsten I (2006) Measuring the meaning of words in contexts: An automated analysis of controversies about "Monarch butterflies", "Frankenfoods", and "stem cells". Scientometrics 67(2):231–258
105. Aizen J et al (2004) Traffic-based feedback on the web. Proc Nat Acad Sci USA 101(Suppl 1):5254–5260
106. Cavaller V (2009) Scientometrics and patent bibliometrics in RUL analysis: a new approach to valuation of intangible assets. Vine 39:80–91
107. Cavaller V, Aubertin C (2008) Elements of scientometrics and patent bibliometric-analysis for the estimated remaining useful life (RUL) in the valuation of intangible assets. In: Proceedings of the 5th international conference on intellectual capital and knowledge management and organisational learning, New York, pp 87–95
108. Laporte RE et al (2002) Papyrus to powerpoint (P 2 P): metamorphosis of scientific communication. Brit Med J 325(7378):1478–1481
109. Sa ER et al (2003) Open source model for global collaboration in higher education. Int J Med Inform 71(2–3): 165–165
110. Linkov F et al (2003) Globallsation of prevention education: a golden lecture. Lancet 362(9395):1586–1587
111. Linkov F, The I (2006) Intemet-based supercourse system. J Public Health Policy 27(4):442–443
112. Laporte RE et al (2002) Infopoints – Whisking research into the classroom. Brit Med J 324(7329):99–99
113. Laporte RE et al (2006) A scientific supercourse. Science 312(5773):526–526
114. Sauer F, Bennett S, Cha M, Linkov F, LaPorte R (2010) Supercourse, Bibliotheca Alexandrina, and the educator as catalyst. Educause Quart 33(3)
115. Ammann K (2011) Presentations for conferences etc. with powerpoint slides. In Audio-Visual Material 20110904, Ammann K, Neuchatel
116. Adly N (2009) Bibliotheca alexandrina: a digital revival. Educause Rev 44(6):8–9
117. Craig W et al (2008) An overview of general features of risk assessments of genetically modified crops. Euphytica 164(3):853–880

118. Leicht EA, Newman MEJ (2008) Community structure in directed networks. Phys Rev Lett 100(11):118703
119. Newman MEJ (2003) The structure and function of complex networks. Siam Rev 45:167–256
120. Saner M (2007) A map of the interface between science & policy, staff papers. Council of Canadian Academies, Ottawa, p 15
121. Rith C, Dubberly H (2007) Horst W. J. Rittel's writings on design: select annotations. Des Issues 23(1):75–77
122. Rittel H, Weber M (1973) Dilemmas in a general theory of planning. Policy Sci 4:155–169
123. Ammann K, Papazova Ammann B (2004) Factors influencing public policy development in agricultural biotechnology. In: Shantaram S (ed) Risk assessment of transgenic crops. Wiley, Hoboken, p 1552
124. Gasson M, Burke D (2001) Scientific perspectives on regulating the safety of genetically modified foods. Nat Rev Genet 2(3):217–222
125. Phillips PWB (2003) Traceability and trade of genetically modified food. Biotechnol Sci Soc Crossroad 5:141–154
126. Sheehy RE, Kramer M, Hiatt WR (1988) Reduction of polygalacturonase activity in tomato fruit by antisense rna. Proc Nat Acad Sci USA 85(23):8805–8809
127. Redenbaugh K et al (1994) Regulatory Assessment of the Flavr-savr tomato. Trends Food Sci Technol 5(4):105–110
128. Kramer MG, Redenbaugh K (1994) Commercialization of a tomato with an antisense polygalacturonase gene – the Flavr Savr(Tm) tomato story. Euphytica 79(3):293–297
129. Krieger EK et al (2008) The Flavr Savr tomato, an early example of RNAi technology. Hortscience 43(3):962–964
130. Graff G, Zilberman D (2004) Explaining Europe's resistance to agricultural biotechnology. Agric Resour Econ 7(5):4
131. Lawrence F (2009) It is too late to shut the door on GM foods consumers said no to the GM farming giants a decade ago, but that didn't stop millions of tonnes of their soya entering the food chain, in The Guardian. The Guardian and Observer, London
132. Flachowsky G et al (2007) Studies on feeds from genetically modified plants (GMP) – Contributions to nutritional and safety assessment. Anim Feed Sci Technol 133(1–2):2–30
133. Aumaitre A (2004) Safety assessment and feeding value for pigs, poultry and ruminant animals of pest protected (Bt) plants and herbicide tolerant (glyphosate, glufosinate) plants: interpretation of experimental results observed worldwide on GM plants. Italian J Anim Sci 3(2):107–121
134. Paarlberg R (2006) Are genetically modified (GM) crops a commercial risk for Africa? Int J Technol Globalisation 2(1–2):81–92
135. Cohen JI, Paarlberg R (2002) Explaining restricted approval and availability of GM crops in developing countries. AgBiotechNet 4:1–6
136. Gruere GP, Carter CA, Farzin YH (2008) What labelling policy for consumer choice? The case of genetically modified food in Canada and Europe. Canad J Econom-Revue Canadienne D Economique 41(4):1472–1497
137. Gruere GP, Rosegrant MW (2008) Assessing the implementation effects of the biosafety protocol's proposed stringent information requirements for genetically modified commodities in countries of the Asia Pacific economic cooperation. Rev Agric Econom 30(2):214–232
138. Gruere GP, Sengupta S (2009) Biosafety decisions and perceived commercial risks, The role of GM-free private standards. In: IFPRI Discussion Paper 00847, Environment and Production Technology Division. FPRI, Washington DC, p 40
139. Greenpeace (2007) Contamination Report 2006, annual review of cases of contamination, illegal planting and negative side effects of genetically modified organisms. Greenpeace International, Amsterdam, p 24
140. Greenpeace (2008) Contamination Report 2007, annual review of cases of contamination, illegal planting and negative side effects of genetically modified organisms. Greenpeace International, Amsterdam, p 48
141. ISAAA (2011) Cotton (*Gossypium hirsutum l.*) events. ISAAA 2011 11. Oct 2011. Available from: http://www.isaaa.org/gmapprovaldatabase/cropevents/default.asp?CropID=6
142. Sadashivappa P, Qaim M (2009) Bt cotton in India: development of benefits and the role of government seed price interventions. AgBioForum 12:172–183
143. Mueller-Jung J (2007) Wie verpackt man eine Kulturrevolution in Watte? How to wrap up a cultural revolution in cotton wool?. In: Frankfurter Allgemeine Zeitung. Frankfurt. p N1
144. Gruere G, Methta-Bhatt P, Sengupta D (2008) Bt cotton and farmer suicides in India, reviewing the evidence. IFPRI-Discussion Paper 2008, 00808
145. Gruere G, Sengupta D (2011) Bt cotton and farmer suicides in India: an evidence-based assessment. J Develop Stud 47(2):316–337
146. Shiva V (2004) The suicide economy of corporate globalisation. Z Net – The spirit of resistance lives 2004. Available from: http://www.zcommunications.org/the-suicide-economy-of-corporate-globalisation-byvandana2-shiva
147. Sunilkumar G et al (2006) From the cover: engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proc Natl Acad Sci 103(48):18054–18059. doi:10.1073/pnas.0605389103
148. Choudhary B, Gaur K (2011) Bt cotton in India, a multipurpose crop. In: ISAA (ed) Celebrating 10 years. International Service for the Aquisition of Agri-Biotech Applications, Biotech Information Center, New Delhi, p 6
149. Graff G, Hochman G, Zilberman D (2009) The political economy of agricultural biotechnology policies. AgBioForum 12:1–13, http://www.agbioforum.org/v12n1/v12n1a04-graff.htm and http://www.botanischergarten.ch/Regulation/Graff-Political-Economy-Policies-2009.pdf
150. Ayal S, Hochman G (2009) Ignorance or integration: the cognitive processes underlying choice behavior. J Behav Decis Mak 22(4):455–474
151. Ministerio da Ciencia e Technologia (2005) CTN Bio, Biosafety Law Nº 11.105, of 24 March 2005. Ministerio da Ciencia e Technologia, Brazilia, p 17

152. European Parliament and European Council (2003) Regulation (EC) No 1829/2003. Off J Euro Union L 268(1):1–23, 20030922
153. The European parliament and the council of the European union (2010) EU-Regulation-GMO-free regions, GMOs: Member states to be given full responsibility on cultivation in their territories, IP/10/921. 20100713, The European parliament and the council of the European union, Brussels, p 2
154. James C (2009) Global status of commercialized biotech/GM Crops. In: ISAA (ed) AAA briefs. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca
155. Galvao A (2010) Celeres, Biotechnology Report 2010. 20100809, Uberlandia, Matto Grosso, Celeres, p 7
156. Marques R, Neto CGA (2007) The Brazilian system of innovation in biotechnology: a preliminary study. J Technol Manag Innov 2(1):55–63
157. Mendonca-Hagler L et al (2008) Trends in biotechnology and biosafety in Brazil. Environ Biosaf Res 7(3):115–121
158. Silveira JM, Ferreira J, Dal Poz ME, Alssad A (2004) Biotecnologia e recursos genéticos: desafios e oportunidades para o Brasil/Biotechnology and genetic resources: challenges and opportunities for Brazil. Campinas Instituto de Economia, Rio de Janeiro, p 412
159. Frohme M et al (2000) Mapping analysis of the Xylella fastidiosa genome. Nucl Acids Res 28(16):3100–3104
160. Simpson AJG et al (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406(6792):151–157
161. Vasconcelos A (2003) The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. Proc Nat Acad Sci USA 100(20): 11660–11665
162. Magnani GS et al (2010) Diversity of endophytic bacteria in Brazilian sugarcane. Genet Molec Res 9(1):250–258
163. Mendes R et al (2007) Diversity of cultivated endophytic bacteria from sugarcane: Genetic and biochemical characterization of *Burkholderia cepacia* complex isolates. Appl Environ Microbiol 73(22):7259–7267
164. Editorial N (2010) Brazil's biotech boom. Nature 466(7304): 295–295
165. Bonfim K et al (2007) RNAi-mediated resistance to bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). Molec Plant Microbe Interact 20(6):717–726
166. Oda L (2011) Approval of Brazilian transgenic beans has social importance, says ANBio in the Sacramento Bee. PRN Newswire, AnBio, Sao Paulo, p 2
167. Paganelli A et al (2010) Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem Res Toxicol
168. Chassy B, Parrott W (2009) Is this study believable? Examples from animal studies with GM foods. Agric Biotechnol 9. doi: http://www.agribiotech.info/details and http://www.botanischergarten.ch/Peer-Review/Chassy-Parrott-Believable-2009.doc
169. Antoniou M et al (2010) GM Soy, sustainable?, responsible?, GV-SOJA, Nachhaltig? Verantwortungsbewusst? German, p 11
170. Martin-Orue SM et al (2002) Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. Brit J Nutr 87(6):533–542
171. Netherwood T et al (2004) Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. Nat Biotechnol 22(2):204–209
172. Netherwood T et al (1999) Gene transfer in the gastrointestinal tract. Appl Environ Microbiol 65(11):5139–5141
173. Zhang L et al (2011) Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res
174. GMwatch (20110921) We incorporate genetic information from the food we eat – new study GMwatch website. http://www.gmwatch.org, DOI: http://www.gmwatch.org/index.php?option=com_content&view=article&id=13423:we-incorporate-genetic-information-from-the-food-we-eat-new-study
175. Auer C, Frederick R (2009) Crop improvement using small RNAs: applications and predictive ecological risk assessments. Trends Biotechnol 27(11):644–651
176. Fransen R (2007) Peer review: too much of a good thing? Scientist 21(9):18–18
177. Waltz E (2009) Battlefield, papers suggesting that biotech crops might harm the environment attract a hail of abuse from other scientists, News feature. Nature 461:27–32
178. Ammann K (2011) Review: is the impact of Bt maize on non-target insects significantly negative?. ASK-FORCE contribution AF-8 *AF-8*, 23, 20111002, DOI: http://www.ask-force.org/web/AF-8-Lovei/AF-8-Lovei-Non-Target-20111002-opensource.pdf
179. Reiss T, Lacasa ID (2007) Benchmarking national biotechnology policy across Europe: a systems approach using quantitative and qualitative indicators. Res Eval 16(4):331–339
180. Linkov F, Lovalekar M, LaPorte R (2006) Scientific journals are "faith based": is there science behind peer review? J Roy Soc Med 99(12):596–598
181. Lubchenco J (1998) Entering the century of the environment: a new social contract for science. Science 279(5350):491–497
182. Linkov F, Lovalekar M, LaPorte R (2007) Quality control of epidemiological lectures online: scientific evaluation of peer review. Croatian Med J 48(2):249–255
183. Ammann K (2007) Evaluations faculty of 1000, manuscript and links. p 3
184. Hansen M (2009) Statement from Michael Hansen, CEO of Elsevier's health sciences division, regarding Australia based sponsored journal practices between 2000 and 2005. Elsevier Website 7 May 2009, doi: http://www.elsevier.com/wps/find/authored_newsitem.cws_home/companynews05_01203 and http://www.ask-force.org/web/Peer-Review/Hansen-Statement-ELSEVIER-2009.pdf
185. Goldacre B (2009) Peer review is flawed but the best we've got. In: The Guardian.
186. Smith R (2005) Medical journals are an extension of the marketing arm of pharmaceutical companies. Plos Med 2(5):364–366

187. Smith R (2003) Medical journals and pharmaceutical companies: uneasy bedfellows. Brit Med J 326(7400):1202–1205
188. Scott A (2007) Peer review and the relevance of science. Futures 39(7):827–845
189. Graff GD, Newcomb J (2003) Agricultural biotechnology at the crossroads, Part 1: the changing structure of the industry. Bio-era, p 26
190. Kostoff R (2002) Citation analysis of research performer quality. Scientometrics 53(1):49–71
191. Rosi-Marshall EJ et al (2007) Toxins in transgenic crop byproducts may affect headwater stream ecosystems. Proc Nat Acad Sci USA 104:16204–16208
192. Tank JL et al (2010) Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. Proc Nat Acad Sci
193. Beachy RN et al (2008) The burden of proof: a response to Rosi-Marshall et al. Proc Nat Acad Sci 105:16204–16208
194. Parrott W (2008) Study of Bt impact on caddisflies overstates its conclusions: response to Rosi-Marshall et al. Proc Nat Acad Sci 105: E10
195. McHughen A et al (2007) Letter to the editor of PNAS, related to the publication of Rosi-Marshall, E. PNAS, Washington
196. Velimirov A et al (2008) Biological effects of transgenic maize NK603xMON810 fed in long term reproduction studies in mice, Report, in Forschungsberichte der Sektion IV Band 3/2008, Bundesministerium für Gesundheit Familie und Jugend Sektion IV (ed) Herausgeber, Medieninhaber und Hersteller: Bundesministerium für Gesundheit, Familie und Jugend, Sektion IV Radetzkystraße 2, 1031 Wien, p 109
197. Ammann K (20100407) Review: the Austrian experiment with mice fed with a hybrid GM maize from Monsanto, Part 1: background and Part 2: experiment. ASK-FORCE contribution AF-5 **AF-5**, Experiment: 19p and Background 8p doi: http://www.ask-force.org/web/AF-5-Austrian-Micestudy/AF-5-Austrian-Experiment-20100407-opensource.pdf, http://www.ask-force.org/web/AF-5-Austrian-Micestudy/AF-5-Austrian-Experiment-20100407-web.doc, http://www.ask-force.org/web/AF-5-Austrian-Micestudy/AF-5-Austrian-Exp-Background-20090807-opensource.pdf, http://www.ask-force.org/web/AF-5-Austrian-Micestudy/AF-5-Austrian-Exp-Background-20090828-web.pdf
198. Sinha G (2009) Up in arms. Nat Biotechnol 27(7):592–594
199. Dona A, Arvanitoyannis IS (2009) Health risks of genetically modified foods. Crit Rev Food Sci Nutr 49(2):164–175
200. Tang H, Tan S, Cheng X (2009) A survey on sentiment detection of reviews. Exp Syst Appl 36:10760–10773
201. Ammann K (20090828) Review: genomic misconception of transgenesis, The difference between GM- and non-GM-crops on the level of molecular processes has been overestimated. ASK-FORCE contribution AF-7 AF-7, 57 DOI: http://www.ask-force.org/web/AF-7-Dona-rebuttal/AF-7-Dona-20090828-opensource.pdf
202. Chassy BM (2009) Global regulation of transgenic crops. In: Kriz AL, Larkins BA (eds) Molecular genetic approaches to maize improvement. Springer, Berlin, pp 107–124
203. Intemann KK, de Melo-Martin I (2008) Regulating scientific research: should scientists be left alone? Faseb J 22(3):654–658
204. de Melo-Martin I, Meghani Z (2008) Beyond risk – A more realistic risk - benefit analysis of agricultural biotechnologies. Embo Reports 9(4):302–306
205. Seralini GE, Cellier D, de Vendomois JS (2007) New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch Environ Contamin Toxicol:596–602
206. EFSA (2007) Safety and nutritional assessment of GM plants and derived food and feed: the role of animal feeding trials. Food Chem Toxical 46(Suppl 1):S2–S70
207. EFSA (2007) Statement of the scientific panel on genetically modified organisms on the analysis of data from a 90-day rat feeding study with MON 863 maize. European Food Safety Authority, p 5
208. EFSA (2007) Press release: EFSA reaffirms its risk assessment of genetically modified maize MON 863 maize. European Food Safety Authority, p 5
209. Imposteurs. Tout (ou presque) sur le CRIIGEN 2011 (cited 12. Oct 2011. Available from: http://imposteurs.over-blog.com/pages/Tout_ou_presque_sur_le_CRIIGEN-4536267.html
210. Kuntz M (2011) Seralini critique: the latest opus of "parallel science" of Criigen from March 2011. Parallel Science (Website) 2011 (cited 12 Oct 2011). Available from: http://ddata.over-blog.com/xxxyyy/1/39/38/37/Critical_views_on_Seralini_20110710.pdf
211. Watson JD, Crick FHC (1953) Genetical implications of the structure of deoxyribonucleic acid. Nature 171(4361):964–967
212. Watson JD, Crick FHC (1953) Molecular structure of nucleic acids - a structure for deoxyribose nucleic acid. Nature 171(4356):737–738
213. Wilkins MHF et al (1953) Helical structure of crystalline deoxypentose nucleic acid. Nature 172(4382):759–762
214. Berg P et al (1975) Summary statement of asilomar conference on recombinant DNA-molecules. Proc Nat Acad Sci USA 72(6):1981–1984
215. Berg P, Singer M (1995) The recombinant-DNA controversy - 20 years later. Bio-Technol 13(10):1132–1134
216. Friedberg EC (2007) The writing life of James D. Watson. Adler Museum Bull 33(2):3–16
217. Klug A (2004) The discovery of the DNA double helix. J Molec Biol 335(1):3–26
218. Bennett D, Glasner P, Travis D (1986) The politics of uncertainty. Routledge and Kegan Paul plc, London, p 218
219. NRC (National-Research-Council) (1989) Field testing genetically modified organism. Framework for decisions. In: National Research Council (ed) Committee on scientific evaluation of the introduction of genetically modified microorganisms and plants into the environment, NAO Sciences. The National Academy Press, Washington, DC, p 184
220. Lehrman S (1992) Overregulation could damage united-states biotechnology, says report. Nature 359(6396):569–569
221. Mundell I (1992) Britain wrestles with EC rule on modified organisms. Nature 359(6396):569–569

222. McClintock B (1930) A cytological demonstration of the location of an interchange between two non-homologous chromosomes of Zea mays. Proc Nat Acad Sci USA 16: 791–796
223. McClintock B (1953) Induction of instability at selected loci in Maize. Genetics 38(6):579–599
224. Fedoroff N (1994) Mcclintock, Barbara (June 16, 1902 September 2, 1992). Proc Am Philos Soc 138(3):431–445
225. Fedoroff N, Schlappi M, Raina R (1995) Epigenetic regulation of the maize Spm transposon. Bioessays 17(4):291–297
226. Shapiro JA (1997) Genome organization, natural genetic engineering and adaptive mutation. Trends Genet 13(3): 98–104
227. Lewin R (1983) A naturalist of the genome. Science 222(4622):402–405
228. Arber W (2000) Genetic variation: molecular mechanisms and impact on microbial evolution. Fems Microbiol Rev 24(1):1–7
229. Arber W (2002) Roots, strategies and prospects of functional genomics. Curr Sci 83(7):826–828
230. Arber W (2003) Elements for a theory of molecular evolution. Gene 317(1–2):3–11
231. Arber W (2004) Biological evolution: lessons to be learned from microbial population biology and genetics. Res Microbiol 155(5):297–300
232. Arber W (1994) Molecular evolution: comparison of natural and engineered genetic variations. Pontifical Acad Sci Scripta Varia 103:90–101
233. Hackett P (2002) Genetic engineering: what are we fearing? Trans Res 11(2):97–99
234. Ghatnekar L, Jaarola M, Bengtsson BO (2006) The introgression of a functional nuclear gene from Poa to Festuca ovina. Proc Biol Sci 273(1585):395–399
235. Baudo MM et al (2006) Transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. Plant Biotechnol J 4(4):369–380
236. Batista R et al (2008) Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion. Proc Nat Acad Sci USA 105(9):3640–3645
237. Shewry PR et al (2007) Are GM and conventionally bred cereals really different? Trends Food Sci Technol 18(4): 201–209
238. Ammann K (2009) Feature: why farming with high tech methods should integrate elements of organic agriculture. New Biotechnol 25:378–388
239. Barnabás B, Obert B, Kovács G (1999) Colchicine, an efficient genome-doubling agent for maize (Zea mays L.) microspores cultured in anthero. Plant Cell Reports 18(10):858–862
240. Awoleye F et al (1994) Nuclear-DNA content and in-vitro induced somatic polyploidization cassava (*Manihot-Esculenta* crantz) breeding. Euphytica 76(3):195–202
241. Reynolds MP, van Ginkel M, Ribaut JM (2000) Avenues for genetic modification of radiation use efficiency in wheat. J Exp Botany 51:459–473
242. Molnar I, Benavente E, Molnar-Lang M (2009) Detection of intergenomic chromosome rearrangements in irradiated *Triticum aestivum* – *Aegilops biuncialis* amphiploids by multicolour genomic in situ hybridization. Genome 52(2):156–165
243. Schouten HJ, Jacobsen E (2007) Are mutations in genetically modified plants dangerous? J Biomed Biotechnol
244. Latham JR, Wilson AK, Steinbrecher RA (2006) The mutational consequences of plant transformation. J Biomed Biotechnol 2006:1–7
245. Wilson A, Latham J, Steinbrecher R (2006) Transformation-induced mutations in transgenic plants: analysis and biosafety implications. Biotechnol Genet Eng Rev 23(11):1–26
246. Baarends WM, van der Laan R, Grootegoed JA (2001) DNA repair mechanisms and gametogenesis. Reproduction 121(1):31–39
247. Dong CM, Whitford R, Langridge P (2002) A DNA mismatch repair gene links to the Ph2 locus in wheat. Genome 45(1):116–124
248. Morikawa K, Shirakawa M (2001) Three-dimensional structural views of damaged-DNA recognition: T4 endonuclease V, E coli Vsr protein, and human nucleotide excision repair factor XPA (vol 460, pg 257, 2000). Mutation Res DNA Repair 485(3):267–268
249. Lammerts van Bueren ET, Struik PC, Jacobsen E (2002) Ecological concepts in organic farming and their consequences for an organic crop ideotype. Netherlands J Agric Sci 50(1):1–26
250. Lammerts van Bueren ET, Struik PC, Jacobsen E (2003) Organic propagation of seed and planting material: an overview of problems and challenges for research. Njas-Wageningen J Sci 51(3):263–277
251. Anonymous P (1992) Pose no special risks just because of the processes used to make them. Nature 356(6364):1–2
252. Andree P (2002) The biopolitics of genetically modified organisms in Canada. J Canad Stud Revue D Etudes Canadiennes 37(3):162–191
253. Berwald D, Carter CA, Gruere GP (2006) Rejecting new technology: the case of genetically modified wheat. Am J Agric Econ 88(2):432–447
254. Macdonald P, Yarrow S (2002) Regulation of Bt crops in Canada. In: 8th international colloquium on invertebrate pathology and microbial control/35th annual meeting of the SIP/6th international conference on Bacillus Thuringiensis. Academic Press Inc Elsevier Science, Iguassu Falls, Brazil
255. Ramjoue C (2007) The transatlantic rift in genetically modified food policy. J Agric Environ Ethics 20(5):419–436
256. Ramjoue C (2007) The transatlantic rift in genetically modified food policy. Thesis presented to the Faculty of Arts. University of Zurich, Zurich, p 263
257. Kalaitzandonakes N, Marks L, Vickner SS (2005) Sentiments and acts towards genetically modified foods. Int J Biotechnol 7(1–3):161–177
258. Snyder LU et al (2008) European union's moratorium impact on food biotechnology: a discussion-based scenario. J Nat Resour Life Sci Educ 37:27–31

259. Herman RA, Chassy BM, Parrott W (2009) Compositional assessment of transgenic crops: an idea whose time has passed. Trends Biotechnol 27(10):555–557, Corrected proof
260. Romeis J et al (2008) Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nat Biotechnol 26(2):203–208
261. Raybould AF (2010) Reducing uncertainty in regulatory decision-making for transgenic crops: more ecological research or shrewder environmental risk assessment? GM crops 1(1):1–7
262. Raven P et al (2006) Where next for genome sequencing? Science 311(5760):468–468
263. Kesavan PC, Swaminathan MS (2008) Strategies and models for agricultural sustainability in developing Asian countries. Philos Trans Roy Soc B-Biol Sci 363:877–891
264. Plan D, van den Eede G (2010) The EU legislation on GMOs. JRC Scientific and Technical Reports. European Commission Joint Research Denter, JRC, and Institute for Health and Consumer Protection IHCP. Publications Office of the European Union, © European Union, Luxembourg
265. McLean MA et al (2002) A conceptual framework for implementing biosafety: linking policy, capacity, and regulation. In: ISNAR briefing papers. ISNAR, International Service for National Agricultural Research, Washington DC, pp 1–12
266. Graff GD, Zilberman D, Bennett AB (2009) The contraction of agbiotech product quality innovation. Nat Biotechnol 27(8):702–704
267. Miller JK, Bradford KJ (2010) The regulatory bottleneck for biotech specialty crops. Nat Biotechnol 28(10):1012–1014
268. Strauss SH et al (2009) Strangled at birth? Forest biotech and the convention on biological diversity. Nat Biotech 27(6):519–527
269. McLean MA, Charest PJ (2000) The regulation of transgenic trees in North America. Silvae Genetica 49(6):233–239
270. Kalaitzandonakes N, Alston JM, Bradford KJ (2007) Compliance costs for regulatory approval of new biotech crops. Nat Biotechnol 25(5):509–511
271. Morandini P (2007) (20071211) A serious cover up story unveiled in Italy concerning a GM crop field trial, Press release. DOI: http://www.botanischergarten.ch/ASK-FORCE-NEWS-Maize-Lombardia/Morandini-press-release-20071211.pdf
272. Morandini P (2008) Al contadino non far sapere. Espansione n. 5–41, Polenta, May 2008, p 3
273. Marshall A (2007) Another inconvenient truth. In Europe, no one apparently wants to listen if you have good news about genetically modified organisms (GMOs). Nat Biotechnol 25(12):1330
274. Ammann K (2009) Biodiversity and GM crops. In: Ferry N, Gatehouse AMR (eds) Environmental impact of genetically modified/novel crops, released in March, 423p. CAB International, Wallingford, p 28
275. Ronald PC, Adamchak RW (2008) Tomorrow's table: organic farming, genetics, and the future of food. Oxford University Press, Oxford, p 232
276. deRenobales-Scheifler M (2009) More sustainable food: genetically modified seeds in organic farming. Junta General del Principado de Asturias Sociedad Internacional de Bioética (SIBI), Gijon, p 119
277. Paarlberg R (2009) The ethics of modern agriculture. Society 46(1):4–8
278. Herring RJ (2007) The genomics revolution and development studies: science, poverty and politics. J Develop Stud 43(1):1–30
279. Paarlberg RL (2002) The real threat to GM crops in poor countries: consumer and policy resistance to GM foods in rich countries. Food Policy 27(3):247–250
280. Driessen PL (2006) Eco-imperialism: green power–black death. Academic Foundation, New Delhi
281. Alene AD, Coulibaly O (2009) The impact of agricultural research on productivity and poverty in sub-Saharan Africa. Food Policy 34(2):198–209
282. Gruère G, Sengupta D (2009) GM-free private standards and their effects on biosafety decision-making in developing countries. Food Policy 34(5):399–406
283. Spielman DJ, Cohen JI, Zambrano P (2007) Are developing-country policies and investments promoting research and research partnerships in agricultural biotechnology? Int J Biotechnol 9(6): ISSN 0963-6048(print)|1741-5020(electronic)
284. Cohen JI (2005) Poorer nations turn to publicly developed GM crops. Nat Biotechnol 23(1):27–33
285. Alhassan WS (2002) Agrobiotechnology application in West and Central Africa (2002 Survey outcome). CORAF/WECARD–IITA International Institute of Tropical Agriculture, Ibadan, p 107
286. Gruere G, Bouët A, Mevel S (2007) Genetically modified food and international trade: the case of India, Bangladesh, Indonesia and the Philippines. In: IFPRI Discussion Paper 00740. IFPRI, Washington, p 60
287. Smale M et al (2008) The economic impact of transgenic crops in developing countries: a note on the methods. Int J Biotechnol 10(6):519–555
288. Falck-Zepeda JB, Traxler G, Nelson RG (2000) Surplus distribution from the introduction of a biotechnology innovation. Am J Agric Econ 82(2):360–369
289. Pray CE et al (2006) Costs and enforcement of biosafety regulations in India and China. Int J Technol Globalisation 2(1–2):137–57
290. Antle JM (1999) Benefits and costs of food safety regulation. Food Policy 24(6):605–623
291. Shelton AM (2003) Considerations for conducting research in agricultural biotechnology. J Invertebr Pathol 83(2):110–112
292. Kochetkova T (2006) The transatlantic conflict over GM food: cultural background. In: Kaiser M, Lien M (eds) Ethics and the politics of food. Wageningen Academic, Wageningen, pp 325–329
293. Laget P, Cantley M (2001) European responses to biotechnology: research, regulation, and dialogue. Issues Sci Technol 17(4):37–42
294. Ramessar K et al (2010) Going to ridiculous lengths (mdash) European coexistence regulations for GM crops. Nat Biotech 28(2):133–136

295. Bradford KJ et al (2005) Regulating transgenic crops sensibly: lessons from plant breeding, biotechnology and genomics. Nat Biotechnol 23(4):439–444
296. Stein AJ et al (2007) Plant breeding to control zinc deficiency in India: how cost-effective is biofortification? Public Health Nutr 10(5):492–501
297. Stein AJ, Qaim M (2007) The human and economic cost of hidden hunger. Food Nutr Bull 28(2):125–134
298. Stein AJ, Sachdev HPS, Qaim M (2007) What we know and don't know about golden rice. Nat Biotechnol 25(6):624–624
299. Humphrey JH et al (1998) Neonatal vitamin A supplementation: effect on development and growth at 3 y of age. Am J Clin Nutr 68(1):109–117
300. Humphrey JH, West KP, Sommer A (1992) Vitamin-a-deficiency and attributable mortality among under-5-year-olds. Bull World Health Organization 70(2):225–232
301. Depee S et al (1995) Lack of improvement in vitamin-a status with increased consumption of dark-green leafy vegetables. Lancet 346(8967):75–81
302. Mayer JE, Pfeiffer WH, Beyer P (2008) Biofortified crops to alleviate micronutrient malnutrition**.** Genome studies Molec Genet edited by Juliette de Meaux and Maarten Koornneef/ Plant Biotechnol, edited by Andy Greenland and Jan Leach 11(2):166–170
303. Miller HI (2009) A golden opportunity, squandered. Trends Biotechnol 27(3):129–130
304. Potrykus I (2003) Nutritionally enhanced rice to combat malnutrition disorders of the poor. Nutr Rev 61(6):S101–S104
305. Stein AJ et al (2008) Potential impacts of iron biofortification in India. Soc Sci Med 66(8):1797–1808
306. Stein AJ, Sachdev HPS, Qaim M (2006) Potential impact and cost-effectiveness of Golden Rice. Nat Biotechnol 24(10):1200–1201
307. Qaim M, Stein AJ (2008) Economic consequences of Golden Rice. In: Invited presentation at the fourth conference of the European plant science organisation. Toulon (Cote d'Azur), France
308. Qaim M, Stein AJ, Meenakshi JV (2007) Economics of biofortification. In: Otsuka K, Kalirajan K (eds) Contributions of agricultural economics to critical policy issues. Blackwell, Malden, pp 119–133
309. Qaim M, Pray CE, Zilberman D (2008) Economic and social considerations in the adoption of Bt crops. In: Romeis J, Shelton AM, Kennedy GG (eds) Integration of insect-resistant genetically modified crops within IPM programs. Springer, Dordrect, pp 329–356
310. Bouis HE (2007) The potential of genetically modified food crops to improve human nutrition in developing countries. J Develop Stud 43(1):79–96
311. Atanassov AB, Brink A, Burachik J, Cohen M, Dhawan JI, Ebora V, Falck-Zepeda RV, Herrera-Estrella J, Komen L, Low J, Omaliko FC, Odhiambo E, Quemada B, Peng H, Sampaio Y, Sithole-Niang MJ, Sittenfeld I, Smale A, Sutrisno M, Valyasevi R, Zafar Y, Zambrano P (2004) To reach the poor: results from the ISNAR-IFPRI next harvest study on genetically modified crops, in EPTD Discussion Paper No. 116. 2004, ISNAR-IFPRI, International Food Policy Research Institute, Washington DC
312. Potrykus I (2010) Regulation must be revolutionized. Nature 466(7306):561–561
313. Dhlamini Z et al (2005) Status of research and application of crop technologies in developing countries, preliminary assessment. In: FAO (ed) FAO Reports, FAO, Rome, p 62
314. Krattiger A, Mahoney RT (2006) Intellectual property and public health. Bull World Health Organization 84(5):340–340
315. Atkinson RC et al (2003) Public sector collaboration for agricultural IP management including corrigendum of fig. on front page of text, vol. 302, 5648, pp 1152–1152. Science 301(5630):174–175
316. Beachy R et al (2002) Divergent perspectives on GM food. Nat Biotechnol 20(12):1195–1196
317. Krattiger A, Mahoney RTL, Nelsen L, Thompson GA, Bennett AB, Satyanarayana K, Graff GD, Fernandez C, Kowalsky SP (2007) Intellectual property management in health and agricultural innovation a handbook of best practice. MIHR/PIPRA, Oxford/Davis, pp 1539–1559
318. Singh A, Hallihosur S, Rangan L (2009) Changing landscape in biotechnology patenting. World Patent Information, pp 219–225
319. Wright B (2008) Plant genetic engineering and intellectual property protection. Agricultural Biotechnology in California Series Publication, no. 8186
320. Lawson C (2004) Patents and the CGIAR system of international agricultural research centres' germplasm collections under the International Treaty on Plant Genetic resources for food and agriculture. Aust J Agric Res 55(3):307–313
321. Delmer DP et al (2003) Intellectual property resources for international development in agriculture. Plant Physiol 133(4):1666–1670
322. Lempert DH (2009) A dependency in development indicator for NGOs and international organizations.Global Jurist 9(2): Article 6
323. Neidecker-Gonzales O, Nestel P, Bouis H (2007) Estimating the global costs of vitamin A capsule supplementation: a review of the literature. Food Nutr Bull 28:307–316
324. Gressel J, Zilberstein A (2003) Let them eat (GM) straw. Trends Biotechnol 21(12):525–530
325. Potrykus I (2010) Constraints to biotechnology introduction for poverty alleviation. New Biotechnol 27(5):447–448
326. Ademola AA (2011) Global capture of crop biotechnology in developing world over a decade. J Genet Eng Biotechnol (in press)
327. Taverne D (2007) The March of unreason. Oxford University Press, Oxford, p 320
328. Durant J (2005) The march of unreason: science, democracy, and the new fundamentalism. Nature 435(7040):277–278
329. Taverne D (2005) The new fundamentalism, Commentary. Nat Biotechnol 23(4):415–416
330. Herring RJ (2008) Whose numbers count? Probing discrepant evidence on transgenic cotton in the Warangal district of India. Int J Mult Res Approach 2:145–159

331. Marris E (2006) Environmental activism: in the name of nature. Nature 443(7111):498–501
332. Atkinson HJ, Urwin PE (2008) Europe needs to protect its transgenic crop research. Nature 453(7198):979–979
333. Leader SH, Probst P (2003) The earth liberation front and environmental terrorism. Terrorism Polit Violence 15(4):37–58
334. Finkel E (2011) Vandals attack transgenic wheat test plot. Science Insider, July 2011
335. Bettles C (2011) Scientist distances himself from activists. Farm online. DOI: http://sl.farmonline.com.au/news/nationalrural/grains-andcropping/cereal/scientist-distances-himself-from-activists/2239218.aspx?storypage=0 and http://www.ask-force.org/web/Field-Destruction/Bettles-Scientist-Distances-Schubert-20110728.pdf
336. Kuntz M (2011) Academic and governmental research on GMOs has been the target of numerous acts of vandalism in Europe. OGM, environnement, santé et politique. DOI: http://www.marcel-kuntz-ogm.fr/article-news-55055856.html, news in English, French and Spanish and http://ddata.over-blog.com/xxxyyy/1/39/38/37/public-research-vandalized.pdf and http://www.marcel-kuntz-ogm.fr/article-news-55055856.html and http://www.ask-force.org/web/Field-Destruction/Kuntz-Public-Government-Research-Vandalism-Europe-2011.pdf
337. Da Silva W (2011) In focus: the sad, sad demise of Greenpeace in cosmos. About Luna Media Pty Ltd, the boutique publishing company behind COSMOS. Sidney, Australia
338. Gough M (2011) Greenpeace destroys CSIRO wheat GM trial in Cosmos. About Luna Media Pty Ltd, the boutique publishing company behind COSMOS. Sidney, Australia
339. Smith J (2003) Seeds of deception. Yes! Books, Lowa, p 304
340. Smith J (2007) Genetic roulette, the documented health risks of genetically engineered foods. YES ! Books and Chelsea Green, Fairfield Iowa, p 319, second printing edn
341. Miller H (2008) Auf wiedersehen, academic freedom. Wall Street J Europe. p 3
342. Rao CK (2010) Moratorium on Bt Brinjal, a review of the order of the Minister of Environment and Forests, Government of India. Foundation for biotechnology awareness and education, Bangalore, p 74
343. Weese TL, Bohs L (2010) Eggplant origins: out of Africa, into the orient. Taxon 59(1):49–56
344. PRRI (2006) Correspondence between PRRI (Public Research and Regulation Initiative) and FoE (Friends of the Earth). www.pubresreg.org. DOI: http://www.ask-force.org/web/PRRI-FoE/PRRI-FoE-Corresp-Letter-to-FoE-20060629.pdf and http://www.ask-force.org/web/PRRI-FoE/PRRI-FoE-Corresp-Answer-to-FoE-20060926.pdf and http://www.ask-force.org/web/PRRI-FoE/PRRI-FoE-Corresp-Answer-FoE-to-PRRI-20060703.pdf
345. Apel A (2010) The costly benefits of opposing agricultural biotechnology. New Biotechnol 27(5):635–640
346. Borlaug NE (2000) Ending world hunger. The promise of biotechnology and the threat of antiscience zealotry. Plant Physiol 124(2):487–490
347. Hemming D (2006) Swiss vote encourages Austria's anti-GM stance. Outlook Agric 35(1):82–82
348. Motion J, Weaver CK (2005) The epistemic struggle for credibility: rethinking media relations. J Commun Manag 9(3):246–255
349. Burke D (2004) GM food and crops: what went wrong in the UK? Many of the public's concerns have little to do with science. Embo Reports 5(5):432–436
350. Blas X (2009) Bill Gates shifts focus to fighting hunger. Financial Times, London, p 1
351. Miller H, Morandini P, Ammann K (2008) Is biotechnology a victim of anti-science bias in scientific journals? Trends Biotechnol 26(3):122–125, Electronic Prepublication 17 Feb 2008, Hardcopy available in March
352. Horton R (1999) Secret society – Scientific peer review and Pusztai's potatoes. Tls-the Times Literary Suppl 5046:8–9
353. Horton R (1999) GM food debate – Editors reply. The Lancet 354(9191):1729–1729
354. Horton R (1999) Genetically modified foods: "absurd" concern or welcome dialogue? The Lancet 354(9187): 1314–1315
355. Horton R (1999) Health risks of genetically modified foods, editorial, reply to Mitchell and Bradbury, Lancet, p. 1769. The Lancet 353(9167):1811–1811
356. Horton R (1999) Scientific misconduct: exaggerated fear but still real and requiring a proportionate response. The Lancet 354(9172):7–8
357. Ammann K (20110111) Review: Arpad Pusztai's feeding experiments of GM potatoes with lectins to rats: anatomy of a controversy 1998–2009. ASK-FORCE contribution AF-2 AF-2, 46. DOI: http://www.ask-force.org/web/AF-2-Pusztai/AF-2-Pusztai-Food-Safety-20110111.opensource.pdf
358. Quist D, Chapela IH (2001) Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. Nature 414(6863):541–543
359. Campbell P (2002) Quist-chapela paper: editorial note 2. Nature 417(6892):897–897, 27 June 2002
360. Pineyro-Nelson A et al (2009) Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. Molec Ecol 18(4): 750–761
361. Schubert D, Tribe D. comments (2006) Three faces of science fraud. GMO Pundit, DOI: http://gmopundit.blogspot.com/2006/02/david-schubert-alleges-systematic.html.
362. Bradford KJ et al (2005) Regulatory regimes for transgenic crops – Response. Nat Biotechnol 23(7):787–789
363. Schubert D (2005) Regulatory regimes for transgenic crops. Nat Biotechnol 23(7):785–787
364. Punnett RC (1928) Scientific papers of William Bateson. Cambridge University Press, Cambridge
365. Strick J (1999) Darwinism and the origin of life: the role of H.C. Bastian in the British spontaneous generation debates, 1868–1873. J History Biol 32(1):51–92
366. Chassy BN (2002) Food safety evaluation of crops produced through biotechnology. J Am Coll Nutr 21(3):166S–173S

367. Chassy B et al (2007) Nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology: case studies. J Food Sci 72:R131–R137
368. Shelton AM et al (2009) Appropriate analytical methods are necessary to assess nontarget effects of insecticidal proteins in GM crops through meta-analysis (Response to Andow et al. 2009). Environ Entomol 38(6):1533–1538
369. Duan JJ et al (2010) Extrapolating non-target risk of Bt crops from laboratory to field. Biol Lett 6(1):74–77
370. Broer I et al (2011) Response to the criticism by Taube et al. in ESE 23:1, 2011, on the booklet "Green Genetic Engineering". German Research Foundation (DFG), Environmental Sciences Europe, vol 23, issue 1, p 16
371. Green JM, Owen MDK (2010) Herbicide-resistant crops: utilities and limitations for herbicide-resistant weed management J Agric Food Chem
372. Johnson WG et al (2009) Influence of glyphosate-resistant cropping systems on weed species shifts and glyphosate-resistant weed populations. Euro J Agron 31(3):162–172
373. Duke SO, Powles S (2009) Glyphosate-resistant crops and weeds: now and in the future. AgBioForum 12(3&4):346–357
374. Vila-Aiub MM et al (2008) Glyphosate-resistant weeds of South American cropping systems: an overview. Pest Manag Sci 64(4):366–371
375. Powles SB (2008) Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Manag Sci 64(4):360–365
376. Powles SB, Preston C (2006) Evolved glyphosate resistance in plants: biochemical and genetic basis of resistance. Weed Technol 20(2):282–289
377. Neve P (2008) Simulation modelling to understand the evolution and management of glyphosate resistant in weeds. Pest Manag Sci 64(4):392–401
378. Mikulka J, Chodova D (2000) Long-term study on the occurrence of weeds resistant to herbicides in the Czech Republic. (Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz) J Plant Dis Protect 107:373–376
379. Hilbeck A, Schmidt JEU (2006) Another view on Bt proteins – how specific are they and what else might they do? Biopest Int 2(1):1–50
380. Hilbeck A, Meier M, Raps A (2000) Review on non-target organisms and Bt-plants. Ecostrat GmbH, Ecological Technology Assessment Consulting, Amsterdam, p 80
381. Hilbeck A et al (1998) Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environ Entomol 27(5):1255–1263
382. Hilbeck A et al (1999) Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator Chrysoperla carnea. Entomol Exper Et Applicata 91(2):305–316
383. Romeis J, Dutton A, Bigler F (2004) Bacillus thuringiensis toxin (Cry1Ab) has no direct effect on larvae of the green lacewing Chrysoperla carnea (Stephens) (Neuroptera: Chrysopidae). J Insect Physiol 50(2–3):175–183
384. Marshall A (2007) GM soybeans and health safety – a controversy reexamined, additional texts. Nat Biotechnol 25(9):981–987
385. Ammann K (2009) Review web version: are rat organs damaged after feeding on GM soybeans? The Ermakova Case. ASK-FORCE contribution No. 4, 20, 20090801. DOI: http://www.ask-force.org/web/AF-4-Ermakova/AF-4-Ermakova-20090828-web.pdf
386. Seralini GE, Cellier D, de Vendomois JS (2007) New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch Environ Contamin Toxicol 52:596–602
387. OECD (1998) 408 repeated dose 90-day oral toxicity study in rodents (Updated Guideline, Adopted 21st Sept 1998)
388. OECD (1998) 407 repeated dose 28-day oral toxicity study in rodents, Adopted by the Council on 27th July 1995, in OECD Guideline for the testing of chemicals
389. Ammann K (20110921) Summary of 11 ASK-FORCE contributions on biosafety of biotechnology crops. ASK-FORCE contributions AF summary, 69. DOI: http://www.ask-force.org/web/ASK-FORCE-Summary/ASK-FORCE-Summary.pdf
390. Candolfi MP et al (2004) A faunistic approach to assess potential side-effects of genetically modified Bt-corn on non-target arthropods under field conditions. Biocontrol Sci Technol 14(2):129–170
391. Marvier M et al (2007) A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316(5830):1475–1477. doi:10.1126/science.1139208
392. Wolfenbarger LL et al (2008) Bt crop effects on functional guilds of non-target arthropods: a meta-analysis. PLoS ONE 3(5):e2118
393. Naranjo SE (2009) Impacts of Bt crops on non-target invertebrates and insecticide use patterns. CAB Rev Perspect Agric Veterinary Sci, Nutr Nat Resour 4:11–23
394. Duan JJ et al (2008) A meta-analysis of effects of Bt crops on honey bees (Hymenoptera: Apidae). PLoS ONE 3(1):e1415
395. Ammann Ki et al (2004) Biosafety in agriculture: is it justified to compare directly with natural habitats? Frontiers in Ecology, Forum: GM crops: balancing predictions of promise and peril, vol 2, pp 54–160
396. Ammann K (2005) Effects of biotechnology on biodiversity: herbicide-tolerant and insect-resistant GM crops. Trends Biotechnol 23(8):388–394
397. PRRI Public Research and Regulation Initiative(2009) Letter to CBD: LMOs that are likely to have adverse environmental impacts. 20090914, Downloads of PRRI www.pubresreg.org, 3 DOI: http://www.pubresreg.org/index.php?option=com_docman&task=doc_download&gid=490
398. Gupta A (2010) Transparency to what end? Governing by disclosure through the biosafety clearing house. Environ Plann C-Govern Policy 28(1):128–144
399. Felke M et al (2010) Effect of Bt-176 maize pollen on first instar larvae of the Peacock butterfly (Inachis io) (Lepidoptera; Nymphalidae). Environ Biosafety Res 9(1):5–12, Received: 20 November 2008, Accepted: 5 December 2009, online publication 28. October 2010
400. Ammann K (20090911) ASK-FORCE structure and possible contributions. ASK-FORCE contributions, 12 DOI:

http://www.botanischergarten.ch/ASK-FORCE-Strategy/ASK-FORCE-General-List-20090911.pdf
401. Freeland C A hard-pressed trade. In: Financial times, 20070504. Financial Times, London
402. Moore A (2006) Bad science in the headlines – Who takes responsibility when science is distorted in the mass media? Embo Reports 7(12):1193–1196
403. Erjavec K, Erjavec E (2009) Changing EU agricultural policy discourses? The discourse analysis of Commissioner's speeches 2000–2007. Food Policy 34(2):218–226
404. Brabeck-Lemathe P (2008) Nestlé Chairman calls on European policymakers to reconsider opposition to genetically modified (GM) crops; Says 10,000 liters of water required to produce as little as one to two liters of biodiesel. In: Finfacts Business and Finace Portal. Finfacts, Irland, 6 pp. http://www.ask-force.org/web/Nestle/Brabeck-Finfact-Irland-Interview-2009.pdf
405. Rogers C, Farson RE (2007) Active listening, excerpt 1957. University of Chicago Industrial Relations Center, Gordon Training International, Chicago
406. Conklin J (2005) Wicked problems and social complexity. In: Conklin J (ed) Dialogue mapping: building shared understanding of wicked problems. Wiley, Chichester, p 20
407. Peter LJ, Hull R (2009) The Peter principle, why things always go wrong. HarperCollins, New York, 192 pp
408. Blackmore C (2007) What kinds of knowledge, knowing and learning are required for addressing resource dilemmas?: a theoretical overview. Environ Sci Policy 10(6):512–525
409. Zwart NH (2007) Genomics and self-knowledge: implications for societal research and debate. New Genet Soc 26(2):181–202
410. Ikerd JE (1993) The need for a system approach to sustainable agriculture. Agric Ecosyst Environ 46(1–4):147–160
411. Fairclough N (2009) Language and globalization. Semiotica 173(1–4):317–342
412. Scoones I (2008) Mobilizing against GM crops in India, South Africa and Brazil. J Agrarian Change 8(2–3):315–344
413. Weissmann G (2006) DDT is back: let us spray! Faseb J 20:2427–2429
414. WHO (2005) WHO position on DDT use. In: WHO (ed) Disease vector control under the stockholm convention on persistent organic pollutants. WHO, Geneva, p 2
415. Tren R, Bate R (2001) Malaria and the DDT story. In: T.I.o.E.A (ed) The Institute of Economic Affairs, London, 112 pp
416. Losey JE, Raynor LS, Carter ME (1999) Transgenic pollen harms Monarch larvae. Nature 399:214
417. Ammann K (2007) Reconciling traditional knowledge with modern agriculture: a guide for building bridges. In: Krattiger A, Mahoney RTL, Nelsen L, Thompson GA, Bennett AB, Satyanarayana K, Graff GD, Fernandez C, Kowalsky SP (eds) Intellectual property management in health and agricultural innovation a handbook of best practices, Chapter 16.7. MIHR, PIPRA, Oxford/Davis, pp 1539–1559
418. Gerhards J (1997) The discursive versus the liberal public sphere: an empirical critique of Jurgen Haberma' concept of the public sphere. Kolner Zeitschrift Fur Soziologie Und Sozialpsychologie 49(1):1–34
419. Conklin J (2003) Wicked problems and fragmentation. (cited 2003; White Papers, This paper is Chapter 2 in Dialog mapping: making sense of project fragmentation Conklin J, forthcoming). Available from: http://www.cognexus.org/id29.htm
420. Fischer G (2000) Symmetry of ignorance, social creativity, and meta-design. Knowledge-Based Syst 13(7–8):527–537
421. Ammann K (2004) The role of science in the application of the precautionary approach. In: Fischer R, Schillberg S (eds) Molecular farming, Plant-made Pharmaceuticals and Technical Proteins. Wiley-VCH Verlag GmbH & Co KGaA, Weinheim, pp 291–302
422. Rittel H (1984) Second generation design methods. In: Cross N (ed) Developments in design methodology. Wiley, New York, pp 317–327
423. Rith C, Dubberly H (2007) Why Horst W. J. Rittel Matters. Des Issues 23(1):72–74
424. Rith C et al (2007) Bibliography of Horst W.J. Rittel. Des Issues 23(1):78–88
425. Schmidt I et al (2004) SEEbalance reg – managing sustainability of products and processes with the socio-eco-efficiency analysis by BASF. Greener Manag Int 45:79–94
426. Renn O (2008) Risk Governance, coping with uncertainty in a complex world. Earthscan, London
427. Moirand S (2003) Communicative and cognitive dimensions of discourse on science in the French mass media. Discourse Stud 5(2):175–206
428. Chiapello E, Fairclough N (2002) Understanding the new management ideology: a transdisciplinary contribution from critical discourse analysis and new sociology of capitalism. Discourse Soc 13(2):185–208
429. Motion J, Leitch S (1996) A discursive perspective from New Zealand: another world view. Public Relat Rev 22(3):297–309
430. Clark CE (2000) Differences between public relations and corporate social responsibility: an analysis. Public Relat Rev 26(3):363–380
431. Galtung J, Ruge MH (1965) The structure of foreign-news – the presentation of the congo, cuba and cyprus crises in 4 norwegian newspapers. J Peace Res 2(1):64–91
432. Renn O (2006) Risk communication – consumers between information and irritation. J Risk Res 9(8):833–849
433. Chen GKC (1975) What is systems-approach. Interfaces 6(1):32–37
434. Priest SH, Bonfadelli H, Rusanen M (2003) The "trust gap" hypothesis: predicting support for biotechnology across national cultures as a function of trust in actors. Risk Anal 23(4):751–766
435. Iyengar S et al (2009) "Dark areas of ignorance" revisited comparing international affairs knowledge in Switzerland and the United States. Commun Res 36(3):341–358
436. Bonfadelli H, Dahinden U, Leonarz M (2002) Biotechnology in Switzerland: high on the public agenda, but only moderate support. Public Understand Sci 11(2):113–130
437. von Grebmer K, Omamo SW (2007) Options for a rational dialogue on the acceptance of biotechnology. Biotechnol J 2(9):1121–1128

438. Huang JC, Newell S (2003) Knowledge integration processes and dynamics within the context of cross-functional projects. Int J Project Manag 21(3):167–176
439. Beer S (2004) Reflections of a cybernetician on the practice of planning. Kybernetes 33(3–4):767–773
440. Feldman M, Lowe N (2008) Consensus from controversy: Cambridge's biosafety ordinance and the anchoring of the biotech industry. Euro Plann Stud 16(3):395–410
441. Bogner A (2010) Participation as a laboratory experiment paradoxes of deliberation on technology issues by lay people. Zeitschrift Fur Soziologie 39(2):87–105
442. Moore P (2000) Trees are the answer. Forest Prod J 50(10):12–19
443. Moore P (2000) A challenge: protect biodiversity and produce wood. J Forestry 98(8):A2–A3
444. Moore P (2002) Communication through participation, getting it right, environmentalism for the 21st Century. In: Ammann K, Papazova AB (eds) 1st dialogue on science. Academia Engelberg, Engelberg
445. Kahane A (2004) Solving Tough Problems: An Open Way of Talking, Listening, and Creating New Realities. In: Baetz BW (ed) Berrett-Koehler Publishers, San Francisco, 150 pp
446. Schenkel R (2010) The challenge of feeding scientific advice into policy-making. Science 330(6012):1749–1751
447. Rogers-Hayden T, Campbell JR (2003) Re-negotiating science in environmentalists' submissions to New Zealand's royal commission on genetic modification. Environ Values 12:515–534
448. Reich KH (2008) Science-and-religion/spirituality/theology dialogue: what for and by whom? Zygon 43(3):705–718
449. Papazova AB (2010) What do we need as visionaries: progress or development? abstract biovision 2010. Biovision 2010, DOI: http://www.bibalex.org/bva2010/speakers/SpeakerDetails.aspx?m=1&sp=XOHvrH47wZRXTXP5lzyEvA

Grain Quality in Oil and Cereal Crops

Déborah P. Rondanini[1,2], Lucas Borrás[2,3], Roxana Savin[4]
[1]Department of Crop Production, University of Buenos Aires, Buenos Aires, Argentina
[2]CONICET, National Council of Scientific and Technical Research, Buenos Aires, Argentina
[3]Departamento de Producción Vegetal, Universidad Nacional de Rosario, Zavalla, Santa Fe, Argentina
[4]Department of Crop and Forest Sciences, University of Lleida, Lleida, Spain

Article Outline

Glossary

Cereals Monocotyledon plant grains that accumulate starch as the main storage substance for subsequent germination. Two types have been distinguished – cereals that contain gluten and are used for bread-making (wheat, oats, barley, rye) and cereals that do not contain gluten (rice, maize).

Genotype × environment interaction Relative changes in genotype performance when grown under different environments.

Grain development Structural and functional changes that occur in the fertilized flower producing a mature grain capable of germinating.

Grain growth Irreversible increase in grain weight and size caused by cell division, expansion, and reserves accumulation.

Grain quality Group of grain characteristics and measurable attributes (objectively or subjectively) to meet the clients' requirements (i.e., customer, industry, consumers).

Oilseeds Dicotyledon plant grains that accumulate oil as the main storage substance for subsequent germination. Oilseed crop seeds (sunflower, rapeseed, ground pea) are composed of 40–50% oil and 20–30% protein while proteo-oil crop seeds (soybean, lupine) comprise 15–30% oil and 30–40% protein.

Photoassimilates Carbohydrates (sugars, starch, or fructans, depending on the species) synthesized by the green plant parts and translocated to actively growing organs, like grains. Photoassimilates may originate from current photosynthesis or reserve remobilization.

Source–sink balance Quantitative relationship between plant photosynthetic capacity (source) and number of organs under active growth (sink) that are sustained by the former.

Plant stress Changes in plant metabolism in response to environments that endanger plant survival or hinder reaching maximum reproductive capacity.

Definition of the Subject

Grain quality is frequently regarded by agronomists and breeders to be as important as yield. Quality characteristics are the reason why only few plant species are used to satisfy most human requirements for food and fiber [1]. Grain quality comprises a group of characteristics that collectively determine the usefulness of the harvested grains for a particular end use. Therefore, to breed and manage grain crops to achieve a specific quality standard and to be able to predict the quality of a particular crop in a particular growing environment is rather important. Achieving this objective is dependent upon the knowledge of the factors modifying grain composition, and consequently grain quality.

As grain markets have become more specialized, there is a growing pressure on farmers to produce grains with greater uniformity and with certain characteristics [2]. Appropriate husbandry to obtain grains with high and stable "quality" will likely be of increasing importance in achieving economic benefits. It is well known that grain quality is modified by the environment and the crop management practices used by farmers. However, the strategies and tools required to produce grains with certain quality characteristics

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

are not as well established as the ones for achieving high yields. In this context, improving the understanding of the factors that determine grain quality has become increasingly important.

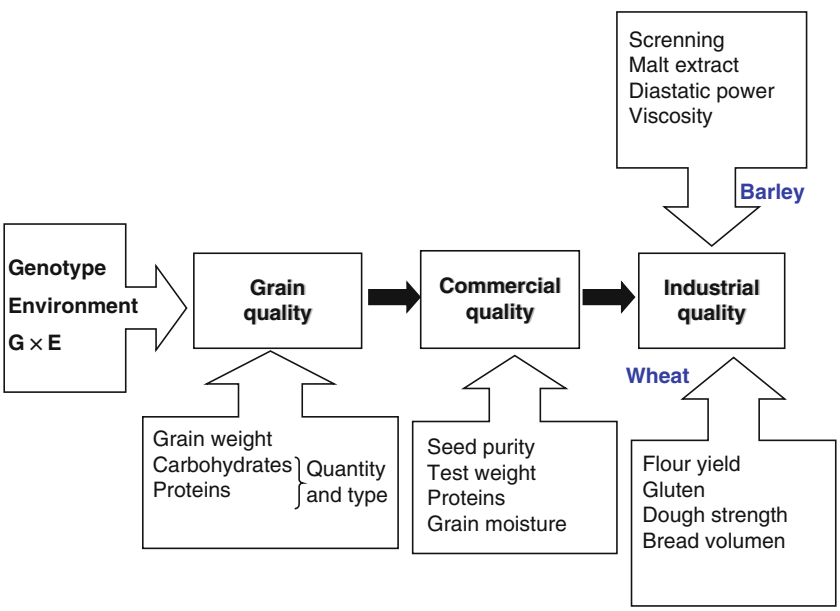
Grain Quality: Concept and Importance

In field crops, the quality of the end product is related to the composition and structure of the seed at harvest maturity. Seed composition and structure at harvest are determined by the genotype, the environment, and the crop management practices used during the crop growing cycle. It is not possible to propose a unique grain quality definition for any specie because it changes depending on the product end use. There is a proper criterion on the concept of quality for each specific end use and for each stage of the commercial chain in every crop (i.e., from harvest in the field, through grain dealers to the industry, Fig. 1). In this context, quality will be considered in relation to the criteria used by those involved in the various aspects of growth and utilization of the grain. As an example, for wheat and barley (Fig. 1), grain quality at the moment of harvest in the field is related to grain size (and weight) and the carbohydrates and protein composition. When the grain is sold to the grain dealer, seed purity, test weight, grain moisture, and protein percentage are the main characteristics that are taken into account for the prize (Fig. 1). After this stage, other attributes may be relevant and they will depend on the involved industry. For baking industry, flour yield and dough strength will be of maximum importance in wheat, while barley for producing beer will take into account the screening percentage, malt extract, and diastatic power, which in turn is related with nitrogen content.

This article aims to summarize key elements of grain structure, grain growth, and synthesis of major grains components in field crops in order to highlight the main attributes which modify grain quality.

Grain Structure

Harvested cereal and oilseed organs may comprise true seeds (soybeans, rapeseed) or fruits (seeds and maternal-accompanying structures, like sunflower achenes or wheat, barley, rice, maize, and sorghum caryopses). Seeds develop from fertilized ovules and consist of three genetically different tissues: (a) the embryo developed from a zygote (diploid, representing the next



Grain Quality in Oil and Cereal Crops. Figure 1
Schematic postharvest processing and storage of wheat and barley production and main quality attributes in each step

generation), (b) the endosperm (usually triploid), and (c) the seed coat formed out by integuments, representing the maternal tissues of the ovule [3]. The proportion of these three components differs in mature seeds of cereals and oilseeds; endosperm is preponderant in cereals while the embryo prevails in oilseeds. With a few exceptions, the development of the endosperm always precedes that of the embryo; and the seed coat development precedes both. These genetically different parts interact closely during development and germination, and recent studies demonstrate the complexity of the connections and regulations among the different seed tissues [4, 5]. After fertilization and seed setting, grains are the primary sink in the plant. Grain filling requires important amounts of photoassimilates supplied by the mother plant through actual photosynthesis and/or the remobilization of stored carbohydrates from vegetative structures. No vascular connection exists between the mother plant and the developing embryo [5, 6] so grain growth is therefore sustained by water and solute movement through cell membranes regulated by both mother plant and seed.

Seed-attached structures include coats (testa and tegmen) and other diverse maternal-originated structures, like the lemma and palea in cereals, pods in soybeans, siliques in rapeseed, and hull (ovary wall attached to the floral receptacle) in sunflower. These structures can greatly influence grain quality appreciation. The seed coat color in different types of beans (*Phaseolus*) impacts consumers differently according to the region, causing rejection of some genotypes albeit their good nutritional properties. Sorghum caryopsis with or without tannins are another example of the importance of grain coats affecting seed quality. Some seed coats can provide nutrients, like the B-group vitamins and micronutrients in cereal brans. In addition, they contribute to other important biological and technological functions, protecting the seed from mechanical damage in postharvest, or by affecting the industrial grain processing (wheat grinding, barley malting, rice parboiling). Seed coats can also impact seed dormancy and germination processes [7]. During recent years, seed-attached structures have received special attention as influencing the potential grain size and volume [8–11].

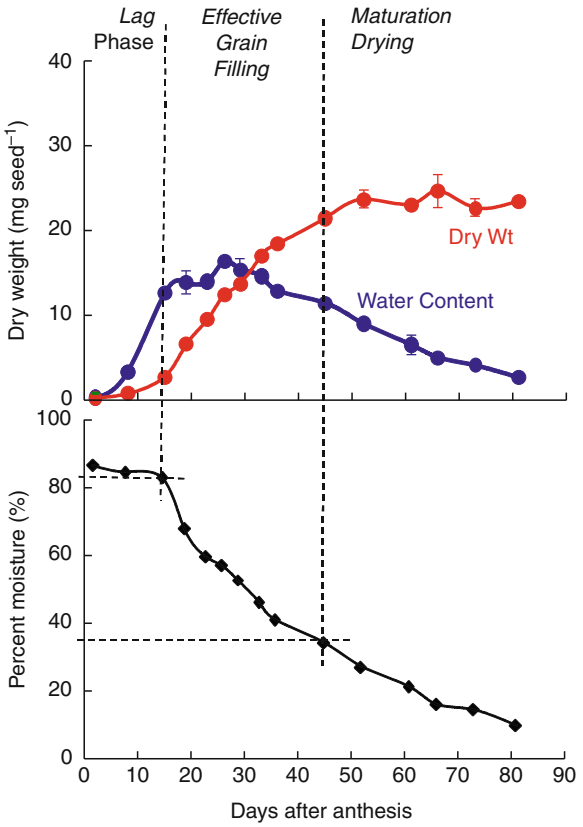
Seeds store carbohydrates (starch, oil) and proteins (soluble and insoluble). The places where these reserves are accumulated vary widely between cereals and oilseeds. In cereals, the tissue that specializes in storing starch and protein is the endosperm. In contrast, oil seeds do not have a specialized storage tissue; oil and protein accumulate in embryo and cotyledon cells. The well-developed starchy endosperm of cereals, with an outer aleurone layer, can comprise as much as 80% of the dry weight of the mature seed. The mature endosperm consists of dead cells packed with starch granules embedded in a protein matrix. The embryo is relatively small, accounting for only about 1–2% of the seed dry weight in wheat, and is usually located on one side of the seed near the point of attachment of the seed to the mother plant [3, 6]. The non-endospermic true seed of oilseeds consists of a large embryo with two cotyledons and the embryo axis. The majority of the reserve materials are stored in the cotyledons, which make up as much as 70% (sunflower) to 90% (soybean, rapeseed) of the total seed dry weight [6].

Grain structure is important since it determines grain and industrial processing quality. Cereal endosperm structure is defined by the number, shape, and size of the starch granules, together with the quantity and type of proteins in the protein matrix. Endosperm structure is used to classify wheat according to its hardness (soft, hard), thus affecting its industrial processing quality (milling capacity and flour yield). In addition, endosperm structure is used to separate dent and flint maize according to the quantity and partitioning of the floury and horny endosperm (greater proportion of horny endosperm in flint maize). Other endosperm structure characteristics that affect grain quality are vitreousness and color, both important for maize, rice, and bread and pasta wheat. Grain structure is also important for defining oilseed quality. In sunflower, the proportion of hull and embryo is an important attribute that defines oil yield, since the hull does not store oil and therefore reduces the oil concentration in the embryo. In the past 30 years, genetic improvement has reduced the hull proportion of sunflower oilseed, increasing the oil percentage on the whole seed [12]. However, thin hulls are usually harder to remove during industrial processing, so other improvement strategies are needed to increase the percentage of sunflower oil in the future. Grain structure has, therefore, a strong impact on the commercial and industrial quality of the grain, and

for this reason its attributes are present in grain marketing regulations worldwide.

Grain Growth and Source–Sink Balance

Seed Biomass During grain filling, the pollinated flower undergoes cell division and differentiation and forms a mature grain (development), which increases in size and weight (growth), reaching mature grain dry weights of 30–50 mg (wheat-barley), 250–400 mg (maize), 20–25 mg (rice), 30–50 mg (sunflower), 150–400 mg (soybeans), and 2–5 mg (rapeseed). Growth and development dynamics can be described by analyzing the rate and time period of grain growth (Fig. 2). The latter are useful tools to explain changes in the final grain weight due to genotypic and environmental factors. Species differ in their biomass per seed, and ample intra-specific differences are also observed [6].



Grain Quality in Oil and Cereal Crops. Figure 2
Dynamics of individual seed dry weight (Dry Wt), water content per seed, and seed moisture of wheat seed

Commercial genotypes used by farmers in maize, wheat, and soybean show differences in seed size, and this variability is even larger when exotic material is considered.

Seed biomass accumulation is commonly partitioned into three phases: the lag phase, the effective seed-filling period, and the maturation drying phase (Fig. 2). The lag phase is a period of active cell division. It is characterized by a rapid increase in water content with almost no dry matter accumulation. Following the lag phase, cells within the seed enter a differentiation and maturation phase, and a period of rapid dry matter accumulation resulting from the deposition of seed reserves. This phase is generally referred to as the effective seed-filling period. As in the lag phase, water content continues to increase rapidly and eventually establishes the maximum volume of the seed. Species vary considerably as to when maximum seed water content is achieved during seed filling [13]. In maize kernels, maximum water content occurs near mid seed filling [14], while in soybean seeds maximum water content is achieved at a later stage, when 70–80% of the final seed size has been achieved [15] and conversely, sunflower reaches it earlier with only 30% of final grain dry weight [10]. During the third phase of development, seeds loose water content, reach "physiological maturity" (maximum dry matter accumulation), and enter a quiescent state [3]. Seed water concentration declines throughout the three stages of seed development (Fig. 2). This decline is most obvious after seeds reach physiological maturity, but it also occurs during rapid seed filling as water is displaced by reserves [14–16].

The progress of dry matter accumulation in developing seeds and the concurrent loss of water are closely related phenomena. Studies with maize, wheat, soybean, and sunflower [17–20] have shown that final seed size is achieved at, or near, a minimum water concentration. Also, results from several studies have shown that seed water concentration accurately predicts the percent of maximum seed size achieved at any moment during seed filling in wheat, soybean, maize, and sunflower [17–20]. Such results support the notion that the duration of seed filling is determined by the interaction between reserve deposition and declining cellular water content, where deposition of reserves such as starch, protein, or lipids replace water

until a critical minimum water concentration is reached [6, 20, 21]. Species differ in the seed water concentration when they achieve maximum seed biomass [13]. For example, soybean seeds reach maturity at ~62%, maize seeds at ~36%, and wheat seeds at ~37% moisture. Although minor compared to differences across species, it has been shown that when an ample set of cultivars within a species is analyzed, variability for this trait can also be observed [22].

The rate of seed growth during the effective seed filling is highly dependent upon the number of sites for reserve deposition. The usual estimate of seed sink capacity is the number of differentiated cells during the lag phase. In maize, wheat, and other cereals, the number of endosperm cells is highly related to the rate of seed growth during rapid seed filling. In legumes such as soybean or pea, the number of cotyledon cells is highly related to the rate of seed growth. Thus, rate and duration of grain filling are important to define the final grain weight, an important attribute of grain quality.

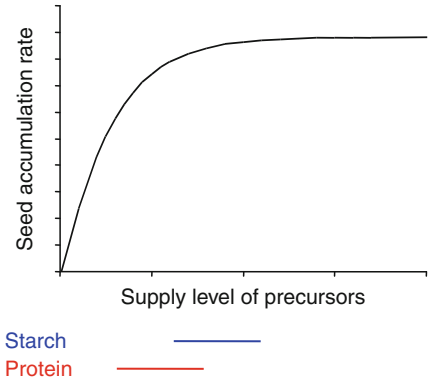
Source–Sink Balance In higher plants, nutrients from assimilation sites (sources) are delivered to sites of nutrient utilization (sinks) through an interconnected network of sieve elements. Partitioning of phloem-delivered nutrients between competing sinks is governed by their relative ability to unload major osmotic species from the importing phloem sieve elements [23]. This process depends upon a set of intercellular (post-sieve element) transport events which are integrated with growth or storage functions of the recipient sink tissues [24].

Species differ on the seed size at maturity [25], and this interspecific variability is more related the amount of assimilates available per seed during the early lag phase than during the effective seed-filling period [21]. At flowering, plants adjust the number of seeds and the potential seed size to the growth environment [21], and species differ in how they distribute available assimilates into more seeds or more potential seed size at around the period when seed number is being determined [26]. Seed size is mainly determined by the genotype, although the environment can affect the final size as well. Water availability and temperature are two environmental conditions that can create important changes in the size of the seeds at maturity.

The amount of assimilates available per seed is usually referred as the source–sink balance, and is used to describe the relation between the total amount of available assimilates and the sink number. This ratio is used to simplify the idea of assimilate availability per sink, and the way the source–sink ratio has been estimated can vary widely. Different researchers have used plant growth per seed, green leaf area per seed, sucrose availability per seed, and alternative approaches including plant growth per day per unit of sink growth per day. The source–sink balance that the seeds experience during their growth is adjusted at around flowering, when plants are setting the number of seeds.

Because plants grow in a nonuniform environmental condition, the source–sink balance during the period when seeds are accumulating biomass can change. An example can be a defoliation caused by an insect eating leaves attacking the crop at mid grain filling (which would reduce the source–sink balance of the crop) or a drought stress reducing plant growth (also reducing the source–sink balance). The source–sink balance becomes relevant because not all seed components vary to the same degree when assimilate availability per seed is altered, so the seed composition and quality may change [27–29].

Jenner and coworkers developed a theoretical model to understand how changes in the amount of assimilates available per seed can affect seed composition [28]. Their model is based on the idea that each one of the seed components can be more or less affected by changes in the level of precursors available for the growing seed because not all components are receiving from the mother plant the same level of precursors needed for their deposition within the seed. An example is illustrated in Fig. 3, where changes in the level of precursors will most surely not affect the starch content of the seed but changes in the level of precursors needed for protein synthesis will affect the protein content and the final protein concentration. This model helps explain why changes in plant growth (that affect the precursor levels) will most surely not affect starch content but will surely affect the protein content of the seed. Recent studies conducted on soybean [30] and maize compositions [27, 31] agree with this model.



Grain Quality in Oil and Cereal Crops. Figure 3
Level of precursors available per seed to synthesis of different components of seeds (Adapted from [28])

Synthesis of Major Components

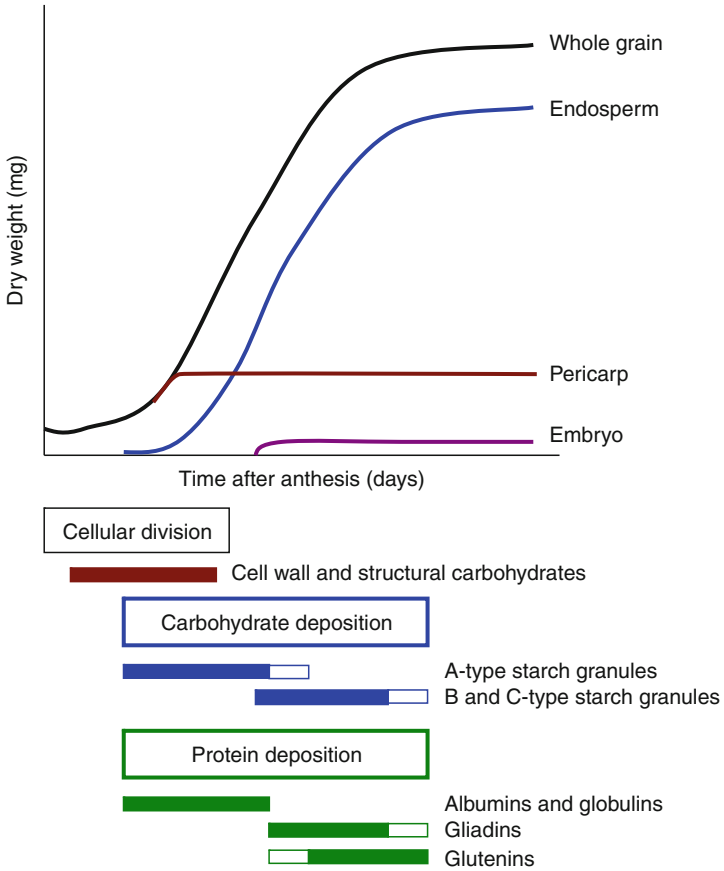
Grain growth involves growth processes of various structures (seed coats, embryo, endosperm) and accumulation of different substances (starch, oil, protein). Grain components are not synthesized simultaneously nor do they occur at the same rate; thus, physical and chemical grain composition varies during grain filling. This is an important aspect when dealing with industrial and nutritional grain quality.

As an example, the different parts of a wheat grain and the main components synthesized during grain filling are shown in Fig. 4. Starch is the main component in wheat, comprising 70–80% of the final grain weight. Different types of starch granules of varying numbers, shapes, and sizes are synthesized into the endosperm cells. The A-type granules are bigger and quantitatively more important than the succeeding B- and C-type granules. Starch is composed of amylose and amylopectin at a 3:1 ratio, except in waxy genotypes where amylopectin is more abundant.

In wheat bread, proteins comprise 5–20% of the final grain dry weight and include albumins and globulins (30–40%); gliadins and glutenins (60–70%). Albumins and globulins have enzymatic and metabolic functions and are located in the embryo and aleurone layer; gliadins and glutenins form the gluten and are reserve proteins, confined in the endosperm [32]. During grain filling, metabolic proteins are synthesized first and predominate until 10–15 days after anthesis. Reserve proteins accumulate during the effective filling phase; gliadins are the first to be detected (10–15 days after anthesis) while glutenins are deposited later (15–20 days after anthesis). Gliadins give viscosity to the mass while the glutenins confer elasticity, and both result in viscoelastic gluten appropriate for a good loaf volume. Since the gliadins:glutenins ratio changes during grain filling, crop exposure to stressful conditions (i.e., high temperature, water stress) during this phase will modify the total protein mass and the gliadins: glutenins ratio, affecting baking quality.

Grains from other species have different grain component synthesis patterns. For example, in maize there is only one type of starch granule and although starch is synthesized through the entire effective filling phase amylose accumulation occurs after amylopectin. Reserve proteins (5–14% of the final grain weight) are accumulated during the entire effective filling period as well, forming protein bodies in the endosperm cells. Oil accumulation (3–15% of the final grain weight) takes place at the end of the filling phase and most of the oil is found in the embryo. In oilseeds, oil and protein are the main reserve substances; carbohydrates are scarcely accumulated (<20%). Sunflower seeds contain 40–55% oil and 10–20% protein, while soybean seeds contain greater protein percentages (35–50%) and lower oil percentages (10–25%). Both protein and oil are deposited in the embryo cells during the linear grain filling phase. Oil deposits form oleosomes or lipid bodies of spherical shapes, while reserve proteins form dense and irregular protein bodies [33, 34].

During grain filling oilseeds, protein synthesis usually occurs after oil synthesis. Oil is formed by triglycerides, which are composed of one glycerol molecule combined with three fatty acids. Fatty acids differ in the number of carbon atoms (typically between 14 and 22 in vegetable oils) and the number of double bonds between carbon atoms. The different proportions of fatty acids modify the physicochemical and industrial properties of oils. Oils with high saturated fatty acid percentages (without double bonds; like palmitic and stearic acids) are semisolid at room temperature, with a high melting point and a higher resistance to oxidation (fat degradation due to oxygen presence). However, consumption of these oils (especially palmitic acid) increases cholesterol levels in the bloodstream. On the contrary, oils with high proportions of monounsaturated fatty acids (like oleic acid) and



Grain Quality in Oil and Cereal Crops. Figure 4
Grain filling dynamics of a cereal (wheat) and synthesis of major grain components (Adapted from [29])

polyunsaturated fatty acids (like linoleic and linolenic acids with two and three double bonds, respectively) are liquid at room temperature, have a lower melting point, and a higher susceptibility to oxidation as the number of double bonds increase; these oils are healthier than saturated fatty acids. During grain filling, the proportion of fatty acids varies according to the species and crop varieties. In traditional sunflower genotypes, the oleic:linoleic ratio decreases during grain filling while total oil accumulation increases. In contrast, in "high oleic" sunflowers, the oleic proportion is high and constant during grain filling due to the low activity of the enzyme responsible for the linoleic synthesis deriving from oleic acid [35, 36]. Oil final composition varies greatly among oilseed species, and genetic improvement has achieved a wide variety of fatty acid compositions within the same species resulting from physical and chemical mutagenesis that affect specific enzyme functions responsible for the presence of double bonds in fatty acids [37, 38]. These enzymes are also affected by the environment, producing changes in grain oil content and composition.

Main Factors Affecting Grain Quality

Genotypic Effects

Some few grain attributes are mainly driven by the genotype, and the environment has relatively low influence. For example, the color of the wheat grain (white, yellow) is strongly determined by the ability of genotype to accumulate lutein, and the character "high oleic" in oilseed genotypes is associated with genetic mutations defective for the enzyme that desaturates oleic acid. Genotypes within the same species can

Grain Quality in Oil and Cereal Crops. Table 1 Grain composition ranges reported in different species

Species	Grain composition range	Source
Soybean	33–42% protein	[35]
Sunflower	20–30% oil (confectionary type)	[33, 38]
	40–55% oil (oil type)	
Wheat bread	5–20% protein	[40]
Corn	5–14% protein	[31]
	8–12% protein (pop corn type)	[41]
	5–10% oil (high oil content)	[42]

present large differences in grain composition. For example, in commercial genotypes of soybean, the difference in protein percentage can easily vary from 33% to 42% [39]. This variability can be observed for any seed component in any species [40–42], as ample natural variation is common (Table 1).

Earlier studies working on understanding the genetic basis of genotypic differences in seed composition were based on the use of mutants, and these usually yielded qualitative differences in seed composition. For example, a commercial genotype of maize usually contains ~40% amylopectin and ~60% amylose, and a waxy mutant contains 100% amylopectin and no amylose. At present time, a large number of mutants have been discovered and used within any species as specialty quality genotypes.

The modification of the fatty acid profile of oil seeds has been one of the main tasks faced by oilseed breeders over the past 40 years. Success in this field has been of paramount importance for the worldwide expansion of some oilseed crops. The elimination of erucic acid (a harmful fatty acid) from rapeseed oil was the first step toward the development of canola (zero-erucic, low-glucosinolate rapeseed) as one of the major sources of vegetable oil in the world. Other landmarks in oilseed breeding for seed oil quality have been the development of high oleic, low linolenic acid canola, low linolenic acid linseed and soybean, high oleic acid sunflower, high saturated sunflower, and sunflower lines with modified tocopherol (antioxidant compounds) composition. Most of these traits defining seed oil quality have been found to be governed by a reduced number of genes (one to three major genes, with several alleles for each locus in most cases), and this fact implies that the practical management of single quality traits in breeding programs is relatively easy if compared with polygenic traits (as grain yield, grain weight or protein and oil content). Additionally, the fatty acid composition of the seed oil is determined by the genotype of the developing embryo (not the whole plant), so mutagenesis and selection can be carried out at a single-seed level, using the half-seed technique.

In wheat, improving yield potential without negatively affecting grain quality is difficult, mainly because increases in grain yield are generally accompanied by a decrease in grain protein content, which is strongly associated with bread-making quality. Wheat breeders give grain quality the same level of importance as yield potential and disease resistance. In contrast to the low heritability of protein content, grain hardness and yellow pigment are highly heritable and can be readily improved through conventional breeding. Plant breeders select at least one parent with the desired quality when designing their crossing strategies, particularly as end-use requirements frequently determine the fate of potential new cultivars, but the stage in the breeding process at which quality determination takes place will influence which tests (micro or macro tests) are applied, according to the sample size available.

At present, natural variation for seed composition is being studied identifying quantitative trait loci (QTLs) for different seed components (oil, protein), as any seed component is a quantitative trait governed by many genes and each one with an individual small effect. The study by Blanco and coworkers can be mentioned as an example, where the authors studied seed protein concentration in wheat where three major QTLs were detected [43]. This methodology is currently becoming very popular and has yielded molecular markers associated with seed component traits that can help understand the genetic bases of the trait and be used by breeders and the industry.

Environmental Effects

As mentioned earlier, the majority of quality traits are greatly modified by the environment and by genotype–environment interactions. Grain weight and protein concentration are found within this group of traits.

Environment variables like high temperature, water and nitrogen (N) availabilities have been the most studied modifying grain quality.

The response in grain composition to a particular stress depends mainly on the stress characteristics (i.e., intensity, duration of the stressful period, opportunity of occurrence, and the interaction that this stress may have with other stresses to which the crop is exposed to). The relevance of the intensity and duration of the stress on the magnitude of the change in grain composition is self-explained (the more severe and the longer a stress is, the greater change in composition produced, though not necessarily this implies that the relationship is linear). The timing of occurrence is also critical, as shown in Fig. 4 not all stages are equally critical for the final determination of grain quality: if the stress coincides with critical stages for synthesis and deposition of the components, the changes will result far stronger than that of stresses occurring is less-critical stages. Therefore, the grain composition responses to stressful factors may range from virtual insensitivity (if punctual synthesis reductions are compensated by recovering when no stress occurs) to different ranges of quality reductions to even crop failure to produce a certain quality level.

Seed growth and development are responsive to temperature, but their responses vary with the temperature range considered [44]. As a general rule, the rate of seed development increases as temperature increases, reducing the duration of seed-filling period. At lower temperatures, seed growth rates decrease linearly as temperatures fall below 15°C in wheat, soybean, rice, sunflower, and maize. Seed growth rates increase when temperatures rise from 20°C to 30°C; however, this increase does not offset the linear decrease in seed-filling duration, resulting in lower grain weights [44]. In most cases, moderately high temperatures (20–30°C) prevail during grain filling, although short periods of very high temperatures (>30–32°C) may occur reducing seed growth rate and causing the early end of grain filling period. In addition, the earlier the heat stress, the greater the impact on grain weight [45, 46]. Brief periods of high temperatures can cause reductions in grain weight, but these effects can be overlooked if only the average temperature during post-flowering period is considered. Thus, moderately high temperatures (20–30°C) during the post-flowering period reduced grain weight mainly through shortening the grain filling period, while very high temperatures (>30–32°C) even for a few days can reduce grain weight by reducing grain filling rate and the early cessation of grain growth period. Both aspects of post-flowering temperature should be considered especially because climatic change could bring about high-temperature scenarios in the next decades, together with an increase in heat-stress events [47, 48].

Grain quality and composition are also affected by temperature. Several experiments suggest that the temperature effects on seed composition are related to dry matter metabolism and accumulation. The timing, intensity, and duration of occurrence of heat stress may alter final grain quality according to the grain component synthesis process involved (carbohydrates, proteins, oils). Interestingly, there are some reports on the possibility of recovery post-stress [49, 50]. In wheat and barley, protein percentage increases with increasing temperatures (15–30°C) because the negative impact of high temperatures on starch synthesis is greater than the impact on protein synthesis, thus decreasing the starch proportion in the grains [28]. High temperatures also affect protein quality, generally increasing gliadin:glutenin ratio, which causes weak dough with a low bread-making quality. The temperature impact on wheat grain quality will therefore depend on the balance between the positive (higher protein) and negative (greater gliadin:glutenin ratio) effects. Temperature also affects oil fatty acid composition in oilseeds [51]. The higher the temperatures during grain filling, the higher the fatty acid saturation (i.e., greater proportions of oleic acids and lower proportions of linoleic and linolenic acids) due to the reduced activity of unsaturation enzymes in grains [52]. Temperatures registered during the night in early grain filling phases have shown to have the best predictive values for modeling the final oil composition in sunflower [53]. Progress in modeling the quality of other grains is underway [54].

In field crops, high-temperature occurrences are commonly associated to water stress, increasing the negative temperature effects. Drought stress produces a shortage of assimilates and often reduced N availability, which cause a reduction in grain growth. In general, a drought episode occurring after flowering

has a similar effect as an increase in temperature – the quantity (mg grain^{-1}) of protein per grain remains stable, while starch accumulation in grain is significantly reduced, resulting in smaller grains with a greater protein percentage [55]. In oilseeds, post-flowering droughts decrease grain oil percentages and increase protein percentages [56, 57] indicating that carbon metabolism is affected to a greater extent than N metabolism. Water stress has a smaller impact on fatty acid composition; in general, droughts do not modify the saturation degree in oils except under severe stress conditions which produce an early grain-filling cessation [58].

N availability also affects final cereal and oilseed grain composition. In general, when soil N availability is low, cereal crop yields respond positively to N fertilization. A dilution effect occurs when N taken up by the crop is partitioned in a greater number of grains, which reduces grain protein percentage. If N availability is further increased, both crop yield and grain protein percentage are increased. In addition, the stage of development when N is added is important in defining wheat grain quality. N applications around flowering increase nitrogen availability per grain, increasing protein percentage. It is reported that increases in N availability result in increases of gliadin:glutenin ratio, which in turn produce a weakening of the dough [59]. In oilseeds, a greater soil N availability increases crop yield and grain protein percentage. Consequently, oil percentages in grain decrease due to the negative relationship between oil and protein (expressed as a percentage of the grain weight). Nitrogen application effects on the grain fatty acid composition are smaller and more variable compared to temperature and water stress effects [60, 61]. A greater knowledge on the physiological processes that regulate the responses to these environmental factors is essential to decide the management of the crop to produce grain for a specific end use.

Management Strategies

Although both grain yield and quality are determined throughout the growing season, important decisions that will strongly affect them should be taken before planting [62]. The farmer's choice of genotype and the amount of nitrogen available are central for successfully combining the genotype potential for yield and quality with the environmental availability of resources. As stated earlier, final grain quality is the result of the interaction between the genotype, the natural environment, and the crop management practices [63]. In extensive production systems, it is not possible to provide each stage of the crop cycle with the optimal combination of environmental factors to reach the highest possible yield and quality, therefore, a trade-off is to make preplanting decisions to ensure that critical crop stages for the definition of yield and quality are given a preferential environment [62]. Nevertheless, knowledge of the effects of environment and G × E interaction is still rather imprecise, so management strategies with the objective of increasing yields, while obtaining high quality, are difficult to design.

There are a number of grain quality attributes that are strongly governed by the genotype and therefore choosing the proper genotype in relation to the final end use of the grain is critical. In several countries, for trading purposes wheat is classified into distinct categories of endosperm hardness (soft, semihard, and hard). Grain hardness is determined by the packing of grain components in the endosperm cells [40] and according to this attribute, the end product can vary from pasta (hard endosperm), biscuits (soft endosperm), to bread (hard endosperm). Usually, this classification can be more detailed and complex [64]. In the case of sunflower, oil fatty acid composition is genetically controlled [65], and the oil composition has been modified mostly by altering the function of major genes through mutagenesis [38].

Addition of nitrogen fertilizer is one of the most frequent management practices for altering grain quality (and of course grain yield). It is difficult a priori to know the effect of adding nitrogen to grain quality as many other factors are intervening and modify the final expected result. In the case of wheat crops, the initial amount of nitrogen in the soil, the specific moment of fertilization, the amount of available water, and rain pattern during the growth cycle as well as plant density at sowing and genotype nitrogen use efficiency are the main factors that interact and may modify the final response in grain quality. In general, it is accepted that regardless of the species, the increase in grain yield leads to a decrease in the protein to starch or oil ratio. This negative relationship between yield and grain

N concentration reflects the fact that carbon assimilation and accumulation during the grain filling period is sink-limited [66] while nitrogen accumulation in grains is usually source-limited [67], as a result of dilution effects. The final protein concentration will thus depend on the balance between the source capacity to provide nitrogen and the strength of the sink for accumulating carbohydrates [68].

Future Directions

The compositional requirements for a particular grain vary from one product to the other depending on its end use. In addition, grain quality is a dynamic concept as it changes constantly as new uses can be developed for particular grains. The three major pillars of grain composition are: the genotypes, the environments during grain growth, and their interaction.

On the genetic pillar, the knowledge gained in the recent past has been extraordinary. Based on the molecular tools developed, a number of genes and QTLs involved in the determination of particular grain components (in turn determining grain quality attributes) have been identified and mapped in several crops, and it seems easy to predict that in the near future almost any breeding program in the world will be able to manipulate these genetic factors with certainty.

Regarding the environment during grain filling, important and useful findings have been reported in relation to high temperatures, and in lesser extent in water stress, and nitrogen availability. Few studies have attempted to examine the interactions between these environmental factors on grain quality attributes. It has been recently reported that high-temperature stress effects may be mitigated under high nitrogen availability for wheat and barley [69–71].

Undoubtedly, the challenge for breeders and agronomist is dealing with G × E interactions [72]. Therefore, there is a need for increasing current knowledge on the physiology of quality traits in order to obtain both high yield and high quality through breeding and management strategies. This will also help predict grain composition through a series of genotypes and environments.

Using agronomic simulation models properly calibrated and validated for the target population of environments can be a tool for understanding and predict final grain composition. The incorporation of grain quality modules into crop simulation models is increasing (European Journal of Agronomy 25, 2006). Grain protein content was the first trait incorporated into modeling as well as grain size (grain weight) which is a quality criteria especially valued by millers in the case of cereals but also for oil extraction in oil crops. Recently, more detailed concepts have been incorporated such as the type of protein [73] and oil quality [74]. It is expected that incorporating genetic data into simulation routines will be done in the near future.

Bibliography

Primary Literature

1. Slafer GA, Satorre EH (1999) Wheat production systems of the Pampas. In: Satorre EH, Slafer GA (eds) Wheat: ecology and physiology of yield determination. Food Product Press, New York, pp 333–343
2. Wrigley CW (1994) Developing better strategies to improve grain quality for wheat. Aust J Agric Res 45:1–7
3. Boesewinkel FD, Bouman F (1995) The seed: structure and function. In: Kigel J, Galili G (eds) Seed development and germination. Marcel Dekker, New York, pp 1–24
4. Berger F, Grini PE, Schnittger A (2006) Endosperm: an integrator of seed growth and development. Curr Opin Plant Biol 9:664–670
5. Meyer CJ, Steudle E, Peterson CA (2007) Patterns and kinetics of water uptake by soybean seeds. J Exp Bot 58:717–732
6. Egli DB (1998) Seed biology and the yield of grain crops. CAB International, New York, 178 p
7. Baskin JM, Baskin CC (2004) A classification system for seed dormancy. Seed Sci Res 14:1–16
8. Millet E, Pinthus MJ (1984) The association between grain volume and grain weight in wheat. J Cereal Sci 2:31–35
9. Calderini DF, Abledo LG, Slafer GA (2000) Physiological maturity in wheat based on kernel water and dry matter. Agron J 92:895–901
10. Rondanini DP, Mantese AI, Savin R, Hall AJ (2009) Water content dynamics of achene, pericarp and embryo in sunflower: associations with achene potential size and dry-down. Eur J Agron 30:53–62
11. Lizana XC, Riegel R, Gomez LD, Herrera J, Isla A, McQueen-Mason SJ, Calderini DF (2010) Expansins expression is associated with grain size dynamics in wheat (*Triticum aestivum* L.). J Exp Bot 61:1147–1157
12. Putt ED (1997) Early history of sunflower. In: Schneiter AA (ed) Sunflower technology and production. American Society of Agronomy, Madison, pp 1–19

13. Egli DB, TeKrony DM (1997) Species differences in seed water status during seed maduration and germination. Seed Sci Res 21:289–294
14. Westgate ME, Boyer JS (1986) Water status and the developing grain of maize. Agron J 78:714–719
15. Egli DB (1990) Seed water relations and the regulation of the duration of seed growth in soybean. J Exp Bot 41:243–248
16. Borrás L, Westgate ME, Otegui ME (2003) Control of kernel weight and kernel water relations by post-flowering source-sink ratio in maize. Ann Bot 91:857–867
17. Borrás L, Westgate ME (2006) Predicting maize kernel sink capacity early in development. Field Crop Res 95: 223–233
18. Swank JC, Egli DB, Pfeiffer TW (1987) Seed growth characteristics of soybean genotypes differing in duration of seed fill. Crop Sci 27:85–89
19. Rondanini DP, Savin R, Hall AJ (2007) Estimation of physiological maturity in sunflower as a function of fruit water concentration. Eur J Agron 26:295–309
20. Schnyder H, Baum U (1992) Growth of the grain of wheat (*Triticum aestivum* L.): the relationship between water content and dry matter accumulation. Eur J Agron 1:51–57
21. Gambín BL, Borrás L (2010) Resource distribution and the trade-off between seed number and weight: a comparison across crop species. Ann Appl Biol 156:91–102
22. Borrás L, Zinselmeier C, Senior ML, Westgate ME, Muszynski MG (2009) Characterization of grain filling patterns in diverse maize germplasm. Crop Sci 49:999–1009
23. Patrick JW (1997) Phloem unloading: sieve element unloading and post-sieve element transport. Annu Rev Plant Biol 48:191–222
24. Patrick JW, Offler CE (2001) Compartmentation of transport and transfer events in developing seeds. J Exp Bot 52:551–564
25. Egli DB (1981) Species differences in seed growth characteristics. Field Crop Res 4:1–12
26. Sadras VO (2007) Evolutionary aspects of the trade-off between seed size and number in crops. Field Crop Res 100:125–138
27. Borrás L, Curá JA, Otegui ME (2002) Maize kernel composition and post-flowering source-sink ratio. Crop Sci 42:781–790
28. Jenner CF, Ugalde TD, Aspinall D (1991) The physiology of starch and protein deposition in the endosperm of wheat. Aust J Plant Physiol 18:211–226
29. Savin R, Molina-Cano JL (2002) Changes in malting quality and its determinants in response to abiotic stress. In: Slafer GA, Molina-Cano JL, Savin R, Araus JL, Romagosa I (eds) Barley science: recent advances from molecular biology to agronomy of yield and quality. Food Product Press, New York, pp 523–544
30. Rotundo JL, Borrás L, Westgate ME, Orf JH (2009) Relationship between assimilates supply per seed and soybean seed composition. Field Crop Res 112:90–96
31. Seebauer JR, Singletary GW, Krumpelman PM, Ruffo ML, Below FE (2010) Relationship of source and sink in determining kernel composition of maize. J Exp Bot 61:511–519
32. Shewry PR, Napier JA, Tatham AS (1995) Seed storage proteins: structures and biosynthesis. Plant Cell 7:945–956
33. Mantese AI, Medan D, Hall AJ (2006) Achene structure, development and lipid accumulation in sunflower cultivars differing in oil content at maturity. Ann Bot 97:999–1010
34. Tanaka W, Mantese AI, Maddonni GA (2009) Pollen source effects on growth of kernel structures and embryo chemical compounds in maize. Ann Bot 104:325–334
35. Garcés R, Mancha M (1989) Oleate desaturation in seeds of two genotypes of sunflower. Phytochemistry 28:2593–2595
36. Ohlrogge J (1997) Regulation of fatty acid synthesis. Annu Rev Plant Physiol Plant Mol Biol 48:109–136
37. Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. Biochim Biophys Acta 1301:7–56
38. Velasco L, Perez-Vich B, Fernández-Martínez JM (2004) Grain quality in oil crops. In: Benech-Arnold RL, Sánchez RA (eds) Handbook of seed physiology: applications to agriculture. Food Products Press/The Haworth Press, New York, pp 389–405
39. Rotundo JL, Westgate ME (2009) Meta-analysis of environmental effects on soybean seed composition. Field Crop Res 110:147–156
40. Peña RJ, Trethowan R, Pfeiffer WH, van Ginkel M (2002) Quality (end-use) improvement in wheat compositional, genetic, and environmental factors. J Crop Prod 5:1–37
41. Park D, Allen KGD, Stermitz FR, Maga JA (2000) Chemical composition and physical characteristics of unpopped popcorn hybrids. J Food Comp Anal 13:921–934
42. Thomison PR, Geyer AB (1999) Evaluation of TC-Blend7 used in high oil maize production. Plant Var Seeds 12:99–112
43. Blanco A, Simeone R, Gadaleta A (2006) Detection of QTLs for grain protein content in durum wheat. Theor Appl Genet 112:1195–1204
44. Wardlaw IF, Wrigley C (1994) Heat tolerance in temperate cereals: an overview. Aust J Plant Physiol 21:695–703
45. Savin R, Nicolas M (1999) Effects of timing of heat stress and drought on growth and quality of barley grains. Aust J Agric Res 50:357–364
46. Stone PJ, Nicolas ME (1996) Effect of timing of heat stress during grain filling on two wheat varieties differing in heat tolerance. II. Fractional protein accumulation. Aust J Plant Physiol 23:739–749
47. Easterling D, Horton B, Jones P, Peterson T, Karl T, Parker D, Salinger M, Razuvayev V, Plummer N, Jamason P, Folland C (1997) Maximum and minimum temperature trends for the globe. Science 277:364–367
48. Meehl GA, Tebaldi C (2004) More intense, more frequent, and longer lasting heat waves in the 21st century. Science 305:994–997
49. Hawker JS, Jenner CF (1993) High temperature affects the activity of enzymes in the committed pathway of starch synthesis in developing wheat endosperm. Aust J Plant Physiol 20:197–209
50. Stone PJ, Gras PW, Nicolas ME (1997) The influence of recovery temperature on the effects of a brief heat shock on wheat. III.

Grain protein composition and dough properties. J Cereal Sci 25:129–141
51. Canvin D (1965) The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. Can J Exp Bot 43:63–69
52. Garcés R, Mancha M (1991) In vitro oleate desaturase in developing sunflower seeds. Phytochem 30:2127–2130
53. Izquierdo N, Aguirrezábal LAN, Andrade F, Cantarero M (2006) Modeling the response of fatty acid composition to temperature in a traditional sunflower hybrid. Agron J 98:451–461
54. Martre P (2006) Modelling quality traits and their genetic variability for wheat. Eur J Agron 25:75–78
55. Triboï E, Martre P, Triboï-Blondel AM (2003) Environmentally-induced changes in protein composition in developing grains of wheat are related to changes in total protein content. J Exp Bot 54:1731–1742
56. Champolivier L, Merrien A (1996) Evolution de la teneur en huile et de sa composition en acides gras chez deux variétés de tournesol (oléique ou non) sous l'effet de températures différentes pendant la maturation des graines. Oleagineux Corps Gras Lipides 3:140–145
57. Rotundo JL, Westgate ME (2010) Rate and duration of seed component accumulation in water-stressed soybean. Crop Sci 50:676–684
58. Flagella Z, Rotunno T, Tarantino E, Di Caterina R, De Caro A (2002) Changes in seed yield and oil fatty acid composition of high oleic sunflower (*Helianthus annuus* L.) hybrids in relation to the sowing date and the water regime. Eur J Agron 17:221–230
59. Payne PI, Holt LM, Worland AJ, Law CN (1982) Structural and genetical studies on the high-molecular-weight subunits of wheat glutenin. Theor Appl Genet 63:129–138
60. Steer BT, Seiler GJ (1990) Changes in fatty acid composition of sunflower (*Helianthus annuus*) seeds in response to time of nitrogen application, supply rates and defoliation. J Sci Food Agric 51:11–26
61. Zheljazkov VD, Vick BA, Baldwin BS, Buehring N, Astatkie T, Johnson B (2003) Oil content and saturated fatty acids in sunflower as a function of planting date, nitrogen rate, and hybrid. Agron J 101:1003–1011
62. Calderini DF, Dreccer MF (2002) Choosing genotype, sowing date and plant density for malting quality. In: Slafer GA, Molina-Cano JL, Savin R, Araus JL, Romagosa I (eds) Barley science. Recent advances from molecular biology to agronomy of yield and quality. Food Product Press/The Haworth Press, New York, pp 413–444
63. Gooding MJ, Davies WP (1997) Wheat production and utilization. Systems, quality and the environment. CAB International, Wallingford, 355 p
64. Wrigley CW, Bekes F (2004) Processing quality requirements for wheat and other cereal grains. In: Benech-Arnold R, Sanchez RA (eds) Handbook of seed physiology: applications to agriculture. The Haworth Press, New York, pp 389–405
65. Garcés R, Mancha M (1989) Oleate desaturation in seeds of two genotypes of sunflower. Phytochem 28:2593–2595
66. Borrás L, Slafer GA, Otegui ME (2004) Seed dry weight response to source-sink manipulations in wheat, maize and soybean: a quantitative reappraisal. Field Crop Res 86: 131–146
67. Savin R, Prystupa P, Araus JL (2006) Hordein composition as affected by post-anthesis source-sink ratio under different nitrogen availabilities. J Cereal Sci 44:113–116
68. Stone PJ, Savin R (1999) Grain quality and its physiological determinants. In: Satorre EH, Slafer GA (eds) Wheat: ecology and physiology of yield determination. Food Product Press/ The Haworth Press, New York, pp 85–120
69. Zahedi M, Mc Donald G, Jenner CF (2004) Nitrogen supply to the grain modifies the effects of temperature on starch and protein accumulation during grain filling in wheat. Aust J Agric Res 55:551–564
70. Dupont FM, Hurkman WJ, Vensel WH, Tanaka C, Kothari KM, Chung OK, Altenbach SB (2006) Protein accumulation and composition in wheat grains: effects of mineral nutrients and high temperature. Eur J Agron 25:96–107
71. Passarella VS, Savin R, Slafer GA (2008) Are temperature effects on weight and quality of barley grains modified by resource availability? Aust J Agric Res 59:510–516
72. Aguirrezábal LAN, Martre P, Pereyra-Irujo G, Izquierdo N, Allard V (2009) Management and breeding strategies for the improvement of grain and oil quality. In: Sadras VO, Calderini DF (eds) Crop physiology: applications for genetic improvement and agronomy. Academic/Elsevier, New York, pp 387–410
73. Martre P, Porter JR, Jamieson PD, Triboï E (2003) Modeling grain nitrogen accumulation and protein composition to understand the sink/source regulations of nitrogen remobilization for wheat. Plant Physiol 133:1959–1967
74. Pereyra-Irujo GA, Aguirrezábal LAN (2007) Sunflower yield and quality interactions and variability: analysis through a simple simulation model. Agr For Meteorol 143:252–265

Books and Reviews

Aguirrezábal LAN, Andrade FH (1998) Calidad de productos agrícolas. Bases ecofisiológicas, genéticas y de manejo agronómico. Unidad Integrada INTA Balcarce, Balcarce, 315 p

Baskin CC, Baskin JM (1998) Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press/ Elsevier, San Diego, 666 p

Basra AS, Randhawa LS (2002) Quality improvement in field crops. Food Products Press/The Haworth Press, New York, 433 p

Benech-Arnold RL, Sánchez RA (2004) Handbook of seed physiology: aplications to agriculture. Food Products Press/The Haworth Press, New York, 483 p

Bewley JD, Black M (1985) Seeds: physiology of development and germination, 1st edn. Plenum, New York, 125 p

Gunstone FD, Harwood JL, Dijkstra AJ (2007) The lipid handbook with CD-ROM, 3rd edn. CRC Press, Boca Raton, 1472 p

Sadras VO, Calderini DF (2009) Crop physiology: applications for genetic improvement and agronomy. Academic Press/ Elsevier, New York, 583 p

Schneiter AA (1997) Sunflower technology and production. ASA, CSSA & SSSA, Madison, 834 p

Simmonds DH (1989) Wheat and wheat quality in Australia. CSIRO, Melbourne, 299 p

Slafer GA, Molina-Cano JL, Savin R, Araus JL, Romagosa I (2002) Barley science: recent advances from molecular biology to agronomy of yield and quality. Food Products Press/The Haworth Press, New York, 551 p

Triboi E, Triboi-Blondel AM (2002) Productivity and grain or seed composition: a new approach to an old problem. Eur J Agron 16:163–186

Increasing Salinity Tolerance of Crops

Stuart J. Roy, Mark Tester
Australian Centre for Plant Functional Genomics, University of Adelaide, Adelaide, SA, Australia

Article Outline

Glossary

Exclusion The ability to maintain low concentrations of toxic ions in the plant shoot.

Ionic stress The stress imposed on a plant by the accumulation of salts to toxic concentrations in cells, particularly those of the shoot, leading to premature death.

Genetically modified plant A plant which has been transformed by artificial means with single or multiple genes from another variety or species.

Osmotic stress The stress imposed on a plant by the accumulation of high concentrations of salt around the root, which reduces plant growth.

Osmotic tolerance The ability of a plant to maintain growth under osmotic stress.

Saline soil Soils affected by excess accumulation of salts. Accumulation of sodium chloride (NaCl) on agricultural land has a severe impact on crop yield.

Salt tolerant plant A plant with the ability to grow and set seed in saline environments without significant reductions in plant biomass or yield.

Selective breeding Where two plant species with desirable phenotypes are bred together in an attempt to produce an offspring with both traits.

Tissue tolerance The ability to withstand high concentrations of toxic ions in the shoot.

Definition of the Subject

Plant growth and yield are severely affected by saline soils. High concentrations of salt in the soil make it difficult for plants to take up water, while the accumulated salts in cells, particularly the sodium (Na^+) and chloride (Cl^-) ions, are toxic to plant metabolism. These two factors result in a reduction in plant growth, an increase in the rate of leaf senescence, and a loss in crop yield. The fact that significant areas of farmland worldwide are affected by salt brings with it potentially serious implications for crop yield.

Introduction

Saline soils have been defined as areas where the electrical conductance (ECe; a means of measuring the amount of ions in the soil) is greater than 4 dS/m. It is at around 4 dS/m (approximately 40 mM NaCl) that most plants start to exhibit significant reductions in yield [1]. Over 800 million hectares of land worldwide are affected by saline soils; this accounts for more than 6% of the total land area of the world [2]. Most of this salt-affected land has arisen from natural causes, such as the weathering of rocks, which releases a variety of soluble salts including Cl^-, Na^+, calcium, magnesium, sulfates, and carbonates [3]. Other sources of salt accumulation include the deposition of salts from seawater that is transported by wind and rain, as well as from salts carried in rainwater. It has been estimated that rainwater contains 6–50 mg/kg of sodium chloride (NaCl) which, over time, results in large-scale salt depositions [1].

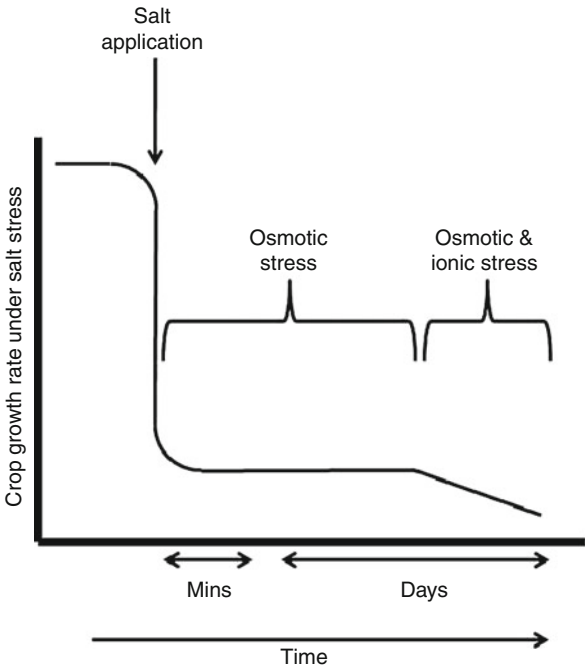
In addition to the natural processes of salinization, farmland areas are affected by secondary types of salinity which are a consequence of human activities such as land clearing and/or irrigation. This secondary form of salinity results in the raising of water tables and an increase in the concentration of salts around plant roots. Approximately 32 million hectares of the 1,500 million hectares farmed by dryland agriculture are affected by secondary salinity, while 45 million hectares of the 230 million hectares of irrigated land are salt

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

affected [2]. Although it accounts for only 15% of the total cultivated area, irrigated land is twice as productive as dryland agriculture. Consequently, losses of yield which result from an increase in soil salinization in irrigated areas have a disproportionally large effect. Unfortunately, the areas of farmland affected by salinization are increasing and irrigated land is particularly at risk [1].

The deleterious effect of soil salinity on agricultural yields is enormous. To solve this problem will require a variety of approaches including altering farming practices to prevent soil salinization; the implementation of remediation schemes to remove salt from soils; and programs aimed at increasing the salt tolerance of crop plants, either through traditional breeding or by genetic manipulation technologies. By increasing crop salinity tolerance, plant varieties can be generated which will grow on marginal saline soils while longer term land management practices are being introduced. However, before crop salinity tolerance can be improved, an understanding is required of the two separate stresses imposed on a plant when it is grown on a saline soil: osmotic stress and ionic stress.



Increasing Salinity Tolerance of Crops. Figure 1
The effect of salt stress on the growth rate of a crop plant. Plants experience an immediate reduction in growth rate after exposure to salt as a result of osmotic stress. Overtime, the effect of ionic stress increases as shoot Na^+ concentrations build to toxic levels (Adapted from [1])

Effects of Salt Stress on Plant Growth

Osmotic Stress

Osmotic stress affects a plant as the salt concentration around the root reaches 4 dS/m and results in an immediate reduction in shoot growth [1, 4, 5] (Fig. 1). Osmotic stress reduces the rate at which growing leaves expand, the rate of emergence of new leaves, and the development of lateral buds. As this stage of salt stress concerns the inability of a plant to maintain water relations, the cellular and metabolic processes involved are similar to those observed in drought-stressed plants [6]. In dicotyledonous crop species, such as soybean, this osmotic stress results in reductions in the size of leaves and the number of branches [1]; in the monocotyledonous cereals, such as wheat, barley, and rice, the major effect is a reduction in total leaf area and number of tillers [6, 7].

Although it is the roots that are initially exposed to the saline soil, it is actually the growth of the shoot which displays a greater sensitivity to salt; root growth recovers quickly even after exposure to high levels of NaCl [4, 8]. The reduction in leaf development has been attributed to the high salt concentration outside the roots and not to toxic levels of Na^+ or Cl^- within the tissues of the plant [9–11]. This is supported by experiments where plants demonstrate reduced shoot growth when grown in a mixture of salts which individually are at concentrations below those necessary for ionic toxicity but together cause osmotic stress [7, 12]. The mechanisms underlying this down regulation of leaf growth and shoot development remain unclear, but a decrease in shoot area is likely to reduce water use by the plant, thereby conserving soil moisture and preventing an increase in the soil salt concentration. It has been suggested that this reduction in growth rate is regulated by long distance signals in the form of plant hormones and, as the reduction in growth rate is independent of carbohydrate or water supply, is not due to nutrient deficiency [13, 14]. However, it is not just vegetative growth that can be affected by osmotic stress but also the reproductive development of a crop

plant – osmotically stressed plants have been found to exhibit either early flowering and/or a reduced number of flowers [1].

Osmotic stress has other detrimental effects on crop plants. On the surfaces of their leaves, plants have stomatal pores, tiny holes through which carbon dioxide (CO_2) enters the leaf for use in photosynthesis and carbohydrate production, and water and oxygen leave the plant. Due to reduced water uptake, osmotically stressed plants close these stomatal pores [1, 15]. The consequent reduction in CO_2 assimilation results in a reduction of carbohydrate production which is detrimental to crop yield. Many plants are able to compensate partially for the reduction in the amount of CO_2 entering the leaf by producing smaller, thicker leaves with more densely packed chloroplasts although this is expensive in terms of expenditure of energy [1]. The decrease in photosynthesis caused by the closure of stomatal pores has the secondary effect of a build up of reactive oxygen species (ROS) [16, 17]. Reactive oxygen species are high energy forms of oxygen, such as superoxide and hydrogen peroxide, which can damage plant DNA and proteins. These ROS accumulate in plant leaves when the energy absorbed from sunlight by chloroplasts cannot be used to synthesize carbohydrates as there is insufficient CO_2 in the leaf to provide the carbon source. If left unchecked these ROS can cause significant damage to plants so cells must produce a range of enzymes, such as superoxide dismutase, ascorbate peroxidase, and catalase, to detoxify and convert the ROS into harmless forms [16, 17]. These detoxifying enzymes are naturally present in plants to protect leaves from sudden burst of sunlight, such as that which occurs when the sun emerges from behind a cloud, but more must be manufactured in response to salt stress, this again being an energy expensive process.

Ionic Stress

Ionic stress has a slower speed of onset than osmotic stress (Fig. 1). It occurs only when the Na^+ or Cl^- accumulation in older leaves reaches a high concentration which results in premature leaf senescence [1, 4, 6, 18]. All salts at high concentrations can affect plant growth but in saline soils it is the Na^+ and Cl^- ions which cause the most detrimental effects on growth. For some plant species, especially citrus, soybean, and grapevines, it is the accumulation of Cl^- ion in the shoot which leads to toxicity, as Na^+ is retained within the roots and the stem [18–22]. However, for most crop plants including the cereals, Na^+ reaches toxic concentrations before Cl^- and is the ion responsible for most of the damage caused to plants [1].

Na^+ and Cl^- are delivered to the shoot in the transpiration stream, that is, in the water which is being transported from the root to the shoot in the xylem of the plant. For most plants, the movement of Na^+ and Cl^- back from the shoot to the roots via the phloem is relatively small, most of the salt delivered to the shoot remaining there [1, 18]. High concentrations of Na^+ in the shoot can cause a range of metabolic and osmotic problems for plants [1, 23]. The metabolic toxicity of Na^+ is largely as a result of its ability to compete with K^+, which is required for many essential cellular functions. Over 50 essential enzymes have been shown to be activated by K^+. Consequently, high levels of cellular Na^+, which will increase the cellular Na^+:K^+ ratio and decrease the availability of K^+, can disrupt a variety of enzymatic processes [24]. In addition, protein synthesis requires high concentrations of K^+ so that tRNA can bind to ribosomes [25]. A reduction in the amount of available cellular K^+ due to high concentrations of Na^+ will disrupt protein synthesis [26]. As older leaves have ceased expanding they cannot use additional water to dilute the salt being transported into, and this leads to an increase in the senescence of older leaves. Consequently, a failure to exclude Na^+ from the shoot over time will result in the accumulation of toxic levels of ions leading to premature senescence and leaf death [4, 27]. If the rate of leaf death is greater than leaf production, the photosynthetic capacity of the plant will be reduced, the plant will be unable to supply carbohydrates to any new leaves, and the growth rate of the plant will decrease.

There is also an osmotic component to ionic stress. During ionic stress, Na^+ and/or Cl^- remain when water from the transpiration stream evaporates and can, therefore, accumulate to high concentrations in the leaf apoplast [5, 28]. These high extracellular concentrations of ions will result in water leaving cells with a consequent severe impact on cellular function. High concentrations of Na^+ and Cl^- in the leaf also present another osmotic problem, that of maintaining cellular water potential below that of the soil, thereby

facilitating water uptake for growth. Under conditions of elevated salt concentrations in the soil, plants need to accumulate solutes in order to maintain water uptake. Under such circumstances, the most readily available and energy efficient solutes are the Na^+ and Cl^- ions; however, high cellular concentrations of these ions are toxic. Although Na^+ and Cl^- can be stored within the vacuole of a cell or in the apoplastic space, plant cells have difficulty in maintaining low cytosolic Na^+ and Cl^- [29–31]. Therefore, in order to reduce the water potential within the cell, plants need to synthesize solutes which can be accumulated at high concentrations in the cytoplasm of cells without interfering with metabolism [32, 33]. The synthesis of such compatible solutes, however, is energetically expensive and can make significant demands on the energy resources of a plant.

Overall, in comparison with non-stressed plants, salt stressed plants grow more slowly and die more rapidly. It has been estimated that, due to its immediate effect on plant growth, the osmotic stress has a greater impact than ionic stress on the growth rate of a crop [1]. Ionic stress affects plants only at a later stage and has a lesser effect than osmotic stress, particularly at moderate salinity levels. Only when salt levels are high or if a plant is extremely salt sensitive will the ionic effect be greater than the osmotic.

Variation in Plant Salinity Tolerance

Plants vary widely in their response to saline soils. Many show reduced rates of growth and yield while others, such as the salt tolerant saltbush (*Atriplex amnicola*), only reach an optimal growth rate when a moderate level of salt is present [1, 34]. Depending on their sensitivity to saline soils, plants can be divided into two groups: the salt sensitive glycophytes, which are relatively easily damaged by salt; and the salt tolerant halophytes which can tolerate, and may even require, high concentrations of salt in the soil. Indeed, the halophytic saltbush has been shown to survive at concentrations of salt similar or higher than that of seawater [34]. It has been estimated that only 2% of plant species are true halophytes, while the majority of species, including most crops, are glycophytes [35]. Within the monocotyledonous cereals, rice (*Oryza sativa*) is one of the most salt sensitive [36–39], and shows a significant decrease in growth and yield when exposed to moderate levels of NaCl. By contrast, barley (*Hordeum vulgare*) is significantly more salt tolerant [1, 40]. While not as tolerant as barley, the hexaploid bread wheat (*Triticum aestivum*), which contains the genomes of three different wheat species (AABBDD), is moderately salt tolerant and is able to exclude 97–99% of Na^+ entering the shoot. The tetraploid durum wheat (*T. Turgidum* ssp *durum*), which has the genomes of two species (AABB), is more salt sensitive than bread wheat as it lacks genes for salinity tolerance found on the bread wheat D genome and can exclude only 94–95% of the Na^+ entering the root [6, 30]. In durum wheat, there is a clear deleterious relationship between the amount of Na^+ that accumulates in its shoot and the yield of the plant: the higher the Na^+ concentration, the lower the yield [41].

The variation in salinity tolerance of dicotyledonous crop species is even greater than that observed in the cereals. On a scale of salt sensitivity, sugar beet has been reported as salt tolerant, cotton and tomatoes intermediate in tolerance, and chickpea, beans, and soybean as sensitive to salt [42, 43]. Many fruit trees, such as citrus, are classified as very salt sensitive [43]. A number of legumes have been shown to be extremely salt sensitive, even more so than rice; others, such as alfalfa (*Medicago sativa*) are more salt tolerant than barley [1]. In addition to this variation in salinity tolerance between different crop species, variation also exists within species, some varieties and lines having significantly greater salinity tolerance than others [40–44].

Mechanisms of Salt Tolerance

Osmotic Tolerance

Osmotic stress immediately reduces the expansion rate of shoots and roots. It also results in the closure of stomatal pores. Plants that are more tolerant to osmotic stress will exhibit greater leaf growth and stomatal conductance. This would be desirable in irrigated farmland where water is not limiting, but may be problematic in dryland agricultural systems if the soil water content is depleted before the end of the growing season.

Although it is believed that considerable variation for osmotic tolerance may exist within crop species, until recently this was not easily measured. The estimation of growth rates requires daily measurements of

leaf growth or measurements of stomatal conductance [7, 41, 45–47]. These methods are usually either time consuming or have required destructive measurements of plant material to ensure accuracy. Nondestructive imaging technologies have been developed which use digital photographs to calculate plant area and mass [48], or infrared thermography to measure leaf temperature and, thereby, stomatal conductance [49]. These technologies have been used to measure the growth rates of plants in saline environments and, hence, measurement of osmotic tolerance. Variation for osmotic tolerance has now been observed in durum wheat [45, 49] and in wild relatives of wheat, such as *T. monococcum* which is a modern day variety of the plant which donated the A genome to both durum and bread wheat [48].

Ionic Tolerance

Na^+ can accumulate in the shoots of plants to reach toxic levels at concentrations which are below those required of Cl^- for toxicity. Consequently, most studies have focused on revealing any variation in shoot Na^+ accumulation and on the transport of Na^+ within the plant. Ion concentrations in specific tissues can easily be measured at a specific developmental age, and either image analysis [48] or a meter that measures chlorophyll content can be used to measure leaf senescence.

Ionic Tolerance: Exclusion A long established mechanism for salinity tolerance in crop plants is the exclusion of ions, particularly Na^+, from the shoot. Due to the ease of experimentation, this is the mechanism perhaps most studied. A strong correlation between salt exclusion and salt tolerance has been shown for many crops, such as in durum wheat [41, 50], rice [51, 52], barley [40, 53, 54], lotus [55], and *Medicago* [56]. Na^+ enters a plant initially from the soil through the root and is then rapidly transported to the shoot in the water of the transpiration stream. Roots are able to maintain relatively constant levels of NaCl by exporting excess salt either back to the soil or to the shoot. As a result, there is a higher accumulation of Na^+ in the shoot compared with the root. If the net delivery of Na^+ to the shoot could be reduced, this may enable a plant to become more salt tolerant. There are four distinct components that can be modified in order to reduce shoot Na^+ and Cl^- concentrations, all of which occur in the root: reduction in the initial influx of ions from the soil into the root; maximization of the efflux of ions from the roots back to the soil; reduction of the efflux of ions from the inner root cells into the xylem cells which are carrying water and ions to the shoot in the transpiration stream; and maximization of ion retrieval from the transpiration stream into root cells thereby retaining Na^+ and Cl^- in the root.

Ionic Tolerance: Tissue Tolerance Tissue tolerance is the ability to accumulate Na^+ or Cl^- ions in the absence of any detrimental effects on plant health. Tolerance requires the toxic ions to be compartmentalized into areas where they can do no damage. At the cellular level, this usually involves avoiding the accumulation of Na^+ and Cl^- in the cytoplasm of the plant cell where most of important metabolic processes occur. One strategy of tissue tolerance involves compartmentalization of ions within the vacuole, a large plant cell organelle which can be used as a storage structure. Employing such a mechanism will allow a plant to accumulate high concentrations of Na^+ and Cl^- within the shoot, while avoiding all of the toxicity effects. There already exists a large body of evidence for variation between different varieties of crops in terms of the rates of accumulation of shoot Na^+ and Cl^-, as well as for the concentrations of these ions which the different varieties can tolerate.

Generation of Salt Tolerant Crops

Salt tolerant crop plants may be generated only once there is a clear understanding of the mechanisms underlying salinity tolerance, and of the variation between plant species in effecting such mechanisms. Once identified, the benefit of introducing these salinity tolerance mechanisms into crops must be considered. For example, there would be little point in introducing into a cereal the salinity tolerance mechanisms from a slow growing highly tolerant halophyte if that mechanism involved a slow growth phenotype which would result in the cereal taking years to reach maturity for flowering. In addition, a salt tolerant crop plant must do as well as a sensitive plant when grown in the absence of salt. A high yielding salt sensitive crop which shows a 50% yield reduction under salt stress

will still be of greater value to a farmer than a salt tolerant variety which displays little reduction in yield but which produces only 40% as much grain as the salt stressed sensitive variety in the first place.

Crop plants developed to have increased tolerance to both ionic and osmotic stresses would be able to grow at productive rates throughout the life cycle, and the severe losses of yield experienced for most crops growing on saline soils would be reduced. It should also be noted that it may be necessary to develop crop plants with different salinity tolerance mechanisms depending on the environment in which the plants will grow. Crops grown by dryland agriculture may benefit particularly from possessing tissue tolerance mechanisms, as the accumulation of high concentrations of ions within the vacuoles of the plant cells may assist the plant in retrieving more water from the soil. By contrast, an osmotic tolerance strategy, combined with Na^+ exclusion, may be more beneficial to crops grown under irrigation so that water availability is not an issue but the Na^+ content in that water may be high.

Two approaches may be taken to improve the salt stress tolerance of current crops: the exploitation of natural variation in salinity tolerance between different varieties and species of crops; or, the generation of transgenic plants with altered gene expression to increase salinity tolerance. Both approaches have advantages and disadvantages as discussed below.

Exploitation of Natural Variation

For many crop species there exists large natural variation in salinity tolerance mechanisms, with some lines and varieties producing significant yields under salt stressed environments. The screening of 5,000 accessions of bread wheat led to the identification of 29 accessions which produced seed when grown in 50% seawater [57], while screening of 400 Iranian wheats identified several accession with high grain yield under both salt stressed and control environments [58]. Varietal differences in yield in saline conditions have been observed in many crops such as durum wheat [41, 45, 59], barley [60, 61], soybean [62], citrus [19, 63], chickpea [42, 64], and rice [65, 66]. The selection and breeding of these salt tolerant varieties with the current elite varieties grown by farmers would be a step forward in the generation of salt tolerant plants.

The selective breeding of lines with desirable salt tolerance traits with those lines possessing desirable traits for yield is an approach for generating salinity tolerant crops that has been practiced for thousands of years. One limitation with this approach is the time and space necessary to grow offspring from these crosses, test their salinity tolerance, obtain viable seed, and then repeat the crossing with a parent to produce the next generation. Recently, new molecular technologies have been developed which have aided this approach considerably. Different varieties and species have different DNA sequence. The difference between the DNA may be subtle, such as between varieties of the same species where there may be a single nucleotide change in the coding sequence of a gene, or the differences can be extreme, such as the gene duplications or deletions observed between species. Modern molecular techniques enable the detection of these differences between individuals, varieties, and species and allow the design of molecular markers which recognize specific differences in the DNA between two individuals. Using these molecular markers as DNA landmarks it is possible to produce a map of plant chromosomes which can be divided into regions. By finding differences in regions of DNA between two varieties of plants and then observing the phenotype of the offspring produced by breeding the two original varieties, it is possible to identify regions in DNA linked to that phenotype. These regions are often called quantitative trait loci (QTL). By identifying two different plant varieties with differences in salinity tolerance and by observing molecular markers that are different between the two parents, it is possible to identify QTL linked to salinity tolerance by screening their offspring. As salt tolerance is a complex trait, both genetically and physiologically, it is not uncommon to observe several QTL associated with tolerance.

QTL have now been identified for salinity tolerance in a number of plant species including barley [67, 68], tomato [69], rice [70], citrus [63], bread wheat [71], and durum wheat [41]. When QTL have been discovered, one approach is to then identify the gene in that region of DNA which is responsible for the salt tolerance phenotype. The *SKC1* QTL identified on chromosome 1 in rice and the *Nax1* and *Nax2* loci observed in durum wheat on chromosomes 2A and 5A, respectively, have been narrowed down to genes belonging

to a family of Na^+ transporters [70, 72, 73], which have been shown previously to be important for exclusion of Na^+ from the shoot [74–79]. Once a QTL has been discovered, the plant which contains that important piece of DNA for salt tolerance can be bred with salt sensitive varieties to introduce into them the salt tolerant phenotype. As a molecular marker will be linked to the QTL, it is not necessary to screen every offspring produced from this cross with a salt sensitivity assay, rather, it is possible to identify which of the offspring have the piece of DNA important for salt tolerance by screening for the molecular marker linked to the salt tolerance QTL. While this does not necessarily speed up the length of time it takes an individual plant to reach maturity, it does reduce the necessity to screen hundreds of plants in saline conditions looking for those that are salt tolerant, so that more focus can be placed on breeding tolerance traits into crops.

While one approach is to identify a variety of a crop with good salt tolerance and then cross it to other members of that species, a second approach is to introduce salt tolerance traits from related species or near-wild relatives.

Bread wheat and durum wheat are two separate plant species, but because of their genetic background there is the possibility of breeding these two species together to exchange valuable traits. Durum wheat is a tetraploid (AABB) containing two genomes from an ancient ancestral cross, the A genome and the B genome. Bread wheat is a hexaploid (AABBDD), with the same A and B genomes as durum wheat and also a third genome, the D genome [80]. It is possible to breed a bread wheat and durum wheat together to produce a pentaploid hybrid offspring, which has an AABBD genome. During sexual reproduction, there is the possibility of the chromosomes from the different wheat backgrounds to swap DNA, a process called recombination, thereby transferring genes from bread wheat to durum wheat and vice versa. Importantly, however, only chromosomes from the same genome can recombine, i.e., durum genome A with bread genome A and not durum genome A with bread genome B. By crossing this offspring with either bread wheat or durum wheat it is possible to re-obtain tetraploid durum wheat and hexaploid bread wheat, only now containing genes from the other species. This has been done successfully to transfer disease resistance genes [80] and could be used for transferring salt resistance traits between the two species. Although difficult, because durum wheat contains no D genome, it is possible to introduce genes from the bread wheat D genome into durum wheat; however, a special wheat plant, with a mutation that affects the way in which chromosomes align in recombination, is required [81]. This technique was used to transfer the K^+/Na^+ discrimination locus *Kna1* from chromosome 4D of bread wheat to chromosome 4B of durum wheat [82]. This new durum wheat line was able to maintain a high K^+/Na^+ ratio in the leaves [82, 83], thereby increasing its salinity tolerance. However, there was no significant difference in grain yield between durum plants with the bread wheat *Kna1* and those without, perhaps due to a yield penalty imposed by having a large section of the bread wheat D genome in durum wheat. Unfortunately, no agronomically acceptable durum variety containing the bread wheat *Kna1* locus has been released [80].

In addition to looking for variation in plant salt tolerance in current cultivars, there is the possibility of introducing salinity tolerance traits to crop from their near-wild relatives. These species may have been evolving in areas of high salinity, away from the selective pressures inflicted on domesticated crops. It is, therefore, likely these relatives have developed novel salt tolerance mechanisms which might be introduced into current crops. This approach is not new and there have been many attempts to introduce genes from salt tolerant wild relatives to current salt sensitive crops. Traits for salt tolerance have been discovered in wild relatives of tomato, [84], potato [85], rice [44], wheat [80, 86–88], and barley [40, 86] and several attempts have been made to introduce them to cultivated crops. Screening of eight wild *Hordeum* species, wild relatives of domesticated barley, revealed that seven of the eight had better Na^+ and Cl^- exclusion than domesticated barley under a variety of salt stressed environments. A number of these relatives, such as *H. spontaneum*, *H. marinum*, and *H. intercedens*, had significantly higher relative growth rates than domesticated barley when grown under high salinity stress [40, 89].

T. urartu (AA) is the modern day ancestor of the species that gave rise to the A genomes of

durum and bread wheat. Both *T. urartu* and other closely related A genome species, such as *T. monococcum* spp. *monococcum* and *T. monococcum* spp. *aegilopoides*, show greater Na^+ exclusion than durum wheat [80, 90]. Lines of *T. monococcum* also show great variation in both osmotic and Na^+ tissue tolerance [48] and are, therefore, a potential source of novel genes for salinity tolerance. It is possible to cross these species with durum wheat and transfer salinity tolerance traits. One success story of breeding a salinity tolerant crop has been the introduction of a Na^+ exclusion trait into durum wheat from a near-wild relative *T. monococcum*. Screening of multiple durum wheat lines for Na^+ exclusion from the shoot identified a durum landrace, line 149, with significantly lower shoot Na^+ than cultivated durum [59]. It was discovered that Na^+ exclusion in these lines was controlled by two major genes, *Nax1* and *Nax2*, which had been introduced into durum from a cross with *T. monococcum* [91]. These two genes have now been introduced separately into the Australian durum wheat Tamaroi and have undergone field trials.

Another wild relative source for wheat is from *Aegilops tauschii*, which is the modern day version of the species that donated the D genome to bread wheat – there appears to be no modern day equivalent of the B genome [80]. Several screens of *Ae. tauschii* have identified lines with lower shoot Na^+ accumulation and enhanced K^+/Na^+ discrimination than durum wheat, although the phenotype was comparable to bread wheat [29, 71, 92]. As the natural environment of *Ae. tauschii* is dry and moderately saline [80], there exists the possibility of introducing novel salinity tolerant genes into wheat. One possibility is the re-creation of the original cross that generated bread wheat by breeding durum wheat (AABB) with *Ae. tauschii* (DD), thereby creating a synthetic wheat (AABBDD) with a genome similar in style to bread wheat. While the technique is tricky, it has been successful in the past [92]. The advantage of this technique is that the Na^+ exclusion locus found on the D is introduced which is not on the A or B genomes of wheat. Synthetic hexaploid lines with enhanced Na^+ exclusion have been created successfully to have Na^+ exclusion similar to that of the parent *Ae. tauschii* and significantly greater exclusion than that of the durum parent [29] at both high and moderate levels of salt. Indeed, some of the synthetic hybrids produced have significantly lower shoot Na^+ accumulation than bread wheat and often greater yield under salt stress conditions [80, 93]. These results indicate that the approach clearly has validity.

The use of wild relatives in breeding programs remains controversial as few salt tolerant crops are released through this approach [39]. Wheat was one of the earliest crops to be crossed with halophytic wild relatives but over 25 years have elapsed since that initial cross, and no new tolerant varieties has yet been released to farmers [39]. However, a recent report of significant yield advantage in a saline field site of durum wheat plants incorporating a Na^+-excluding locus, *Nax2*, from *T monococcum* appears to be particularly promising [94].

A considerable disadvantage with introducing salinity tolerance traits into crops which are already well adapted for cultivation is the introduction of undesirable traits encoded by genes which may be physically close to the desirable gene for salinity tolerance in the plant genome [80]. This is a particular problem when breeding current crop varieties with wild relatives, as cultivated crops have been designed by breeders for thousands of years to have desirable traits such as high grain yield, appropriate height and disease resistance. When new traits are introduced into crops by breeding it is not possible to introduce only the gene responsible for that trait. The piece of DNA introduced from the wild relative can be quite large and will contain many genes, for most of which the functions are unknown. If these genes have an undesirable effect which impacts on the agricultural value of the crop leading, for example, to low yield or incorrect flowering time, the crop will be of no value to a farmer. This phenomenon is known as linkage drag [39, 80]. It is possible to reduce the size of the DNA insertion from the wild species by breeding the line with a cultivated crop as, over time, fragments of the wild species DNA will be replaced by that of the cultivated crop. This process, however, can take many generations and requires the breeder to have a molecular marker specific to the Na^+ tolerance gene that has been inserted into the genome; otherwise this gene also would be lost [80].

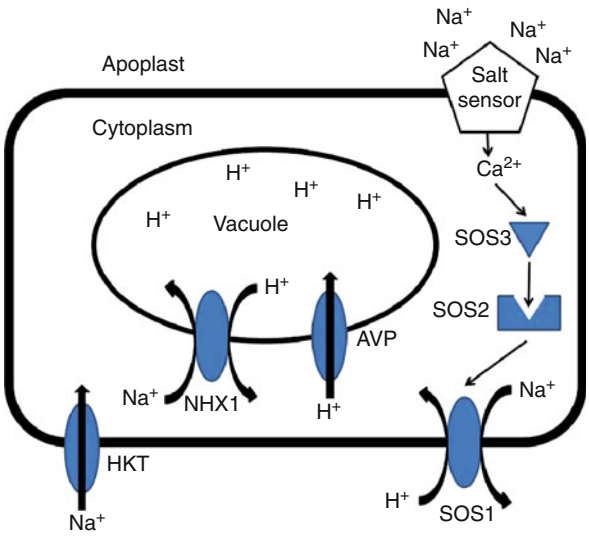
Transgenic Approaches to Generating a Salt Tolerant Crop

Transgenic approaches are attractive in the generation of salinity tolerant plants, as the sequences of genes known to encode proteins involved in salinity tolerance can be artificially introduced directly into the target variety, without the compounding effects of bringing in multiple, and often undesirable, genes through traditional breeding approaches. In theory, the transformation of commercially relevant crop plants directly with genes for salinity tolerance would help to reduce the time required before farmers can use these crops in the field.

There are numerous possibilities for generating transgenic crops with increased salinity tolerance, either by introducing novel genes for salinity tolerance into crops from other plant species, or by altering the expression of existing genes within the crop (see Fig. 2 for examples).

To date, the greatest success with the development of transgenic salinity tolerant crops has been the generation of plants which are better able to compartmentalize Na^+ in the vacuole, where Na^+ can accumulate to high levels without detrimental effects on the plant cells. Central to this process of vacuolar compartmentation is a gene encoding a vacuolar Na^+/H^+ antiporter (NHX), which transports Na^+ into the vacuole in exchange for a proton (H^+) [1, 95, 96] (Fig. 2). The activity of this transporter has been shown to increase under salinity and it is expressed in a variety of different plant species including barley [97], maize [98], sunflower [99], tomato [100], cotton [101], and Arabidopsis [102]. Constitutive expression in yeast of the *NHX* gene from Arabidopsis, *AtNHX1*, had the effect of significantly increasing the salinity tolerance of the yeast [103, 104]. Transgenic plants which have been created to constitutively overexpress the same Arabidopsis *AtNHX* gene, such as Arabidopsis [105], tomato [106], *Brassica napus* [107], and cotton [108], also show increased Na^+ accumulation in the shoot and greater salinity tolerance. These plants are, therefore, Na^+ tissue tolerators. Importantly from a farmer and a consumer point of view, Na^+ accumulation only occurred in the green tissue and not in the fruit, as in the case for tomato [106]. Of particular interest is that both increasing or decreasing the expression of the Arabidopsis *AtNHX* gene has been shown to significantly affect the expression patterns of other genes involved in salinity tolerance mechanisms [96, 109, 110]. This finding has significant implications for the generation of transgenic crops as it indicates that it may not be necessary to transform a plant with multiple salinity tolerant genes but rather with one gene which can regulate others.

Several homologues of the *AtNHX1* gene have now been identified in a number of crops including



Increasing Salinity Tolerance of Crops. Figure 2 Intracellular location of proteins in a plant cell which are encoded by candidate genes for transformation into transgenic crop plants. NHX1 is a transporter which is involved in the compartmentation of Na^+ into the vacuole by swapping a cytoplasmic Na^+ ion with a vacuolar proton (H^+). AVP is a proton pump that uses the energy released from the breakdown of PPi to move protons into the vacuole. These protons can then be used by transporters such as NHX to transport Na^+ into the vacuole. HKT proteins are involved in the transport of Na^+ from the extracellular space (apoplast) into the cytoplasm. In the salt overly sensitive (SOS) pathway, high concentrations of Na^+ are detected by a membrane bound salt sensor, which results in the release of Ca^{2+} to the cytoplasm. This cytoplasmic Ca^{2+} binds to the calcium binding protein SOS3, which activates the protein kinase SOS2. Together SOS3 and SOS2 activate the Na^+ transporting ability of the SOS1, which moves Na^+ out of the cell

wheat [111, 112], barley [113], cotton [101], *Medicago* [114], Maize [98], and rice [115, 116], and the constitutive overexpression of these gene in Arabidopsis [111], rice [115–117], wheat [118], tobacco [101], and barley [113] has also been reported to improve salinity tolerance.

Another candidate gene family for the generation of salinity tolerant transgenic crops are the vacuolar H^+ pyrophosphatase genes [1, 96, 119]. Similar to the *NHX* genes, H^+ pyrophosphatase genes, such as Arabidopsis *AVP1*, are involved in Na^+ sequestration to the vacuole. These genes do not encode proteins that are directly responsible for the transport of Na^+ into the vacuole, but rather ones that use the energy released from the breakdown of the high energy molecule inorganic pyrophosphate (PPi) to pump protons (H^+) into the vacuole (Fig. 2). PPi is produced as a by-product of a wide range of biosynthetic pathways. Use of PPi as an energy donor for the activity of the vacuolar H^+-PPase allows ATP to be conserved and improves plant cell performance under more demanding environmental conditions. Once the vacuolar H^+ pyrophosphatase proteins have transported protons into the vacuole, these protons can then be used by Na^+ transporters such as NHXs to move Na^+ into the vacuole. Analysis of plants that are growing under salt stress, such as barley and Arabidopsis, reveals that these genes are significantly upregulated [102, 113]. Arabidopsis, alfalfa (*Medicago sativa*), tobacco (*Nicotiana tabacum*), bentgrass (*Agrostis stolonifera* L.), and rice plants genetically engineered to either express *AVP1* alone, or in combination with *NHX*, have been shown to have increased salinity tolerance [111, 117, 119–122]. Transgenic alfalfa which was constitutively overexpressing *AtAVP1* maintained a greater shoot biomass than wild type alfalfa when grown on 200 mM NaCl [122]. Similarly, transgenic bentgrass expressing the *AtAVP1* gene was not greatly affected when grown on 100 mM NaCl, and was able to survive salt stress of 200 and 300 mM NaCl, levels which severely reduced the growth of wild type bentgrass [123].

In addition to the success in generating salt tolerant plants using genes involved in the mechanisms for sequestering Na^+ in the vacuole, transformation of plants with genes controlling other processes, such as exclusion of Na^+ from the plant, have also been successful. Other candidate genes for increasing the salinity tolerance of crop plants include members of the Salt Overly Sensitive (SOS) pathway.

Many aspects of plant growth, development, and responses to environmental stresses are mediated by the calcium ion (Ca^{2+}) as a secondary messenger signaling molecule. The external cue is first perceived by receptors on the plant cell membrane and this then activates a signaling cascade, using calcium, which regulates the activities of proteins and gene expression [124–127]. The SOS pathway mediates the response of a plant cell to salinity stress. The SOS pathway was so named due to the extreme salt sensitivity of plants which had mutations in key genes of this pathway [128]. Initially, three genes from these mutants, *AtSOS1*, *AtSOS2*, and *AtSOS3*, were identified in Arabidopsis as being important in salinity tolerance [129]. It should be noted, however, that the SOS name refers to a specific salt sensitive phenotype and that the genes sharing the same SOS identifier are unrelated to each other. Indeed, the proteins encoded by these genes are quite different: AtSOS1 is a plasma membrane Na^+ transporter [130]; AtSOS2 is a protein kinase belonging to a large family of Calcineurin B-like Interacting Protein Kinases (CIPKs) [125, 127]; and AtSOS3 is a plasma membrane bound Ca^{2+} binding protein which belongs to the Calcineurin B-Like proteins (CBL) [125, 127]. However, although they have completely different functions, it is the interactions of these proteins that help a plant cell survive salt stress.

It has been shown in Arabidopsis that under salt stress Ca^{2+} is released into the plant cell cytoplasm from either internal or external cellular stores and it binds to the plasma membrane bound AtSOS3 (AtCBL4). CBL proteins have specific regions which allow them to bind to specific CIPKs, such as SOS2. When Ca^{2+} becomes bound to AtSOS3, it recruits AtSOS2 to the plasma membrane where the kinase phosphorylates the Na^+/H^+ antiporter AtSOS1, thereby activating the transporter and allowing the movement of Na^+ out of the cell [1, 124, 125, 127] (Fig. 2). Although these genes were identified initially in Arabidopsis, homologues for all three genes have now been discovered in a variety of plant species, including crops, such as *Thellungiella halophila* [131], poplar [132], and rice [133]. In all of these species, the genes involved in the SOS pathway have been shown to be significantly upregulated under salt stress,

particularly in the plant roots. This would make them ideal as candidate genes for transformation into transgenic crops to increase salinity tolerance.

Arabidopsis plants that were engineered to constitutively express the *AtSOS1* gene had significantly increased salinity tolerance, showing greater biomass, increased chlorophyll retention, and reduced concentrations of Na^+ in the shoot when compared to wild type plants when grown under high saline conditions [134]. Importantly, these plants did not suffer any yield penalty when grown under non-stressed conditions. The increase in the salinity tolerance of the transgenic plants was attributed to them having a great efflux of Na^+ at the cellular level, when compared to control plants.

It is not always necessary to generate a salt tolerant plant by altering the expression level of a gene that encodes a transporter of ions. The salinity tolerance of a plant can also be increased by overexpressing genes encoding molecules that are involved in signaling or activating genes. Overexpression of the transcription factor *Alfin1* in alfalfa resulted in plants with increased root and shoot growth under both control and salt stressed conditions [135]. Enhanced expression of genes involved in signaling pathways, such as those encoding calcium binding CBL proteins and the protein kinase CIPKs, increases the salt tolerance of Arabidopsis, rice, and tobacco [136–139], presumably through enhancing the signaling response of the cell when it is under salt stress. However, the way in which some genes contribute to overall salt tolerance remains unclear. Transgenic tomato that had been transformed with the yeast gene *HAL1* showed increased salt tolerance under stressed conditions but had reduced shoot weight when grown in control conditions, significantly lower than non-transformed plants [140]. This demonstrates that there remains significantly more to understand about the timing and regulation of genes *in planta* before a transgenic salt tolerant plant can successfully be produced.

Certain genes that have been identified as important for plant salinity tolerance have nevertheless not been shown to increase the salinity tolerance of genetically modified plants when constitutively overexpressed. For example, although the *HKT* gene family has been shown to be important in salinity tolerance, the constitutive overexpression of an *HKT* gene was found to have a detrimental effect. The *HKT* gene family can be divided into those genes encoding a Na^+ transporting protein (subfamily 1) or a K^+/Na^+ transporting protein (subfamily 2) [23, 74]. Members of subfamily 2 are considered to be involved in nutrition and the uptake from the soil of ions essential to plant growth (small quantities of Na^+ can be beneficial to plant growth) [141–144], whereas members of subfamily 1 are believed to be important for plant salt tolerance [1, 23, 96]. Members of the subfamily 1 *HKT* gene family have been shown to encode proteins important for the retrieval of Na^+ from the xylem in both the root and the shoot, thereby reducing the accumulation of Na^+ in the shoot [23, 74, 75, 79, 96, 145]. The protein moves Na^+ from the transpiration stream into the cells surrounding the xylem (Fig. 2). Evidence for this function has now been found in a number of plant species in addition to Arabidopsis, such as rice [70] and wheat [72, 73]. Both naturally occurring ecotypes and mutant lines of Arabidopsis which have reduced expression of this gene show increased shoot Na^+ accumulation [75, 102, 146, 147]. However, constitutive overexpression of this subfamily 1 *HKT* gene also results in higher concentrations of Na^+ and salt sensitive plants [77]. As HKT proteins move Na^+ into cells, the increased salt sensitiveness of constitutive overexpressing plants may be due to the fact that, when the gene is expressed throughout the plant, the protein encoded by the gene transports more Na^+ from the soil into the root, resulting in more Na^+ being transported to the shoot in the transpiration stream. Expression of this gene only in the cells surrounding the xylem would result in a plant being more efficient in retrieving Na^+ from the transpiration stream.

Plants consist of multiple tissues and multiple cells. Each tissue is adapted for a specific purpose – roots for nutrient uptake, leaves for photosynthesis, stems for support – and, therefore, will not necessarily express the same genes. Genes responsible for the maintenance of photosynthesis in the leaves will not be expressed in the roots, and genes for nutrient uptake from the soil will not be expressed in floral tissue. Similarly, not all genes in a plant are expressed all the time; many genes are activated only when required. When growing in non-stressed environments there is little point in a plant using critical energy supplies to generate and

maintain proteins important for salinity tolerance. It is unsurprising, therefore, that the continuous expression throughout a plant of a gene important for salinity tolerance, such as *AtHKT1;1*, often results in detrimental effects [77]. A critical feature in the generation of crops engineered to have increased salinity tolerance is the spatial and temporal control of the transgene which has been introduced.

Recently, transgenic Arabidopsis plants have been produced with cell-specific expression of the *AtHKT1;1* gene in the root cells surrounding the xylem [148]. Unlike plants with constitutive overexpression of *AtHKT1;1*, these cell-specific plant lines showed a significant reduction in shoot Na^+ and increased salt tolerance [148].

In a different approach, rice plants designed to overexpress a gene involved in the synthesis of trehalose only when the plants experienced stress exhibited reduced shoot Na^+ concentrations and better growth in saline conditions than non-transformed plants [149]. Trehalose is a sugar involved in protecting cells from long periods of desiccation and possibly aids salinity tolerance through an ability to scavenge reactive oxygen species, thereby protecting cellular proteins [39]; however, plants with constitutive overexpression of genes for trehalose synthesis display severe stunting [150]. The use of a stress-inducible promoter is, therefore, an important control to minimize growth inhibition of transgenic plants when grown in non-stressed environments. The focus now is the identification of gene promoters (sequences of DNA which are used to activate genes) which allow the cell- and temporal-specific expressions of genes in crops.

In addition to the fine control of genes transformed into transgenic crops, there is also the need to identify gene combinations which may have the potential to increase crop salt tolerance. As has been observed, plants employ multiple salinity tolerance mechanisms to survive saline soils, all of which rely on a variety of different genes and proteins. It seems unlikely, therefore, that the generation of a successful commercial salt tolerant crop will be achieved by the constitutive overexpression of one single gene. Recent research promoting salt tolerance in plants focuses on either boosting the intracellular salt-sequestering processes, or on the Na^+ exclusion mechanisms by transferring into selected crop species genes for salinity tolerance from model organisms (such as *Arabidopsis*) or from salt tolerant plants. A complementary approach focuses on the challenging task of reducing net input of salt into plants by perturbing the function of channels and transporters involved in sodium uptake but without disturbing potassium uptake. An ideal scenario contemplates the generation of transgenic plants with an enhanced capability for vacuolar salt sequestration combined with a reduced uptake of salt. While a number of genes involved in these processes have now been identified, the challenge is to switch these genes on at the appropriate time and in the appropriate tissues where they can be most effective. In order to achieve this aim, improved knowledge is required of gene promoters that are stress-inducible and cell specific.

While it is clear that there are potentially many avenues for the generation of a genetically modified salt tolerant plant, there remain significant challenges. Although it is now possible to generate salt tolerant plants in a laboratory, it has yet to be shown whether this relates to actual yield improvements in the field. There are cases where using genetic modification to generate a salt tolerant plant has a negative effect on yield when no stress is present. It is clear, therefore, that more information on gene promoters is required to enable the activation of salt tolerant genes in specific tissues/cell types only when plants are grown in salt. Furthermore, there are areas of the world, such as Europe, where there remains considerable resistance to the acceptance of genetically modified plants. This may well be due to the lack of availability to consumers of clear, accurate information as well as the prevalence of extremist views. A more open, transparent approach by scientists is required explaining the potential advantages and disadvantages of this technology. Only then will consumers be able to make their own informed choices about genetically modified organisms.

Future Directions

Crops growing on saline soils suffer severe reductions in yield due to both ionic and osmotic stresses. As considerable areas of farmland are currently affected by saline soils much research has been undertaken to enhance crop salinity tolerance by exploitation of natural variation in salinity tolerance or through the

generation of transgenic plants expressing genes shown to be important for salt tolerance. While salinity tolerant plants have been generated by both approaches, the focus should now be on the production of viable crop plants for farmers to grow in affected areas. For this to occur, the new cultivars of tolerant plants need to be tested under rigorous field conditions and those with enhanced salt tolerance and, as equally important, no yield penalties when grown in nonsaline conditions pass to breeders for incorporation into future crops.

Approaches are still required to help speed up the generation of salinity tolerance crops through the exploitation of natural variation. Sequencing of cereal genomes will greatly speed up the identification of candidate genes underlying salt tolerance QTL, thereby enabling highly specific molecular markers that are tightly aligned to the trait. Using these markers will help reduce the effects of linkage drag bringing undesirable traits into the population.

For transgenic plants, it is clear that refined control over when and where a gene is expressed is essential. As there are now multiple candidate genes, with potential for enhancing salinity tolerance, research should now focus more on identifying the controlling elements in a plant's genome, which dictate when and where a gene is expressed, and less on the identification of candidate genes. In addition, combinations of genes which have additive effects on salinity tolerance need to be identified, thereby allowing the production of the most optimal salt tolerant plants. When these factors are known, crops can be produced which have the ability to activate multiple genes for salinity tolerance in different areas of the plant but only when saline soils are experienced.

Although further research is clearly still required, considerable progress has been made in generating salt tolerant *plants* through the exploitation of natural variations and the generation of genetically modified organisms. The next step is to deliver salt tolerant *crops* to farmers.

Acknowledgments

We thank Christina Morris for her comments on the manuscript, and the Australian Research Council and Grains Research Development Corporation for financial support.

Bibliography

Primary Literature

1. Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681
2. FAO. FAO Land and Plant Nutrition Management Service (2008) Available from http://www.fao.org/ag/agl/agll/spush
3. Szabolcs I (1989) Salt-affected soils. CRC Press, Boca Raton
4. Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25:239–250
5. Flowers TJ, Hajibagherp MA, Yeo AR (1991) Ion accumulation in the cell walls of rice plants growing under saline conditions: evidence for the Oertli hypothesis. Plant Cell Environ 14:319–325
6. Munns R, James RA, Lauchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot 57:1025–1043
7. Yeo AR et al (1991) Short- and long-term effects of salinity on leaf growth in rice (*Oryza sativa L.*). J Exp Bot 42:881–889
8. Frensch J, Hsiao TC (1994) Transient responses of cell turgor and growth of maize roots as affected by changes in water potential. Plant Physiol 104:247–254
9. Hu Y et al (2007) Short-term effects of drought and salinity on mineral nutrient distribution along growing leaves of maize seedlings. Environ Exp Bot 60:268–275
10. Hu Y, Fricke W, Schmidhalter U (2005) Salinity and the growth of non-halophytic grass leaves: the role of mineral nutrient distribution. Funct Plant Biol 32:973–985
11. Fricke W (2004) Rapid and tissue-specific accumulation of solutes in the growth zone of barley leaves in response to salinity. Planta 219:515–525
12. Termaat A, Munns R (1986) Use of concentrated macronutrient solutions to separate osmotic from NaCl specific effects on plant growth. Aust J Plant Physiol 13:509–522
13. Munns R et al (2000) Leaf water status controls day-time but not daily rates of leaf expansion in salt-treated barley. Aust J Plant Physiol 27:949–957
14. Fricke W, Peters WS (2002) The biophysics of leaf growth in salt-stressed barley. A study at the cell level. Plant Physiol 129:374–388
15. Fricke W et al (2004) Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. J Exp Bot 55:1115–1123
16. Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annu Rev Plant Biol 55:373–399
17. Logan BA (2005) Reactive oxygen species and photosynthesis. In: Smirnoff N (ed) Antioxidants and reactive oxygen species in plants. Blackwell, Oxford, pp 250–267
18. Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 91:503–527
19. Storey R, Walker RR (1999) Citrus and salinity. Sci Hortic 78:39–81
20. Flowers TJ, Yeo AR (1988) Ion relations of salt tolerance. In: Baker D, Halls J (eds) Solute transport in plant cells and tissues. Longman, Harlow, pp 392–416

21. Läuchli A (1984) Salt exclusion: an adaptation of legumes for crops and pastures under saline conditions. In: Staples RC (ed) Salinity tolerance in plants: strategies for crop improvement. Wiley, New York, pp 171–187
22. Teakle NL, Tyerman SD (2010) Mechanisms of Cl^- transport contributing to salt tolerance. Plant Cell Environ 33:566–589
23. Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci 14:660–668
24. Bhandal IS et al (1988) Potassium estimation, uptake, and its role in the physiology and metabolism of flowering plants. Int Rev Cytol 110:205–254
25. Wyn Jones RG, Brady CJ, Spears J (1979) Ionic and osmotic relations in plant cells. In: Laidman DL, Wyn Jones RG (eds) Recent advances in the biochemistry of cereals. Academic, London, pp 63–103
26. Blaha G et al (2000) Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. Methods Enzymol 317:292–306
27. Munns R (1993) Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. Plant Cell Environ 16:15–24
28. Öertli JJ (1968) Extracellular salt accumulation, a possible mechanism of salt injury in plants. Agrochimica 12: 461–469
29. Gorham J (1990) Salt tolerance in the *Triticeae*: K/Na discrimination in synthetic hexaploid wheats. J Exp Bot 41:623–627
30. Dubcovsky J et al (1996) Mapping of the K^+/Na^+ discrimination locus *Kna1* in wheat. Theor Appl Genet 92:448–454
31. Maathuis FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann Bot 84:123–133
32. Hu YC, Schnyder H, Schmidhalter U (2000) Carbohydrate deposition and partitioning in elongating leaves of wheat under saline soil conditions. Aust J Plant Physiol 27:363–370
33. Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol 5:250–257
34. Aslam Z et al (1986) Effects of external NaCl on the growth of *Atriplex amnicola* and the ion relations and carbohydrate status of the leaves. Plant Cell Environ 9:571–580
35. Glenn EP, Brown JJ, Blumwald E (1999) Salt tolerance and crop potential of halophytes. Crit Rev Plant Sci 18:227–255
36. Aslam M, Qureshi RH, Ahmed N (1993) A rapid screening technique for salt tolerance in rice (*Oryza sativa* L.). Plant Soil 150:99–107
37. Walia H et al (2005) Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. Plant Physiol 139:822–835
38. Flowers TJ, Yeo AR (1981) Variability in the resistance of sodium chloride salinity within rice (*Oryza sativa* L.) varieties. New Phytol 88:363–373
39. Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307–319
40. Garthwaite AJ, von Bothmer R, Colmer TD (2005) Salt tolerance in wild *Hordeum* species is associated with restricted entry of Na^+ and Cl^- into the shoots. J Exp Bot 56:2365–2378
41. Munns R, James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. Plant Soil 253:201–218
42. Flowers TJ et al (2010) Salt sensitivity in chickpea. Plant Cell Environ 33:490–509
43. Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophytes. Ann Rev Plant Phys 31:149–190
44. Sengupta S, Majumder AL (2010) *Porteresia coarctata* (Roxb.) Tateoka, a wild rice: a potential model for studying salt-stress biology in rice. Plant Cell Environ 33:526–542
45. James RA et al (2008) Genetic variation in tolerance to the osmotic stress component of salinity stress in durum wheat. Funct Plant Biol 35:111–123
46. James RA et al (2002) Factors affecting CO_2 assimilation, leaf injury and growth in salt-stressed durum wheat. Funct Plant Biol 29:1393–1403
47. Silva C, Martínez V, Carvajal M (2008) Osmotic versus toxic effects of NaCl on pepper plants. Biol Plantarum 52:72–79
48. Rajendran K, Tester M, Roy SJ (2009) Quantifying the three main components of salinity tolerance in cereals. Plant Cell Environ 32:237–249
49. Sirault XRR, James RA, Furbank RT (2009) A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. Funct Plant Biol 36:970–977
50. Poustini K, Siosemardeh A (2004) Ion distribution in wheat cultivars in response to salinity stress. Field Crop Res 85: 125–133
51. Lee K-S et al (2003) Salinity tolerance of japonica and indica rice (*Oryza sativa* L.) at the seedling stage. Planta 216: 1043–1046
52. Zhu GY, Kinet JM, Lutts S (2001) Characterization of rice (*Oryza sativa* L.) F3 populations selected for salt resistance. I. Physiological behaviour during vegetative growth. Euphytica 121:251–263
53. Forster B (2001) Mutation genetics of salt tolerance in barley: an assessment of Golden Promise and other semi-dwarf mutants. Euphytica 120:317–328
54. Wei W et al (2003) Salinity induced differences in growth, ion distribution and partitioning in barley between the cultivar Maythorpe and its derived mutant Golden Promise. Plant Soil 250:183–191
55. Teakle N et al (2007) *Lotus tenuis* tolerates the interactive effects of salinity and waterlogging by 'excluding' Na^+ and Cl^- from the xylem. J Exp Bot 58:2169–2180
56. Sibole JV et al (2003) Ion allocation in two different salt-tolerant Mediterranean Medicago species. J Plant Physiol 160:1361–1365
57. Kingsbury RW, Epstein E (1984) Selection for salt-resistant spring wheat. Crop Sci 24:310–315
58. Jafari-Shabestari J, Corke H, Qualset C (1995) Field evaluation of tolerance to salinity stress in Iranian hexaploid wheat landrace accessions. Genet Resour Crop Evol 42:147–156

59. Munns R et al (2000) Genetic variation for improving the salt tolerance of durum wheat. Aust J Agric Res 51:69–74
60. Richards RA et al (1987) Variation in yield of grain and biomass in wheat, barley, and triticale in a salt-affected field. Field Crop Res 15:277–287
61. Slavich P, Read B, Cullis B (1990) Yield response of barley germplasm to field variation in salinity quantified using the EM-38. Aust J Exp Agr 30:551–556
62. Lee J-D et al (2009) Inheritance of Salt Tolerance in Wild Soybean (*Glycine soja* Sieb. and Zucc.) Accession PI483463. J Hered 100:798–801
63. Tozlu I, Guy CL, Moore G (1999) QTL analysis of Na^+ and Cl^- accumulation related traits in an intergeneric BC_1 progeny of *Citrus* and *Poncirus* under saline and nonsaline environments. Genome Biol 42:692–705
64. Vadez V et al (2007) Large variation in salinity tolerance in chickpea is explained by differences in sensitivity at the reproductive stage. Field Crop Res 104:123–129
65. Gregorio GB et al (2002) Progress in breeding for salinity tolerance and associated abiotic stresses in rice. Field Crop Res 76:91–101
66. Sahi C et al (2006) Salt stress response in rice: genetics, molecular biology, and comparative genomics. Funct Integr Genomics 6:263–284
67. Mano Y, Takeda K (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). Euphytica 94:263–272
68. Shavrukov Y et al (2010) *HvNax3* – a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). Funct Integr Genomics 10:277–291
69. Bretó MP, Aśins MJ, Carbonell EA (1994) Salt tolerance in *Lycopersicon* species. III. Detection of quantitative trait loci by means of molecular markers. Theor Appl Genet 88:395–401
70. Ren Z-H et al (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141–1146
71. Gorham J et al (1987) Chromosomal location of a K/Na discrimination character in the D genome of wheat. Theor Appl Genet 74:584–588
72. Huang S et al (2006) A Sodium Transporter (HKT7) Is a Candidate for Nax1, a Gene for Salt Tolerance in Durum Wheat. Plant Physiol 142:1718–1727
73. Byrt CS et al (2007) HKT1;5-like cation transporters linked to Na^+ exclusion loci in wheat, *Nax2* and *Kna1*. Plant Physiol 143:1918–1928
74. Platten JD et al (2006) Nomenclature for HKT transporters, key determinants of plant salinity tolerance. Trends Plant Sci 11:372–374
75. Davenport RJ et al (2007) The Na^+ transporter AtHKT1; 1 controls retrieval of Na^+ from the xylem in Arabidopsis. Plant Cell Environ 30:497–507
76. Huang S et al (2008) Comparative mapping of HKT genes in wheat, barley, and rice, key determinants of Na^+ transport, and salt tolerance. J Exp Bot 59:927–937
77. Rus A et al (2004) AtHKT1 facilitates Na^+ homeostasis and K^+ nutrition in Planta. Plant Physiol 136:2500–2511
78. Rus A et al (2001) AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots. Proc Natl Acad Sci USA 98:14150–14155
79. Sunarpi H et al (2005) Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na^+ unloading from xylem vessels to xylem parenchyma cells. Plant J 44:928–938
80. Colmer TD, Flowers TJ, Munns R (2006) Use of wild relatives to improve salt tolerance in wheat. J Exp Bot 57:1059–1078
81. Dvořák J, Gorham J (1992) Methodology of gene transfer by homoeologous recombination into *Triticum turgidum*: transfer of K^+/Na^+ discrimination from *Triticum aestivum*. Genome Biol 35:639–646
82. Dvořak J et al (1994) Enhancement of the salt tolerance of *Triticum turgidum* L. by the Kna1 locus transferred from the *Triticum aestivum* L. chromosome 4D by homoeologous recombination. Theor Appl Genet 87:872–877
83. Gorham J et al (1997) Genetic analysis and physiology of a trait for enhanced K^+/Na^+ discrimination in wheat. New Phytol 137:109–116
84. Perez-Alfocea F et al (1994) Comparative salt responses at cell and whole-plant levels of cultivated and wild tomato species and their hybrid. J Hortic Sci Biotech 69:639–644
85. Sherraf I et al (1994) Production and characterization of intergeneric somatic hybrids through protoplast electrofusion between potato (*Solanum tuberosum*) and *Lycopersicon pennellii*. Plant Cell Tissue Organ Cult 37:137–144
86. Nevo E, Chen G (2010) Drought and salt tolerances in wild relatives for wheat and barley improvement. Plant Cell Environ 33:670–685
87. Gorham J et al (1986) Salt Tolerance in the Triticeae: Solute Accumulation and Distribution in an Amphidiploid derived from *Triticum aestivum* cv. Chinese Spring and Thinopyrum bessarabicum. J Exp Bot 37:1435–1449
88. King IP et al (1997) Introgression of salt-tolerance genes from *Thinopyrum bessarabicum* into wheat. New Phytol 137:75–81
89. Yan J et al (2008) Phenotypic variation in caryopsis dormancy and seedling salt tolerance in wild barley, Hordeum spontaneum, from different habitats in Israel. Genet Resour Crop Evol 55:995–1005
90. Gorham J et al (1991) The presence of the enhanced K/Na discrimination trait in diploid Triticum species. Theor Appl Genet 82:729–736
91. James RA, Davenport RJ, Munns R (2006) Physiological characterization of two genes for Na^+ exclusion in durum wheat, *Nax1* and *Nax2*. Plant Physiol 142:1537–1547
92. Shah SH et al (1987) Salt tolerance in the Triticeae: the contribution of the D genome to cation selectivity in hexaploid wheat. J Exp Bot 38:254–269
93. Schachtman DP, Lagudah ES, Munns R (1992) The expression of salt tolerance from *Triticum tauschii* in hexaploid wheat. Theor Appl Genet 84:714–719

94. CSIRO. CSIRO develops highest yielding salt tolerant wheat (2010) Available from http://www.csiro.au/news/CSIRO-develops-highest-yielding-salt-tolerant-wheat.html
95. Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. Biochim Biophys Acta 1465:140–151
96. Plett DC, Skrumsager Møller I (2010) Na^+ transport in glycophytic plants: what we know and would like to know. Plant Cell Environ 33:612–626
97. Garbarino J, DuPont FM (1989) Rapid induction of Na^+/H^+ exchange activity in barley root tonoplast. Plant Physiol Biochem 89:1–4
98. Zörb C et al (2005) Molecular characterization of Na^+/H^+ antiporters (*ZmNHX*) of maize (*Zea mays* L.) and their expression under salt stress. J Plant Physiol 162:55–66
99. Ballesteros E et al (1997) $Na^+//H^+$ antiport activity in tonoplast vesicles isolated from sunflower roots induced by NaCl stress. Physiol Plant 99:328–334
100. Wilson C, Shannon MC (1995) Salt-induced Na^+/H^+ antiport in root plasma membrane of a glycophytic and halophytic species of tomato. Plant Sci 107:147–157
101. Wu C-A et al (2004) The cotton *GhNHX1* gene encoding a novel putative tonoplast Na^+/H^+ antiporter plays an important role in salt stress. Plant Cell Physiol 45:600–607
102. Jha D et al (2010) Variation in salinity tolerance and shoot sodium accumulation in *Arabidopsis* ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport. Plant Cell Environ 33:793–804
103. Aharon GS et al (2003) Characterization of a family of vacuolar Na^+/H^+ antiporters in *Arabidopsis thaliana*. Plant Soil 253:245–256
104. Gaxiola RA et al (1999) The *Arabidopsis thaliana* proton transporters, AtNHX1 and AVP1, can function in cation detoxification in yeast. Proc Natl Acad Sci USA 96:1480–1485
105. Apse MP et al (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science 285:1256–1258
106. Zhang H-X, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotechnol 19:765–768
107. Zhang H-X et al (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Natl Acad Sci USA 98:12832–12836
108. He C et al (2005) Expression of an Arabidopsis vacuolar sodium/proton antiporter gene in cotton improves photosynthetic performance under salt conditions and increases fiber yield in the field. Plant Cell Physiol 46:1848–1854
109. Sottosanto J, Gelli A, Blumwald E (2004) DNA array analyses of Arabidopsis thaliana lacking a vacuolar Na+/H+ antiporter: impact of AtNHX1 on gene expression. Plant J 40:752–771
110. Sottosanto J, Saranga Y, Blumwald E (2007) Impact of *AtNHX1*, a vacuolar Na^+/H^+ antiporter, upon gene expression during short- and long-term salt stress in *Arabidopsis thaliana*. BMC Plant Biol 7:18
111. Brini F et al (2007) Overexpression of wheat Na^+/H^+ antiporter *TNHX1* and H^+-pyrophosphatase *TVP1* improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants. J Exp Bot 58:301–308
112. Yu J et al (2007) An Na+/H+ antiporter gene from wheat plays an important role in stress tolerance. J Biosci 32:1153–1161
113. Fukuda A et al (2004) Effect of salt and osmotic stresses on the expression of genes for the vacuolar H^+-pyrophosphatase, H^+-ATPase subunit A, and Na^+/H^+ antiporter from barley. J Exp Bot 55:585–594
114. Zahran HH et al (2007) Effect of salt stress on the expression of NHX-type ion transporters in Medicago intertexta and Melilotus indicus plants. Physiol Plant 131:122–130
115. Fukuda A et al (2004) Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. Plant Cell Physiol 45:146–159
116. Chen H et al (2007) Over-expression of a vacuolar Na^+/H^+ antiporter gene improves salt tolerance in an upland rice. Mol Breed 19:215–225
117. Zhao F-Y et al (2006) Co-expression of the *Suaeda salsa SsNHX1* and Arabidopsis *AVP1* confer greater salt tolerance to transgenic rice than the single *SsNHX1*. Mol Breed 17: 341–353
118. Xue Z-Y et al (2004) Enhanced salt tolerance of transgenic wheat (*Tritivum aestivum L.*) expressing a vacuolar Na^+/H^+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+. Plant Sci 167: 849–859
119. Gaxiola RA et al (2001) Drought- and salt-tolerant plants result from overexpression of the *AVP1* H^+ -pump. Proc Natl Acad Sci USA 98:11444–11449
120. Duan X-G et al (2007) Heterologous expression of vacuolar H^+-PPase enhances the electrochemical gradient across the vacuolar membrane and improves tobacco cell salt tolerance. Protoplasma 232:87–95
121. Gao F et al (2006) Cloning of an H^+-PPase gene from *Thellungiella halophila* and its heterologous expression to improve tobacco salt tolerance. J Exp Bot 57:3259–3270
122. Bao A-K et al (2009) Overexpression of the Arabidopsis H+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (*Medicago sativa* L.). Plant Sci 176:232–240
123. Li ZG et al (2010) Heterologous expression of Arabidopsis H^+-pyrophosphatase enhances salt tolerance in transgenic creeping bentgrass (*Agrostis stolonifera L.*). Plant Cell Environ 33:272–289
124. Zhu J-K (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441–445
125. Luan S (2009) The CBL-CIPK network in plant calcium signaling. Trends Plant Sci 14:37–42
126. Batistic O, Kudla J (2004) Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta 219:915–924

127. Weinl S, Kudla J (2009) The CBL-CIPK Ca^{2+}-decoding signaling network: function and perspectives. New Phytol 184:517–528
128. Wu SJ, Ding L, Zhu JK (1996) SOS1, a genetic locus essential for salt tolerance and potassium acquisition. Plant Cell 8: 617–627
129. Qiu Q-S et al (2002) Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc Natl Acad Sci USA 99:8436–8441
130. Qiu Q-S et al (2003) Na^+/H^+ exchange activity in the plasma membrane of Arabidopsis. Plant Physiol 132:1041–1052
131. Vera-Estrella R et al (2005) Salt stress in *Thellungiella halophila* activates Na^+ transport mechanisms required for salinity tolerance. Plant Physiol 139:1507–1517
132. Wu Y et al (2007) Molecular characterization of PeSOS1: the putative Na+/H+ antiporter of *Populus euphratica*. Plant Mol Biol 65:1–11
133. Kolukisaoglu U et al (2004) Calcium sensors and their interacting protein kinases: genomics of the arabidopsis and rice CBL-CIPK signaling networks. Plant Physiol 134: 43–58
134. Shi H et al (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat Biotechnol 21:81–85
135. Winicov I (2000) Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. Planta 210: 416–422
136. Cheong YH et al (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in Arabidopsis. Plant Cell 15:1833–1845
137. Tripathi V et al (2009) CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants. Plant J 58:778–790
138. Xiang Y, Huang YM, Xiong LZ (2007) Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. Plant Physiol 144:1416–1428
139. Zhao J et al (2009) Cloning and characterization of a novel CBL-interacting protein kinase from maize. Plant Mol Biol 69:661–674
140. Gisbert C et al (2000) The yeast *HAL1* gene improves salt tolerance of transgenic tomato. Plant Physiol 123:393–402
141. Garciadeblás B et al (2003) Sodium transport and HKT transporters: the rice model. Plant J 34:788–801
142. Golldack D et al (2002) Characterization of a HKT-type transporter in rice as a general alkali cation transporter. Plant J 31:529–542
143. Horie T et al (2001) Two types of HKT transporters with different properties of Na^+ and K^+ transport in *Oryza sativa*. Plant J 27:129–138
144. Wang T-B et al (1998) Rapid up-regulation of *HKT1*, a high-affinity potassium transporter gene, in roots of barley and wheat following withdrawal of potassium. Plant Physiol 118:651–659
145. Horie T et al (2006) Calcium regulation of sodium hypersensitivities of sos3 and athkt1 mutants. Plant Cell Physiol 47:622–633
146. Maser P et al (2002) Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na^+ transporter *AtHKT1*. FEBS Lett 531:157–161
147. Rus A et al (2006) Natural variants of AtHKT1 enhance Na^+ accumulation in two wild populations of Arabidopsis. PLoS Genet 2:1964–1973
148. Møller I et al (2009) Salinity tolerance engineered by cell type-specific over-expression of a Na^+ transporter in the Arabidopsis root. Plant Cell 21:2163–2178
149. Garg AK et al (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99:15898–15903
150. Romero C et al (1997) Expression of the yeast *trehalose-6-phosphate synthase* gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta 201: 293–297

Integrated Pest Management

Ramon Albajes
Department of Plant Production and Forest Sciences, Universitat de Lleida, Centre UdL-IRTA, Lleida, Spain

Article outline

Glossary

Agroecosystem, agrosystem – agricultural ecosystem An ecosystem that is managed to optimize the production of a crop plant or part of it.

Biological control Use or manipulation of natural enemies (predators, parasitoids, or diseases of pests) to suppress pest populations.

Crop resistance to pests One or more qualities that some crop plant varieties have resulting in less damage by a number of pest individuals in comparison with a variety without those qualities when it is exposed to the same pest numbers.

Damage caused by a pest Damage is the monetary value lost to the commodity as a result of injury by the pest, for instance by yield reduction.

Economic injury level of a pest The pest population density at which the cost to control the pest equals the amount of damage it inflicts.

Economic threshold of a pest The pest population density at which a control measure has to be taken to prevent population from reaching the economic injury level.

Genetically modified (GM) crop, transgenic crop A crop whose genetic material has been modified by genetic engineering techniques, through which novel genes have been introduced into the crop.

Integrated pest management (IPM) A system for controlling pests in an economically, ecologically, and sociologically sound manner by the use of multiple tactics in a compatible manner. The term has many (if not all) common elements with integrated control.

Metapopulation A set of populations occupying different patches among which individuals can occasionally move.

Pest Any herbivore that feeds and causes damages on crop plants in an agroecosystem resulting in crop damages if control measures are not taken. In this entry the term "pest" includes only insects and mites but for other authors the same term would additionally include plant diseases and weeds.

Pheromones Chemicals that are released in the environment by one individual and trigger a behavioral response in other individuals of the same species.

Precision agriculture Precision agriculture aims to apply inputs only when and where they are needed and at optimal amounts according to variable field or environment characteristics.

Definition of the Subject

In addition to producing food and fiber to satisfy an increasing world population, agriculture is being asked to supply energy at reasonable prices thus contributing to the acceleration of the demand for agricultural commodities. Increase of crop yields may be achieved by maximizing the proportion of sunlight energy that is fixed by the crop plant or by reducing the amount of energy that is lost by insect pests, diseases, and weeds. More than 50% of the potential yield of agricultural crops is lost by the three causes. To diminish losses caused by insect pests

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

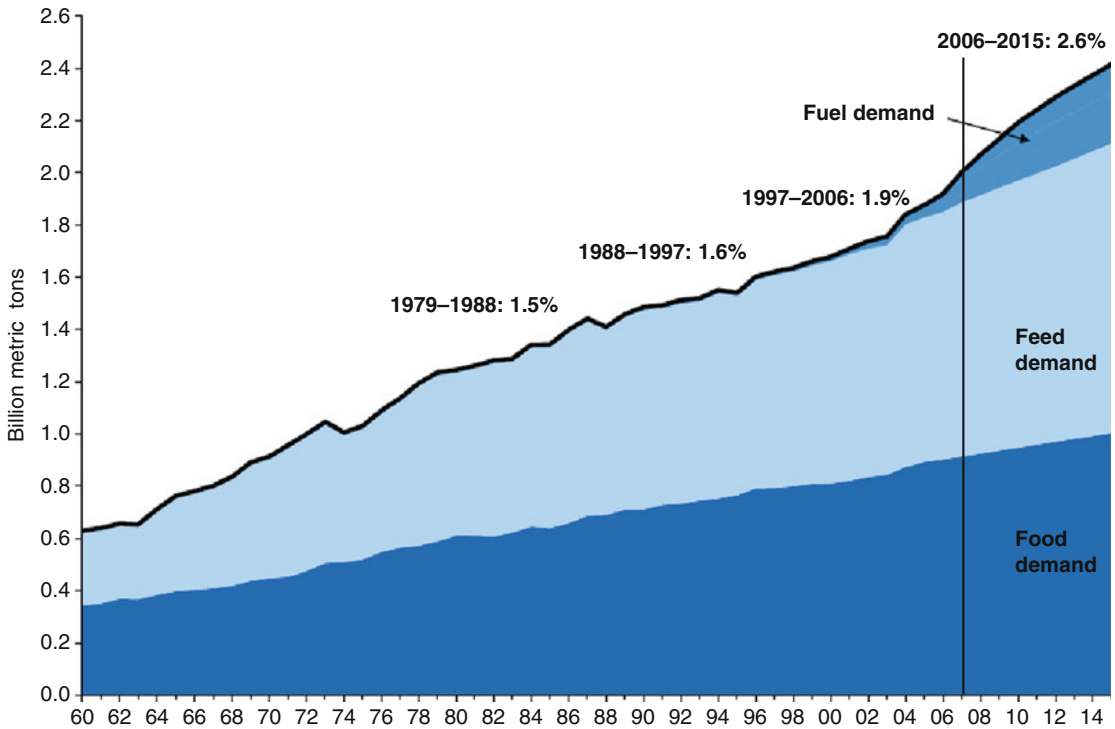
in agriculture in an economically, ecologically, and sociologically acceptable manner is the goal of integrated pest management (IPM). Given the complexity of agricultural ecosystems, IPM has to consider and manage all the elements and relationships involved in agriculture, including those related to nonagricultural ecosystems. Providing a scientific approach to better understand processes in agroecosystems in order to implement more rapidly sustainable IPM systems is a major challenge for ecology.

Introduction: Agriculture and Insect Pests

Agriculture and Productivity

Providing stable food for human subsistence has been the main goal of agriculture throughout the centuries. Probably because more than 50% of world population lives in urban areas and because developed countries do not feel threatened by hunger, society is not aware that agriculture needs to secure the world's food supplies. Increase of both world population and its income lead to increasing rates of food demand and to remarkable changes in the commodity composition of food consumption, particularly a bigger demand for livestock products.

In addition to producing food and fiber to satisfy an increasing world population, agriculture has been asked to supply energy at reasonable prices, mostly for replacing gasoline and petrol for motor vehicles. Although nowadays, as in 2007 and 2008, biofuel demand is linked to rising petrol prices, production of agricultural commodities for bioethanol and biodiesel conversion is expected to increase in the coming years, particularly if their price is competitive with petroleum. Biofuel production has accelerated the demand growth rate for agricultural commodities, which may largely exceed an annual rate of 2% in the coming years (Fig. 1). Reduction of basic food stocks, higher food prices, and increased land use may follow the stronger demand for agricultural commodities.



Integrated Pest Management. Figure 1
Evolution of demand of agricultural commodities for food, feed, and fuel use in the last 50 years (http://www.gceholdings.com/pdf/GoldmanReportFoodFeedFuel.pdf. Accessed 17 October 2009)

Complementarily, agriculture in developed countries faces increasing environmental, human health, traceability, and competition challenges. Only a significant increase of agricultural productivity and sustainable improvement of protection techniques may respond to these challenges.

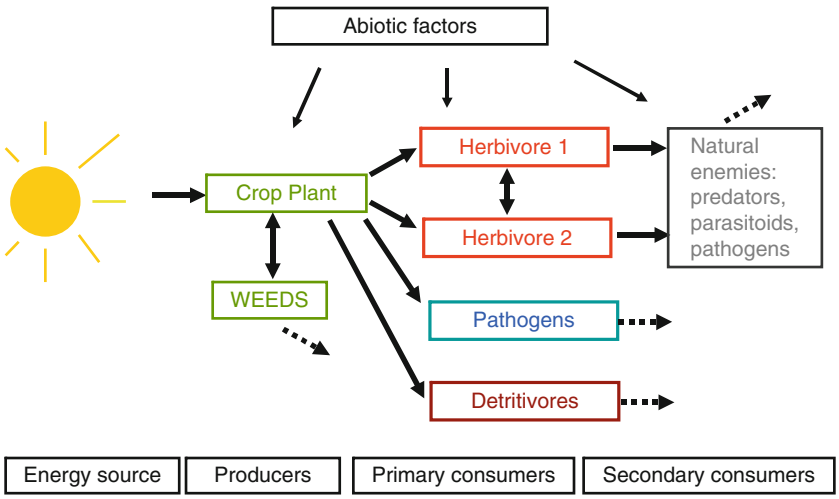
Agricultural Ecosystems: Ecological Basis for an Integrated Approach to Pest Control

It cannot be forgotten that agricultural ecosystems (also called agroecosystems) have their origin in natural ecosystems which human activity has transformed along millennia to better achieve the goal of agriculture: to provide us with sufficient food. Therefore, objectives of agroecosystems are substantially different to those of natural ecosystems; whereas, in the first case, growers manipulate the ecosystem to obtain an optimal amount of one species or part of one species (grain and not plant biomass, e.g., in cereal systems), natural ecosystems tend to perpetuate the system by themselves and do not favor one particular species or group of species. Agroecosystems differ from natural ecosystems in several ways among which two are particularly relevant in this chapter: (a) agroecosystems introduce a certain amount of energy previously processed (e.g., fuel) in comparison with the second which uses almost exclusively the energy provided by the sun; (b) agroecosystems are generally manipulated to deal with a unique plant species (the crop) and this leads to a drastic simplification of biodiversity in producer and ulterior consumer food web levels.

Composition and relationships in an agroecosystem are of crucial importance to the dynamics of its components. Figure 2 represents a simplified scheme of the main components and relationships concerning crop loss agents.

This chapter only deals with the control of those herbivores that cause damage to crops, the so-called pests. In the literature the term "pest" sometimes refers to pathogens and weeds but here the term will be restricted to herbivore species, mainly insects and mites, causing damages to crop yield if preventive measures are not adopted. In order to avoid constant repetition of words such as insects and arthropods the term "pest" will be preferred along all the text.

Maximization of crop yield in Fig. 2 may be achieved by increasing the proportion of sunlight energy that is fixed as biomass by the crop plant or by



Integrated Pest Management. Figure 2
Main components and relationships related to insect pests and their control in an agroecosystem food web. *Arrows* show the direction of energy flow between components of adjacent trophic levels. *Double arrows* show competition between components within a trophic level. *Broken arrows* show that energy flow continues but the further components involved are not represented

reducing the amount of energy that is lost by competition of weeds, by damages caused by pests, or by diseases caused by plant pathogens. The process of accumulating biomass in a crop plant to reach a yield may be compared to the process of filling a water deposit that loses water through three holes (pests, diseases, and weeds). More water may be stocked in the deposit by opening the tap (more photosynthetic production) or by repairing holes. The complex nature of the agroecosystem demands an integrated approach to manage all the components and relationships taking into account that any change in a part of them may lead to undesirable consequences in the whole system. Considering the entire complexity of the agroecosystem is crucial to develop sound integrated pest management programs.

Continuously Increasing New Insect Pests: Pest Invasion

Elements and links in the picture of Fig. 2 may change in space and time. Introduction and establishment of invasive alien species and climatic change are among the most decisive factors influencing the composition and relationships in an agroecosystem. Many of the insect pests in agriculture originated as exotic herbivores introduced in the past; this process continues to accelerate, mainly as a consequence of globalization of agricultural trading. The introduction, establishment, and spread in Europe of the Colorado potato beetle may exemplify the impact of insect pest invasions on agriculture. It originally restricted its distribution to the Rocky Mountains (USA) where it fed on wild plants. In the second half of the nineteenth century, the cultivation of potatoes close to that area allowed the beetle to multiply its populations, spread out of its original area, and distribute around the world where it has become the most harmful insect pest of potatoes in general terms. Hundreds of exotic arthropods have been documented as invading agricultural areas, establishing themselves and becoming important pests. The objective of eliminating or limiting the spread of pests has led several national and international institutions to develop legislation and tools to restrict the movement of insect species with a high potential to become agricultural pests. Regional sections of the International Plant Protection Convention have been particularly active in this direction and its Web site (www.ippc.int) is an important source of information. To categorize the risk of potential invasive alien species is a first step to adopt correct and ad hoc measures. Then, measures of exclusion, early detection, containment, or control of the most risky species may be adopted at the national or international level. Prevention of the introduction and spread of crop pests is an important first step of integrated pest management although it is sometimes difficult to implement due to the ease of international travel of both people and plants, and the increasing trade of agricultural commodities.

Injuries and Losses Caused by Pests

Pest control, and particularly integrated pest management, is therefore necessary to safeguard crop productivity against losses caused by herbivore insects and assure human nutrition. Losses caused by insect and mite pests derive mostly from their feeding activity on plants. Insects consume plant materials with their chewing or sucking mouthparts; in addition to lowering plant vigor, some insects are also able to transmit plant pathogens, with additional losses due to disease development, or injection of toxic substances that interfere with plant physiology. Consequently, potential crop yield is not attained and a variable level of losses results according to the amount of insects feeding on plants, the type of injury caused, and the susceptibility of the plant to the amount of injury infringed.

Potential and actual losses due to animal (mostly insect and mite) pests have been estimated by Oerke and Dehne in major crops [1]. Those were estimated as quite variable according to the crop and geographical area but on average they were 17.6% and 10.1% of the attainable yield in the world that give a control efficacy (percentage of losses prevented) of 42.4%. Efficacy to prevent losses from animal pests was higher than from bacterial and fungal diseases (33.8%) but considerably lower than from weeds (70.1%). These moderate values of efficacy in crop protection, which averaged 52.5% in the world, were reached in spite of particularly high pesticide consumption in Western Europe and North America.

Strategy for Integrated Pest Management

What IPM Is

The necessity to change the strategy of controlling insect pests derived from both theoretical considerations and practical collapse of control systems. Although humans were soon aware of weaknesses in relying on control systems based solely on single tactics, control failure of pesticides due to pesticide resistance in important insect pest species and the consciousness of pesticide transfer to environment might have been the detonators of strong criticism about the mass use of pesticides in the 1950s. Soon after that, scientists, especially applied entomologists, defined new concepts and terms among which were integrated control and integrated pest management (IPM) [2]. These two terms share basically the same underlying concepts although some authors have justified the different use of these two expressions [3]. A broad definition of integrated pest management is this: a pest control system in an economically, ecologically, and sociologically sound manner by the use of multiple tactics in a compatible manner.

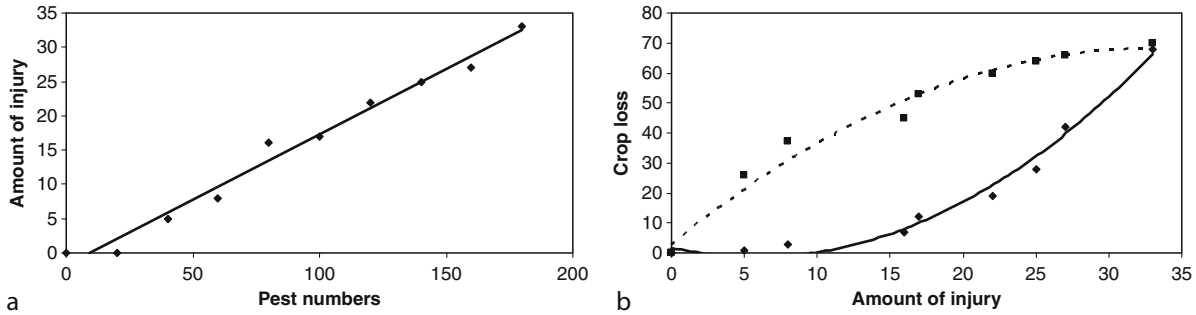
Sustainability should be an inherent qualification of IPM systems. However, development of novel insect pest control methods has often focused more on replacing chemical pesticides than implementing low input tactics, two objectives that do not necessarily progress in parallel ways. Classical biological control provides us with some examples of reduced sustainability when mass-reared natural enemies are repeatedly released to control insect pests in short-lived annual crops as practiced in some Mediterranean greenhouses. Paradoxically, many of pest natural enemies that are mass produced with energetically costly procedures and released into isolated Mediterranean greenhouses are native to the same area and could be managed to enhance their entrance into greenhouses by conservation biological control practices. Early IPM aimed to decrease the use of pesticides and, fortunately, it succeeded in many cases. The novel era of IPM should put emphasis on the agroecosystem management and various entries in the encyclopedia will give some indications on how to proceed in such direction. Nonetheless, some fundamental concepts of IPM as enounced decades ago have kept most of their validity in theory and practice. This is the case of economic threshold which is still a key concept that, under several formulations and criticisms, occupies a central point in IPM development.

Concept of Economic Threshold

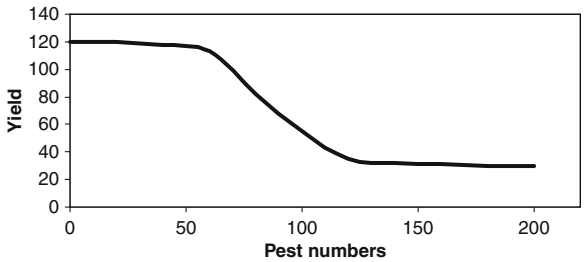
A first question that an IPM practitioner has to face when he/she detects a certain number of pests on the crop is whether intervention is justified. Some criteria to make decisions based on economic, social, ecological, and toxicological needs are necessary. Economic injury level (EIL) and economic threshold (ET) are concepts that primarily were developed to make decisions founded on economic cost-benefit analysis to include later a richer set of the so-called bioeconomic elements that relate pest numbers, crop plants responses to pest injury, and resulting crop losses. With minor modifications these two concepts of EIL and ET still form the basis of the current IPM programs providing the potential to improve economic profits but also reduce environmental impact [4].

Relationships between pest numbers and resulting crop losses are the result of two other subrelationships succeeding each other very closely in time. Pest numbers are grossly related to total injury in a linear manner, whereas the latter relates to crop loss (i.e., lowered yield) by nonlinear figures (Fig. 3) so that the result of combining both curves gives a nonlinear relationship between pest numbers and crop loss (or yield reduction), as that represented in Fig. 4, if crop loss is converted into yield reduction.

As seen in Fig. 4, yield is reduced at low pest numbers very slightly, if at all, but as pest numbers become higher yield is increasingly reduced in a near linear manner until the declining yield per pest number increase decelerates and finally becomes insensitive to pest population growth. Of course the shape and values of this relationship is a function of several factors linked with the plant and pest species. The following are the most significant: yield based on fruit is more sensitive than is biomass, injury on plant tissues contributing directly to yield are more yield reducing, dissimilar host-plant growth stages are differentially sensitive to injury in terms of yield loss in a manner that young plants have a higher capacity to recover or compensate injuries, and finally biotic and abiotic stressors other than the pest may magnify the consequences of injuries.



Integrated Pest Management. Figure 3
Relationship between pest numbers and amount of injury (*left*) and two common relationships between the amount of injury and losses caused on the crop (*right*)



Integrated Pest Management. Figure 4
The relationship between pest numbers and yield as a result of combining the relationships of Fig. 3 and converting crop loss to yield reduction

Knowledge of the curve relating pest numbers and yield is the basis for the calculation of EIL and ET. Simply defined, EIL is the lowest pest population numbers that will cause economic damage this being the amount of damage that equals control cost. EIL shows the amount of pests (and therefore the amount of injury) that can be tolerated by a crop. Operational consequences of the EIL are summarized by the ET, which determines if management action against a pest is needed. As the time needed to make a decision and to take the action may cause pest population to surpass the EIL, decisions have to be made before the population reaches the EIL and thereby prevent economic damage; this value is called economic threshold, ET. When decision making is delayed, pest population continues to grow, control methods are less efficient, and ET is lower in relation to EIL. On the contrary, if pest population is expected to decrease after reaching the EIL, ET may be higher than EIL. There are still many gaps in the knowledge of the mechanisms of herbivorous insect and host–plant relationships and this may be the cause of the insufficient development of ETs in practice; in addition, a rather confusing literature and proliferation of nomenclatures, often with no novel concepts provided, do not help in the implementation of tools for economically, ecologically, and toxicologically sound pest control.

Limitations of Economic Thresholds

In addition to the complexity of determining ET values already stressed, a number of other limitations may constrain the application of criteria for decision making in IPM. One of the limitations derives from the fact that many factors affect the amount of injury (or pest numbers) and yield (crop losses) relationship, and therefore it is quite unrealistic to use deterministic models – as those based on ET – in insect pest control. If different levels of crop loss may be associated to probabilities of occurrence, IPM practitioners may decide on a risk assumption basis. Risk is common in economic activities and growers have to compare the risk of crop losses with control costs. Furthermore, growers may have to choose among a number of control methods, each with a different cost and a different expected efficacy. To further complicate the grower's decision, the benefits of different control options – including no action – may be considered in the short or long term, which are often different. For example, application of an insecticide may be more effective at short time if the chemical has a good

knockdown but less effective later in the season if the insecticide kills most of the pest natural enemies and pest population outbreaks occur soon after chemical application. Finally, decisions concerning pest control may be altered if derived actions are adopted at farm or regional levels; in the first case, market price is unlikely to change as a consequence of actions undertaken; but when pest control actions are adopted at a regional level, resulting yields may affect the price.

Tools for IPM

Precision Agriculture and IPM

The so-called precision agriculture applied to pest control aims to apply control inputs only when and where they are needed and at optimal amounts according to variable field characteristics. Whereas it is understood that decisions must account for temporal changes in population densities, much less attention has been given to spatial heterogeneity probably because pest control actions are mostly decided and implemented at the farm level. For very mobile pests, when control implementation needs to operate over areas larger than just the farm, or simply because pest dynamics is decisively influenced by the spatial structure of the habitat, area wide pest management strategies have to be developed [5]. This has been practiced in the past, such as with human insect-borne diseases or in a few agricultural problems (i.e., locust plagues). Larger and systematic use of area wide control applied to agricultural pests has been implemented only in the last decades.

One of the most common causes for control failures is pest migration into managed areas from unmanaged areas or from areas managed at different times, particularly when the pest is able to move through several kilometers in a short time. Disposal of fields with different crops or different crop phenologies, or those submitted to different abiotic conditions in a patchwork landscape facilitates the movement of flying pests searching for the best environments to develop and reproduce. An area wide IPM approach differs from local pest management in some important characteristics. Whereas local IPM implementation focuses the control in specific parts of the habitat, the area wide approach considers the control in all potential niches. On the other hand, as area wide strategies need to consider and implement the control on a multiyear basis, this is an incentive for building a more permanent organization for such a purpose and thus leading to more professional management tools. These include geographical information systems (GIS), satellite imagery and remote sensing, online processing of climate data and weather forecasting, and kits to detect potential insecticide-resistant populations that can be useful for prevention of spreading resistance genes across the regional population. Finally, area wide implementation of IPM programs may allow the use of methods – sterile insect techniques, mating disruption, and inundative biological control – that are effective only when applied on big surfaces. Even if these and other methods may be used at individual farm level, economies of scale may derive from acquiring large amounts of materials. On the negative side of area wide IPM, there is the increased complexity of decision making if the area covered by the program is not uniform and many inputs are needed to respond with control measures adapted to each condition.

Area wide IPM application is based on a large amount of data referenced to each geographical position which needs to be stored and organized in a systematic and recoverable way. Once data are stored and organized they can be used to monitor some variables, manage resources, and develop forecasting models. GIS technology serves such a purpose. In recent years, GIS techniques have monitored the influence of crop plant, crop phenology, topographical variables, or climatic variability on insect distribution; movement of herbivore insects or predators at landscape scale; impact of crop rotations or cropping system on pest damage; agricultural production techniques, insect community structure and impact of damages caused by insect pests; and invasion and establishment processes by alien invasive insects.

Acquisition of data to feed GIS technology may be a long-lasting and tedious process and it is a major limitation for GIS application in IPM. Remote sensing – acquisition of information on an object without being in contact with it – may reduce the efforts for data acquisition. For instance, remote sensing techniques have been developed to map several types of vegetation or plants that are stressed by damage caused by

different amounts of herbivore insects and which display changes in absorption and reflectance in the visible and near infrared light due to chlorophyll content decrease, alteration of other pigments, or some other changes in the internal plant anatomy. Plants stressed by insects or diseases also may alter their temperature and thermal imagery may be used to detect these plants. Images may be field-based or taken from planes or satellites and thus cover big surfaces. Before using remote sensing to make maps of insects or insect-damaged plant distribution, accuracy of sensing images should be verified by means of ground surveys. Signals from damaged plants may not be very specific and insect populations or damage caused by one species may be overestimated.

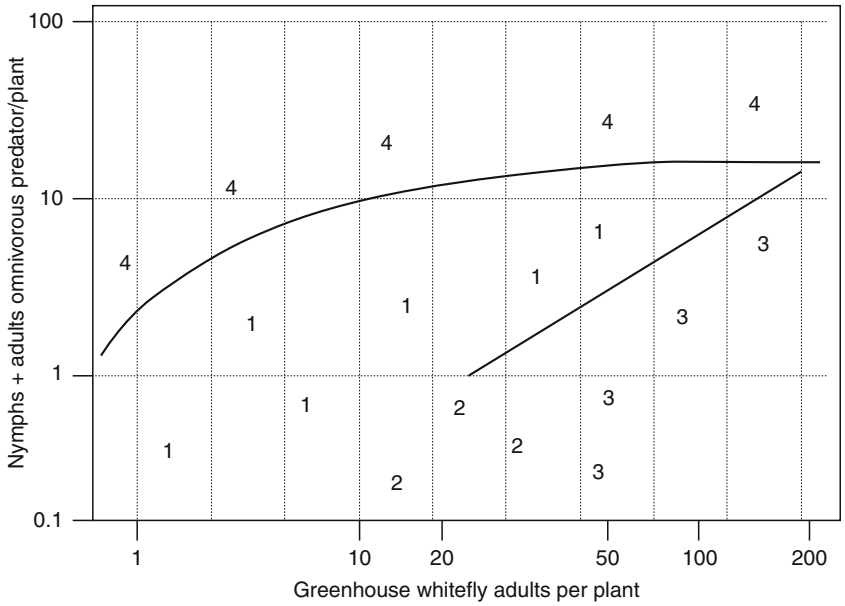
As more data are needed and available for decision making, more sophisticated are the algorithms used to process the information. Processes relating input data with conclusions leading to actions may be performed by expert personnel or by more automatic procedures as computer software; the latter are called expert systems. To consider the role that expert systems may play in IPM, let us recall the steps of any decision in pest control [6]: pest identification, assessment of injury level (density), estimation of likely crop losses, identification of control options, cost/benefit analysis, identification of constraints, integration in the larger framework of crop production [6]. Ideally, expert systems are linked with data bases that allow input and review of historical series of data to analyze past decisions in the light of real posterior events.

Expert systems not only serve for decision making in IPM but may be used as a common base for discussion among scientists or IPM practitioners in order to identify incorrect algorithms or gaps in the current knowledge or incorrect recommendations made in the past. Expert systems can also be used as training tools. Via simulation, students can examine and check situation rules to sample, make calculations, and convert the conclusions into recommendations. When expert systems are used for training or when they have to be presented for demonstration purposes, input variables, algorithms, and logical rules for decision making should be shown in a more explicit format than just computer software, for example, a set of matrices or as a decision chart such as in Fig. 5.

The decision chart in Fig. 5 was designed for field technicians to decide if insecticide treatments are needed in tomato crops. Decision algorithms take into consideration not only the amount of pest (greenhouse whitefly) but also the amount of predators (two mirid bugs) that can keep pest populations under control when the predator–prey ratio is high enough. As mirid bugs are omnivorous predators that may feed on and damage tomato fruit when lacking prey, the strategy adopted was to manage insecticide sprays. This maintained a sufficient number of whiteflies to provide predators with food and prevent tomato damage but was not too high to avoid having honeydew and sooty mold on plants and fruits. Technicians have to sample the field by choosing a number of plants at random and taking seven terminal well-developed leaves to count the number of whitefly adults and predators (adults + nymphs). According to the position in the graph of the values recorded in the field for whiteflies or predators, the decision made is: (a) doing nothing, (b) or spraying against whiteflies, (c) or predators; (d) a four region in the chart does not lead to specific recommendations. Chart is accompanied by keys to identify the targeted whitefly and the predators, some rules to take samples (including a table of random numbers), and an updated list of eligible insecticides with recommendations about their application.

Estimating Insect Population Densities

To make decisions in pest control, pest density needs to be known. As it is normally impossible to count all the insects in a habitat, it is necessary to estimate the population density by sampling. An estimation of absolute or relative population may be needed. In absolute estimations, the estimated number of individuals in a certain surface or volume may be known, whereas in relative estimation a certain and unknown proportion of the population changes in the time or space is determined. The first type of estimate is useful when we want to know if a population has reached the economic threshold or not. The second type serves to compare population numbers in time and space for monitoring purposes. If population numbers may be related to their products – for example, total amount of honeydew excreted by an aphid population – a

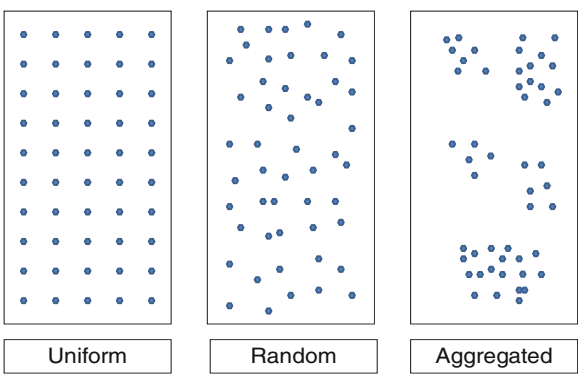


Integrated Pest Management. Figure 5
Decision chart to make recommendations for insecticide spraying in tomato crops against the greenhouse whitefly and the omnivorous predators according to the number of whiteflies and predators recorded on tomato plants. Figure has been simplified to show the most significant elements. Numbers indicate the action to be undertaken after recording densities of the omnivorous predator and the greenhouse whitefly on tomatoes in the field: (1) no action, (2) sample again in one week, (3) spray against whitefly if the recorded numbers remain in zone 2 for two consecutive weeks, and (4) spray against mirids

population index may be more useful than determining the real number of individuals.

An accurate estimate of the density of pests or natural enemies is therefore a major necessity for IPM. To estimate population densities it is necessary to sample the habitat where the population may occur and this may require knowing various aspects. Sampling universe, sampling unit, number of samples to be taken for a certain precision, and data analysis are some of the aspects to be considered in a sampling plan. Readers interested in this concept should consult the Southwood and Henderson book for wider development [7].

How individuals of an insect population are disposed in space – dispersion or distribution of the population – greatly affects the sampling program. Theoretically, three different types of population distributions may be found (Fig. 6). In *uniform distributions* individuals are evenly distributed in space whereas in *random distributions* any point in space has the same probability for occupation by an individual. Finally, most commonly, individuals are clumped on relatively few foci in *aggregated distributions*.



Integrated Pest Management. Figure 6
Three kinds of insect population distributions

Population dispersion has been described by means of aggregation indices or by fitting mathematical

distributions to experimental data. Use of aggregation indices is based on the relationship between variance and mean in the three types of distribution models. In uniform distributions variance is null independently of the mean; in random models mean equals variance; in aggregated distributions variance is higher than mean. The simplest aggregation index uses the variance/mean ratio so that when the ratio is 1 it means that distribution is at random, it is more uniform as the ratio decreases below 1 and, inversely, aggregated models show variance/mean ratios increasing higher than 1 as individuals are more clumped. Other aggregation indices relating variance and mean in different ways have been used – for example Lloyd', Morisita' indices – but most of them have shown a high dependency from sampling unit size and population density, a disturbing inconvenience to characterize a population distribution pattern. Relationship between estimated variance (s^2) and mean (m) has been empirically fitted to a power law: $s^2 = am^b$, where a and b are constants; a is largely a sampling factor while b appears to depend on the degree of aggregation of individuals and thus may be used as an aggregation index. Taylor's power law has shown to overcome some of the limitations mentioned for aggregation indices but usually needs many experimental data to fit a sound power function.

Several mathematical models have been proposed to describe population distributions; for insects many populations have been adequately fitted to the negative binomial distribution which is basically described by the parameter k, which is a measure of the degree of clumping. Although the approach of mathematical models to study population distribution patterns has given more accurate descriptions than aggregation indexes, it often depends on population density and sampling technique. In summary, none of these methods describing population distributions is free from limitations and this can explain why insect ecologists have used so large a variety of approaches to characterize patterns of insect disposition in space.

When populations are very clumped and most individuals occupy a few dense patches among empty patches a question arises: are individuals in one patch moving to another patch? If yes, how often and to which extent does this movement occur? Such a perspective deals with metapopulation ecology. Ecology has classically considered that individuals of a population are capable of unrestricted interaction with each other within a habitat. However, habitats usually occur in a patchwork within a landscape – as islands in the ocean – and populations inhabiting those habitats are consequently patchy. Populations occupying each patch may become extinct but individuals coming from other patches may recolonize that patch and rebuild a new population. Between-patch movement also may be caused by factors other than just extinction and recolonization. In any case, individuals initially belonging to a population in a certain patch occasionally may interact with individuals of other patches. The set of all populations initially occupying different patches is called a metapopulation. There has been a lot of interest in metapopulation ecology in the last decades because of its practical application. In the field of IPM, metapopulation ecology may bring new tools for analyzing the dynamics of insect populations that occur in partially isolated patches (fields or group of fields). Understanding how populations work in a metapopulation may help to increase environmental resistance to pest population development or to favor populations of pest natural enemies. Management of metapopulations and not just local populations may be a valuable approach not only for unstable annual crop systems but also for more permanent crops that are periodically disturbed (i.e., cut, pruned, sprayed with insecticides) so insects are regularly obliged to leave and recolonize fields. In the context of area wide IPM programs, metapopulation ecology may be more predictive than just considering individual populations.

Taxonomic Adscription of Major Pests

Main Animal Taxa with Damaging Species

Most agents causing injuries to crop plants are species belonging to the Phylum Arthropoda and among these the class Insecta includes the majority of arthropods that are crop pests. That is why the discipline dealing with pests and their control very often is called agricultural entomology, the science about insects in relation to agriculture. The class Arachnida, particularly the order Acari (mites), also contains some important pest species. Notice that some herbivorous insects and mites cause injuries to crop plants whereas pathogens are responsible for crop plant diseases.

The class Insecta comprises more than one million identified species and probably even more that have not been identified yet. Insects are grouped in 28 orders – this varies according to the authority– a common taxonomical level to refer to insects. Among those, seven major orders include most of the important insect pests: Orthoptera (grasshoppers and others), Hemiptera (true bugs), Homoptera (hoppers, psyllids, whiteflies, aphids, scale insects), Coleoptera (beetles), Lepidoptera (moths and butterflies), Diptera (mosquitoes, flies), Hymenoptera (ants, wasps, insect parasitoids, and others). Table 1 shows the main families of mites and insects including economically important pests and their most significant pest characteristics.

Other taxa of the animal kingdom include species that can potentially become serious pests. Mollusks (snails and slugs), fishes, reptiles, birds, and mammals may include species that in some circumstances are very damaging.

Identification of Insects and Mites

When a technician observes insects or mites on a crop, the first question that arises is "what are they?" To precisely answer this question is a key starting point for finding an efficient solution if the population grows to become a pest. A wrong answer may lead us to make incorrect decisions as many solutions are specific for each insect pest and crop. Sometimes, even specific identification is needed to adopt correct measures.

Identification of insects and mites is usually done by means of taxonomic keys. Keys are arrangements of related taxa put in clusters. Morphological characters – complemented sometimes with anatomy, appearance, or behavioral features – are mainly used to segregate individuals into clusters. The process goes from more general characteristics (i.e., with wings or wingless) and taxa to more particular characteristics until reaching species-level determination. Even for easy identifications, a certain expertise is needed. For routine identifications field technicians may perform quite well in recognizing common species; when dealing with a new species, correct identification may require sending a sample to a family-level specialist usually working in universities or musea. Availability of good insect taxonomists is therefore critical for developing sound IPM programs, not only to identify insect pests but also their natural enemies (predators and parasitoids).

Molecular techniques are still insufficiently developed to identify insects but rapid progress has been made in recent years. Molecular tools are usually developed to distinguish between two or more taxonomically close species. Molecular identification keys based on a targeted DNA sequence or marker may be useful for such purposes and have some advantages in relation to classical morphological keys. These advantages include: applicability to all developmental stages; less variation than for morphological characters; they may be applied to fragments of the individual to be identified; the technique may be applied for a variety of insects if appropriate specific material and personnel trained in molecular tools are available, in contrast with morphological-based keys that usually require family-level specialists.

Pesticides

Use of Pesticides

In the coming sections, major *control methods* are reviewed in the light of how they can contribute to the sustainability of agriculture. Ecological bases, common applications, and how they can be integrated into IPM systems are presented in each method.

Use of pesticides (mainly herbicides, insecticides, fungicides, and nematocides) in western world agriculture has decreased or been maintained in general. In Fig. 7, the amount of pesticides sold in two main consumer European countries is shown. Whereas in France, the highest consumer of pesticides in Europe, the amount of pesticides sold (mostly applied in France) has decreased significantly, in Italy the amount of pesticides is more or less stable or shows a slight increase taking into account that modern pesticides are used at considerably lower doses than classical active ingredients. Beyond differences due to variable economic and climatic conditions, an important part of the decrease in many countries has been achieved by the progress of application of IPM systems to control insect pests, diseases, and weeds.

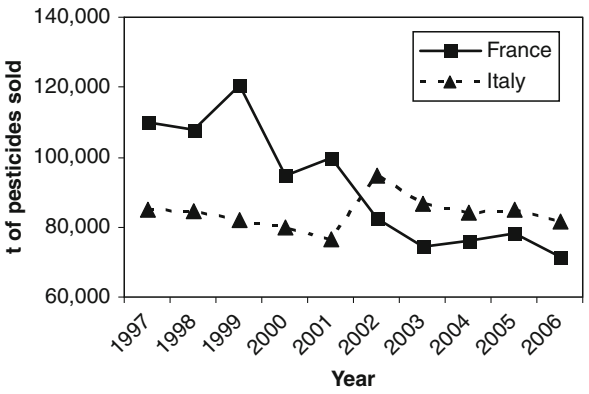
Pesticides are chemicals aimed to kill any kind of plant pest or otherwise lower their populations to prevent their reaching economic thresholds. Pesticides

Integrated Pest Management. Table 1 Main orders and families of arthropods including economically important pest species

Class	Order	Family	Main characteristics and features as pests
Arachnida	Acari	Tetranychidae	Plant-feeding spider mites. They feed on several aerial plant parts but mainly on leaf undersides with loss of photosynthetic products and water
		Eriophyidae	Microscopic mites with only two legs; they feed on plant parts often causing galls
Insecta	Orthoptera	Acrididae	Insects that at high density may aggregate in groups and migrate long distances and become very destructive on many crop and forest plants
	Hemiptera	Pentatomidae	They suck plant sap from several tissues resulting plant wilt, abortion of fruits, or tissue malformations
		Miridae	Numerous, but not only, herbivore species that feed on plant sap causing foliar chlorosis, cankers, abnormal growth, and many kinds of lesions
	Homoptera	Cicadellidae	Usually they feed on leaves where they suck juices and reduce chlorophyll contents and produce small white spots. Plant vigor decrease and disease transmission are common injuries
		Psyllidae	They feed on the phloem causing plant stunting or poor plant growth and sometimes gall forming
		Aleyrodidae	They feed on the phloem and reduce plant vigor, exude honeydew where sooty mold may develop causing fruit depreciation and some species are active plant disease vectors
		Aphididae	As mentioned for Aleyrodidae with special importance for plant virus transmission
		Coccoidea	In addition to the injuries mentioned for the other Homoptera, scale insects may inject saliva into the host causing discoloration, malformations, galls, and also esthetic damages in ornamental plants
		Diaspididae	
		Asterolecaniidae	
		Coccidae	
		Margarodidae	
		Pseudococcidae	
	Coleoptera	Scarabaeidae	As pests they mainly feed on plant roots in larval stages causing plant vigor decrease and even plant death
		Elateridae	Larvae feed below ground on roots and tiller base and kill the plant when it is young. Crops harvested for roots or tubers are more easily injured
		Curculionidae	Many species whose larvae and adults feed on several plant tissues including roots, tillers, leaves, flowers, and fruits. A very damaging family
		Chrysomelidae	Adults and larvae feed on foliage and fruit; in some other species larvae feed on roots
		Scolytidae	Larvae feed internally in tree tissues, below the bark; particularly harmful in forest trees but also in orchard and ornamental trees
	Lepidoptera	Tortricidae	Larvae feed on leaves, fruit, buds, and stems
		Pyralidae	Larvae are leaf-rollers, borers, and detritivorous attacking a large number of crops including stored products
		Crambidae	Larvae are mostly grass stem borers

Integrated Pest Management. Table 1 (Continued)

Class	Order	Family	Main characteristics and features as pests
		Noctuidae	Larvae feed on leaves, stem, and fruits devastating many crops. This is one of the most important families with many economically important species
	Diptera	Cecidomyiidae	Main injury comes from their capacity to cause galls in several plant tissues
		Tephritidae	Larvae feed internally in fruits. They have a high destructive potential
		Agromyzidae	Larvae mine leaves by feeding parenchyma cells between up and down epidermis
	Hymenoptera	Tenthredinidae	Most damage is caused by larvae feeding on leaves that reduce photosynthesis activity and thus plant vigor. They commonly defoliate forest trees but also some agricultural crop plants
		Cephidae	Larvae bore into stems of grass plants and cause their breakage



Integrated Pest Management. Figure 7
Evolution of sales of active ingredients with pesticide activity in two significant consumers in Western Europe (From http://epp.eurostat.ec.europa.eu/tgm.Accessed 6 February 2010)

include four main groups of substances according to their target: herbicides against weeds, insecticides against insects or other pests, fungicides against in general disease-causing agents, and nematocides against plant pathogenic nematodes. A number of characteristics of pesticide use may explain the success of pesticides for pest control in the last decades although probably the amount applied has started to decrease in the developed world in recent years due to the restrictions posed by the legislation and also by the progress experimented by the R&D in implementing IPM systems.

Pesticides are easy to use; growers may fill the tank and spray many hectares while sitting in the tractor and listening to the radio. When used correctly they are effective to lower pest populations and frequently cheaper than other control alternatives. In spite of the better selectivity and lower permanence of modern pesticide active ingredients, at least one pesticide is usually available in the market for each pest. Until the discovery of insecticide properties of DDT in the 1940s, most pesticides were inorganic or extracted from plants. Since the 1940s until the end of the century, the amount of pesticides applied in the world multiplied dramatically per 20 or 30 times according to the country and several chemical families were available to control insect pests. Since the 1950s, however, scientists were aware of the problems derived from the excessive confidence in the efficacy of pesticides. Fewer than 20 years of mass application of pesticides in western agriculture were sufficient to display some of their negative effects. In ulterior years, problems became harder and many field data confirmed first concerns. Development of alternatives to chemical pesticides therefore became the goal of R&D programs in most of those countries.

Problems Associated to Pesticide Use

Problems derived from inadequate and excess pesticide use include (a) risks to public health and environment (e.g., wildlife and groundwater), (b) disturbance within agrosystems due to the common toxicity to natural enemies and secondary pest resurgence,

(c) development of pesticide resistance in the targeted pests (more than 600 pest species related to agriculture and human and livestock health are nowadays confirmed to be resistant to one of more pesticides) (www.pesticideresistance.org accessed on February 10, 2010), and (d) shorter and shorter shelf life and increasing costs to innovate by producing more selective and environmentally friendly new active ingredients. Industry has tried to develop new more compatible chemicals in order to integrate selective chemicals in IPM strategies but innovation is increasingly slow and expensive. Legislation is becoming very strict for registration of new pesticides and obliges repeated registration of old active ingredients for health and environmental safety. As a consequence the number of active ingredients available for chemical pest control is decreasing constantly. It is expected that the number of active ingredients registered as insecticides in the coming years in the EU will be less than a third of those allowed at the end of the twentieth century. Lack of effective insecticides is pressing research in and the development of new and efficient IPM systems.

Controversy on pesticide use in modern agriculture cannot lead us to forget that pesticides, at the moment, have still an important role in IPM systems. Some important pests lack sufficiently effective control methods so that no-chemical methods have to be combined with chemical ones. In other, although few, cases, pests have only chemical insecticides to control them. Invasive exotic insect pests, due to short experience in their control and novelty, may be contained only by the regional application of chemical pesticides. A rigorous analysis of how sustainable is the use of each insecticide for each pest should permit detection of those pest problems in which insecticides are irreplaceable at least at short time and those other pests in which one insecticide is superfluous – therefore that pesticide can be banned – because at least one efficient nonchemical method is available. Unfortunately the control of unnecessary use of chemicals is frequently difficult but should be implemented to speed up the adoption of IPM technology.

Crop Resistance

Ecological Bases of Crop Resistance

Most pests cause plant damage when feeding. However, it is well known by interested observers of nature that not all herbivore insects may feed on any plant. Usually some insects may feed on a few close plant species (monophagous insects), or on plant species belonging to one family or only a few families (oligophagous insects), and finally some others may feed on a broad range of plants (polyphagous insects). Even polyphagous species feeding is usually restricted to a relatively small number of plants available in the habitat. These associations between herbivore insects and host plants are the result of the coevolution of the two components; in such coevolution, plants develop mechanisms to defend themselves from herbivore insects and insects try to develop mechanisms to overcome plant defenses. That a plant possesses some characteristics that diminish its access for one insect pest and that such a trait may be introduced into a host plant for pest control is an old idea. Still, until well into the twentieth century crop resistance to insects was not systematically considered as a universal tool for pest control in spite of early successes in the use of plant resistance. Probably, the most famous early case of using crop plant resistance for pest control was the control of grape phylloxera in European grapevines. Practically all the European wine industry was ruined by the entrance into Europe of the North American phylloxera aphid in the 1860s. Successful control of the pest was achieved at the very end of that century by grafting European vineyards on resistant American rootstocks.

Insect–Plant Relationships and Plant Characteristics for Crop Resistance

Many physical and chemical factors are involved in insect–plant interactions. Plant stimuli and elicited insect responses are usually studied in a sequence of five behavioral steps: (a) host habitat searching, (b) host searching within the habitat, (c) recognition of a host as suitable for feeding and ovipositing, (d) host acceptance, and (e) host suitability. Step (a) is important for species that migrate or disperse over long distances and it is only occasional for pests staying in the crop habitat. Preferred abiotic conditions of the habitat are usually the most involved signals in habitat selection whereas in host selection (step b) visual and olfactory stimuli have a major relevance to bring the herbivore close to the host plant and not only olfactory but also tactile inputs stimulate the herbivore to remain

on the plant. The host is recognized through gustatory receptors that identify particular host substances when bitten by the herbivore or when the female starts ovipositing. Similar mechanisms but different substances in the host plant cause the herbivore to continue or stop feeding or ovipositing after the recognition phase. Host adequacy for the herbivore and descendants is determined by the nutritional value of the plant and the absence of toxic compounds.

Physical and chemical plant characteristics that confer resistance to the host plant against the exploitation by the herbivore may be found in each of the behavioral steps and mechanisms and those traits may be incorporated into the crop plant genotype for pest control. Mechanisms involved in host-plant resistance to herbivores may be grouped into two main categories:

- *Antixenotic* mechanisms prevent herbivores from approaching or establishing on a plant to feed or oviposit on it. There is a varied array of chemical and physical deterrents in plants to prevent or modulate preference of herbivores for feeding/ovipositing. Chemicals may be plant volatiles that act at long distances or nonvolatiles that intervene once the herbivore has landed on the plant or after it has probed the host. Physical characteristics in plants with antixenotic properties include morphological and structural features that interfere with normal feeding or oviposition. For example, plant epidermis hairs and trichomes are common morphological features that impede normal feeding in many herbivore insects and confer resistance to hairy cultivars.
- *Antibiotic* mechanisms cause deleterious effects, including mortality, for the herbivore once it has ingested a certain amount of the host plant. Antibiotic effects on herbivores that have fed on resistant plants may be expressed in a variety of consequences, and not only mortality: lowered development rates, failure of development features like pupation or adult emergence, reduced fecundity or fertility, and irregular behavior. Antibiosis is caused by toxic plant compounds or by other nontoxic plant characteristics like low nutritional quality, unbalanced composition in nutrients, or presence of enzymes interfering with normal insect digestive physiology.

There are mechanisms of plant resistance that are not related to host-plant constitution. These are "ecological resistance," "induced resistance," and "tolerance." Although they have been often neglected in plant breeding programs, their consideration when crop cycling and crop management practices are planned may contribute greatly to reduce crop losses by pests. *Ecological resistance* derives from the phenological asynchrony of crop and pest populations that prevents or diminishes the coincidence of the most susceptible crop growth stages with the most pest stages that are able to attack the host; sowing date may thus be planned for enhancing ecological resistance. *Induced resistance* is the response of a host plant to an environmental stress that reduces herbivore insect fitness or the plant availability for the insect. Once again, many agricultural techniques, like fertilization or irrigation, may alter plant physiology to enhance or decrease induced resistance. *Tolerance* is the capacity of some crop plants or crop cultivars to recover from injuries caused by the pest so that tolerant plants may attain yields that a similar amount of pest on a nontolerant plant would reduce. Several physiological processes have been identified in plants that may compensate for the injury caused by a herbivore insect and those processes may be facilitated by good agricultural practices.

Crop Resistance and IPM

Host crop resistance has been used profusely for disease control but it is increasingly considered and applied for integrated pest management. It is largely *compatible* with other IPM tactics, particularly biological control, although interferences of the host plant in predator–prey relationships have been reported recently. Its effectiveness is cumulative as its effects on pest population is exerted on several pest generations and usually persists after a pest's long exposure to the resistance traits; however, some cases of pests that have overcome resistance barriers at midterm have been described. One of the major advantages of crop resistance as a pest control method in the framework of IPM programs is that growers may easily adopt it as no particular expertise is needed. From a point of view of environment, crop resistance is in general safe, a required trait of any IPM method.

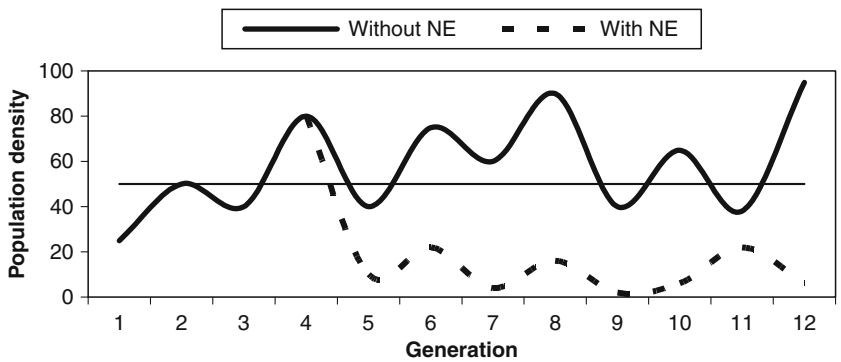
There are also some important limitations for a wider adoption of crop resistance in IPM. Frequently a long time is needed to develop a resistant cultivar, particularly in tree crops, and reaction to new and urgent problems is very slow. Modern biotechnology techniques, particularly genetic engineering (see below), are contributing to mitigate this handicap. Additionally, resistance traits are not always identified and available or they are very difficult to introduce into crop plants (also genetic engineering may favor the transfer of resistance genes between nonsexually compatible organisms). Another difficulty to implement crop resistance for pest control is the incompatibility of the resistance trait and commercial requirements; bad taste for consumers, for example, has been found in some resistant cultivars. In spite of the persistence of crop resistance mentioned as an advantage of the method, and as also mentioned earlier, local biotypes of the pest that are able to overcome or avoid resistance characters in the plant may develop and rapidly multiply as a consequence of their supremacy to exploit the resistant cultivar.

Biological Control

Ecological Basis of Biological Control

Biological control may be defined in general terms as the use or manipulation of natural enemies to suppress pest populations. Some authors include in the term any kind of nonchemical method that is biology-based. However, this is generally considered as incorrect and the narrower meaning given above is preferable. Although biological control may be practiced under several modalities, its principle is unique and responds to the scientific background of predator–prey ecology.

Natural enemies have been signaled as major components of natural control keeping populations within cyclic oscillations between maximal and minimal bounds in the framework of a "spontaneous balance of nature." Therefore the idea that exotic pests greatly increase their densities mainly due to lack of natural enemies in the new habitat led to attempts of reconstituting that balance by importation and release of exotic natural enemies. The ecological basis of biological control may be represented as shown in Fig. 8. A pest population that oscillated around a mean density very often above economic threshold is reduced to oscillations below that threshold after a release of an effective natural enemy. An important part of recent population ecology developments has dealt with prey-predator models that should provide the theoretical basis of biological control allowing it to progress from a rather empirical practice to a scientifically based technology. Unfortunately, the contribution of theoretical developments on predator–prey relationships has not been as fruitful as expected for biological control applications although they have clearly helped to progress beyond the "trial and error" stage of the first half of the twentieth century.



Integrated Pest Management. Figure 8
Fluctuations of a pest population before and after releasing an effective natural enemy. *Broken line* shows the fluctuation under the action of the natural enemy whereas *solid line* shows the dynamics of the pest without the natural enemy. *Horizontal line* represents the value of economic threshold

Understanding relationships between predators and prey and between parasitoids and hosts is important to optimize biological control practices. Prey consumption or host parasitization is the successful result of several behavioral steps of predator/parasitoid including:

- Selection of a *suitable habitat* where prey/host is more likely to occur. Predators and parasitoids may respond to biotic and abiotic characteristics of habitats where prey may be found. Several long-distance visual and olfactive stimuli from habitat components, including the proper prey/host, may be involved in habitat suitability recognition by searching predators and parasitoids.
- Once in the suitable habitat the predator/parasitoid has to *find a prey/host*. Within the habitat vision and olfaction also play a major role, but short distance stimuli may be decisive as adults sometimes look first for a prey in a random way until they come in contact with a potential prey.
- *Acceptance of a prey/host* when it is attacked by the predator/parasitoid is influenced by physical and chemical characteristics of the prey/host but also by hunger of the predator. Abundance may also determine if a prey is attacked or not.
- Prior to the final decision to consume/parasitize requires checking that a *prey/host is suitable* for the predator/parasitoid. Internal composition of prey largely determines if the predator rejects or continues to feed on it.

A relatively low amount of predatory arthropods are quite specific (they feed only on a particular family of prey) whereas generalist predators (they may feed on a wide range of prey taxa) are more common among both insects and spiders. In some groups both juvenile and adult stages are predatory but in some others only juveniles or adults prey. Parasitoids are usually more specific than predators and in general they can parasitize species belonging to one family or to a narrow range of families.

Taxonomic Adscription of Insect Natural Enemies

Natural enemies include three kinds of organisms: predators, parasitoids, and entomopathogens. Biological control only deals with the former two types whereas the third is the subject of microbial control. Predators are organisms that kill and consume a number of other organisms, called prey, along their lifespan from which they obtain the energy needed to grow, develop, and reproduce. Parasites are organisms that usually need to consume only one organism, the host, to develop and reproduce. When the host dies as a result of the action of one parasite this is called parasitoid.

Predaceous habits are relatively common in classes Insecta and Arachnida. Among insects, five orders include particularly important predators in agrosystems: Hemiptera (true bugs), Neuroptera (nerve-winged insects), Coleoptera (beetles), Diptera (flies), and Hymenoptera (wasps and ants). Among Arachnida, the order Acari includes some predatory families, particularly Phytoseiidae, and many predatory species grouped in several families belong to Araneae (spiders). Whereas predators are located in many insect and mite families, parasitoids mainly belong to certain families of Hymenoptera and a few to Diptera. Early on biological control almost exclusively used entomophagous insects but currently a wide range of organisms are being applied or manipulated to control pests. Table 2 shows those predators and parasitoids that are or have been commercially used in Europe and Mediterranean countries (http://archives.eppo.org/EPPOStandards/biocontrol_web) (accessed in December 2009).

The dozens of natural enemies included in Table 2 are only a part of those used in biological control in Europe. At least the same amount can be added when established agents managed for biological control by conservation (conservation and enhancement of natural enemies by crop and habitat management, see biological control by conservation below) are considered. Many other arthropod (e.g., dermapterans, carabids, staphylinids, coccinellids, syrphids, mirids, nabids, lygaeids, spiders, phytoseiids, and stigmaeids) and non-arthropod groups include predatory species that are or have been directly or indirectly managed to suppress pest populations in agriculture in Europe. Similarly, the same or close families to those cited in Table 2 include other species of parasitoids that have been used in biological control by conservation.

Strategies of Biological Control

To achieve pest suppression biological control may follow three main strategies: release of new natural

Integrated Pest Management. Table 2 Main predators and parasitoids that are or have been commercially provided for biological control in Europe and non-European Mediterranean countries

Phylum/order	Family	Species	Main target pests
Predators			
Insecta/Hemiptera	Pentatomidae	*Podisus maculiventris*	Lepidoptera larvae, Colorado potato beetle
		Picromerus bidens	Lepidoptera larvae
	Anthocoridae	*Orius albidipennis*	Thrips
		O. laevigatus	Thrips
		O. majusculus	Thrips
		Anthocoris nemoralis	Psyllidae in orchards
		A. nemorum	Pear psylla
	Miridae	*Macrolophus caliginosus*	Whiteflies
Thysanoptera	Aeolothripidae	*Franklinothrips megalops*	Thrips
		Franklinothrips vespiformis	Thrips
		Karniothrips melaleucus	Scales
Neuroptera	Chrysopidae	*Chrysoperla carnea*	Aphids
Coleoptera	Staphylinidae	*Aleochara bilineata*	Larvae of *Delia* sp. flies in soil
	Coccinellidae	*Adalia 2-punctata*	Aphids
		Chilocorus baileyii	Armored scale insects
		C. bipustulatus	Armored scale insects, olive black scale
		C. circumdatus	Armored scale insects
		C. nigrita	Armored scale insects, pit scales
		Coccinella septempunctata	Aphids
		Cryptolaemus montrouzieri	Mealybugs
		Delphastus catalinae	Whiteflies
		Rhyzobius lophanthae	Armored scale insects
		Rodolia cardinalis	Cottony cushion scale
		Scymnus rubromaculatus	Aphids
		Stethorus punctillum	Red spider mite
Diptera	Cecidomyiidae	*Aphidoletes aphidimyza*	Aphids
		Feltiella acarisuga	Red spider mite
	Syrphidae	*Episyrphus balteatus*	Aphids
Arachnida/Acari	Phytoseiidae	*Amblyseius barkeri*	Thrips, tarsonemid mites
		A. degenerans	Thrips
		Hypoaspis aculeifer	Sciarid flies in soil substrates
		Metaseiulus occidentalis	Spider mites
		Neoseiulus californicus	Spider mites

Integrated Pest Management. Table 2 (Continued)

Phylum/order	Family	Species	Main target pests
		N. cucumeris	Thrips
		Phytoseiulus persimilis	Red spider mite
		Typhlodromus pyri	Some spider and eriophyoid mites
	Laelapidae	*Stratiolaelaps miles*	Sciarid flies in soil substrates
	Cheyletidae	*Cheyletus eruditus*	Storage mites
Parasitoids			
Insecta/Hymenoptera	Mymaridae	*Anagrus atomus*	Leafhoppers
	Encyrtidae	*Anagyrus fusciventris*	Mealybugs
		A. pseudococci	Mealybugs
		Comperiella bifasciata	Armored scale insects
		Encyrtus aurantii	Scales
		E. infelix	Scales
		Gyranusoidea litura	*Pseudococcus longispinus* (mealybug)
		Leptomastidea abnormis	Mealybugs
		Leptomastix dactylopii	*Pseudococcus citri* (mealybug)
		L. epona	Mealybugs
		Metaphycus flavus	Soft scales
		M. helvolus	Soft scales
		M. lounsburyi	Soft scales
		M. swirskii	Soft scales
		Microterys nietneri	Soft scales
		Pseudaphycus maculipennis	Mealybugs
		Tetracnemoidea peregrina	Mealybugs
	Aphelinidae	*Aphelinus abdominalis*	Some aphids
		Aphytis diaspidis	Some armored scales
		A. holoxanthus	Armored scales
		A. lingnanensis	Some armored scales
		A. melinus	*Aonidiella aurantii* (armored scale)
		Coccophagus lycimnia	Soft scales
		C. rusti	Soft scales
		C. scutellaris	Soft scales
		Encarsia citrina	Armored scales
		E. formosa	Greenhosue whitefly
		Eretmocerus eremicus	*Bemisia tabaci* (whitefly)
		E. mundus	*Bemisia tabaci* (whitefly)
		Cales noacki	*Aleurothrixus floccosus* (whitefly)

Integrated Pest Management. Table 2 (Continued)

Phylum/order	Family	Species	Main target pests
	Aphidiidae	*Aphidius ervi*	Aphids
		A. colemani	Aphids
		A. matricariae	Aphids
	Braconidae	*Bracon hebetor*	Lepidoptera
		Cotesia marginiventris	Lepidoptera
		Dacnusa sybirica	*Liriomyza* spp. (leafminers)
		Opius pallipes	*Liriomyza* spp. (leafminers)
		Praon volucre	Aphids
	Eulophidae	*Aprostocetus hagenowii*	Cockroaches
		Diglyphus isaea	*Liriomyza* spp. (leafminers)
		Thripobius javae	Thrips
	Trichogrammatidae	*Trichogramma brassicae*	Lepidoptera
		Trichogramma cacoeciae	Lepidoptera
		Trichogramma dendrolimi	Lepidoptera
		Trichogramma evanescens	Lepidoptera
	Pteromalidae	*Scutellista caerulea*	Soft scales

enemies in a habitat (classical biological control); augmentation of natural enemies in the habitat (augmentative biological control); and conservation of those natural enemies already established in the habitat (conservation biological control) [8].

Classical biological control. Modern biological pest control began at the end of the nineteenth century when an exotic pest, the cottony cushion scale, invaded Californian citrus orchards and caused severe damages. Among other species, a predatory coccinellid beetle, *Rodolia cardinalis*, was imported from its Australian origin and released in some orchards of California. In a few years the predators spread throughout the citrus growing area and greatly reduced scale densities. Success of the strategy created much enthusiasm and many other cases of introduction of exotic natural enemies to control exotic pests followed the release of the Rodolia beetle. Some of them resulted in successful pest suppression but some other failed due to several causes. In spite of this, several benefits derived from successful and failed attempts. First, biological control was shown as a feasible technique to suppress pests. Second, a network of entomology laboratories – mainly American – spread around the world to support expeditions to look for natural enemies and some of them were the embryo of future biological control institutes. Third, successes and failures have pushed entomology science to develop the taxonomy of many families of predators and parasitoids, to furnish an ecological basis of predator–prey relationships for a more theoretically founded biological control and convince governments and policy makers that nonchemical methods may be as efficient as pesticides in controlling insect pests (if not more so). Although there are no reliable records of all introductions of new natural enemies for biological control, more than 1,000 cases have been reported in the last 100 years and about 60% provided a complete control or a substantial reduction of pest damage. A classical reference on the history of biological control in the twentieth century is DeBach [9].

A major concern and limitation of classical biological control is the potential impact that introduced exotic natural enemies may cause on native fauna. Criticism of biological control by introduction of

exotic natural enemies was soon theoretically enounced and several authors have advocated for limiting the trade of commercial biological control agents. In spite of little field data supporting the idea of strong impacts on native fauna, several countries have prepared regulations to forbid the importation of the most risky species of natural enemies. Although some principles have been established to assess risks of classical biological control agents much more knowledge on natural enemy interactions is needed. Two references may be useful for readers interested in environmental impact of biological control agents [10, 11].

Conservation biological control. As shown in Fig. 2, pest population development is the result of interactions among many biotic and abiotic components among which are herbivores and their natural enemies. In fact, pest population outbreaks are often due to agricultural practices which interfere with natural enemies that exert a certain control on herbivore populations in agrosystems. The goal of conservation biological control is to restore or enhance conditions for natural enemy survival, reproduction, and activity.

Conservation biological control may be implemented by manipulating the crop or the habitat. Main concerns of this kind of biological control are the identification of the natural enemies that can play a major role in keeping pest population at tolerable levels and what practices interfere with their functioning. Of course, pesticides are one of the principal interferences with natural enemies either by directly causing mortality or by indirectly influencing their biology, behavior, and movement at sublethal concentrations. Nowadays, data on the effects of pesticides on common natural enemies are needed before a pesticide is authorized; today, selectivity and low persistence in the environment, contrarily to that of years ago, are positive characteristics of pesticides used in pest control. Innovation in pesticide application techniques also tries to reduce the amount of pesticide and concentrates the application on certain sites, two goals that may increase selectivity in relation to natural enemies.

Certain practices to manage soil, water, and crop residues may contribute to natural enemy enhancement. Many insect pests live in the soil or have a part of their life cycle in the soil where they may be attacked by natural enemies; soil tillage has to be practiced in a safe and timely manner for such natural enemies. This includes crop residue management. After harvesting, crop plants may host a high variety of pest natural enemies; destroying crop residues also destroys pest individuals but may inflict a higher damage on natural enemy populations. Water management must provide relative humidity values that are optimal for natural enemies while damaging for pests.

Generally, the more stable is the agrosystem, the more chance natural enemies have to play their role as biological control agents. Whereas permanent crops allow population establishment and increase year after year, annual crops have to be colonized each year by natural enemies. However, cropping patterns may be designed to promote earlier and more abundant field colonization by natural enemies or enhance their survival and reproduction once the crop is established. Manipulation of sowing and harvesting dates may facilitate earlier colonization in the season and maintenance of natural enemies after season. Some other practices may also contribute to stabilize agrosystems. Some examples of crop manipulation to benefit natural enemy survival and activity include strip harvesting, variable crop phenology, and inclusion of banker plants to keep prey and predators in the field between seasons.

Crop diversification seeks to delay crop colonization by insects, both pests and natural enemies, or reduce their retention in the crop. Intercropping – growing two or more crops in the same field at the same time – can cause a more abundant colonization by natural enemies although there are records of the contrary phenomenon. In non-crop-diversified agrosystems, herbivore colonizers arrive earlier and in higher numbers and consequently higher populations of natural enemies may be built up. Crop diversification may be performed by different spatial and temporal patterns of crops in the landscape where one field is the source for colonization of neighboring fields. Diversification of landscape with nonagricultural vegetation is another way to enhance natural enemy presence and activity. Non-crop plants in inter-rows, margins, roads, "fallow" plots, etc., may provide shelter and even food for natural enemies when conditions on crop plants are not suitable for natural enemies and may facilitate the movement of predators and parasitoids among fields in a patchwork landscape.

Biological control by conservation has shown to be very efficient when practiced after a sound knowledge of the agrosystem. Furthermore it is safe as managed natural enemies are already established in the habitat and no negative effects on the environment may be expected. Unfortunately, biological control by conservation is not always possible due to a lack of effective natural enemies in the habitat or a lack of knowledge about how crop or habitat may be managed to effectively enhance predator or parasitoid action. Research needed to implement a program of conservation biological control has to be closely linked with local conditions, and solutions are not universal but related to each particular location.

Augmentative biological control. Although natural enemies exist, sometimes conservation and enhancement practices are not sufficient to increase their populations until reaching levels which are capable to suppress pest populations at desirable levels. In these cases the release of reared natural enemies is needed. Augmentative releases may be necessary when the natural enemy is not present at the place and time needed. There are two types of augmentative releases:

- A relatively low number of individuals of the natural enemy is released and the suppression is expected to be achieved by the first or ulterior descendants: *inoculative augmentation.*
- A high number of individuals are released and control is expected to be exerted by them: *inundative augmentation.*

As in the previously described biological control strategies, augmentative releases use natural enemies that are able to search, locate, recognize as suitable, and attack the prey, but supplementary characteristics in the natural enemy are required in augmentative releases. Biocontrol agents have to be reared, and in inundative releases, they have to be mass reared. Rearing techniques have to meet several economical and quality requirements. The high costs of rearing are mainly due to a lack of true artificial diets but also by the necessity of labor to manipulate materials and individuals and the necessity of producing natural enemies for a rather narrow interval of time in the year. Predators and parasitoids need to be reared on their natural herbivore prey/host or, in some cases, an easily reared alternative prey/host, as in the case of flour moth eggs that can be produced daily for millions at relatively low cost and supplied as food to rear many generalist predators. In addition, herbivores have to be reared on their natural host plant, or similar alternatives, that usually need specific temperature, humidity, and light conditions, sufficient space, and a lot of manpower. Automatic processes to produce plants and natural enemies in biofactories have been designed but much more still has to be done to lower production costs. The extra cost of producing for a few demand peaks in the year may be mitigated if an effective storage method is available. For instance, the egg parasitoids *Trichogramma* spp. may be produced throughout the year and stored for months as diapaused larvae that are reactivated in their development some weeks before they have to be applied in the field.

Continuous rearing of natural enemies in nonnatural conditions and ulterior transport to the field may alter their quality to perform as biological control agent. Genetic uniformity and inbreeding, negative selection for desirable characteristics to perform in the field, high impact of diseases in mass rearing colonies, and lack of learning opportunities of natural features may be some of the problems derived from natural enemy mass rearing. Procedures for quality control are being developed for the most common natural enemies [12]. Dispersal and search capacities, fecundity, health, correct species, and biotype are some of the characteristics checked in natural enemies for quality control.

Biological Control and IPM

Biological control is an important component of many successful IPM programs. The success of biological control has pushed pesticide companies to design new active ingredients with less impact on natural enemies. Whereas a few decades ago long persistence and broad action spectrum were two positive characteristics of new pesticides, current innovation of chemicals for integrated pest management looks for high selectivity and short persistence thus helping pesticides to become more compatible with biological control. The introduction of a new natural enemy in agrosystems to control a certain pest may allow reduced pesticide application and express the full potential of native natural enemies to control other pests.

Predator–prey interactions may be mediated by the host plant so that a natural enemy that is able to successfully control a pest on a certain crop may fail to do so on another crop. Hairy cucumber cultivars, which are more resistant to greenhouse whitefly than glabrous ones, were preferred to control the whitefly before *Encarsia formosa* was profusely used for biological control of this pest. Once the biological control was generally adopted in Dutch greenhouses, objectives of plant breeding shifted 180º and less hairy cultivars were again cultivated because these facilitated the inspection of leaves by the parasitoid to select and parasitize a host, thus improving the efficacy of greenhouse whitefly biological control. Tritrophic relationships – host plant, herbivore, and natural enemy – have to be considered before planning a biological control program. For IPM programs based on biological control, crop plant management and general cultural practices also have to be adapted to enhance natural enemy activity. Detection of those cultural practices that injure natural enemies may allow cultural modification in order to make predators or parasitoids more compatible with agricultural environments. For instance, tomato deleafing to produce more colored fruits has been found to interfere with the establishment of whitefly parasitoids that are inside the host in lower leaves. Tomatoes may be equally deleafed but leaves should be left on the soil between rows for some days until adult parasitoids emerge and fly to upper leaves to parasitize the host there.

Microbial Control

Entomopathogenic Organisms as a Natural Mortality Factor on Insect Populations

Insects are naturally affected in nature and also in agroecosystems by a varied array of pathogenic organisms, called entomopathogens, that cause diseases on insect pests. When naturally occurring epizootics are not efficient enough to lower pest populations under economic thresholds or they occur too late (natural epizootics are very dependent on natural abiotic conditions), the entomopathogen can be released into the environment at the time needed or manipulate the habitat to enhance the impact of the disease on the pest population. Entomopathogens may be mass produced and formulated for applying as a chemical pesticide. Many aspects described and discussed in biological control may be applied to microbial control. For instance, microbial control can be employed with classical, conservation, and augmentation techniques. In classical microbial control, a pathogen is isolated in a foreign insect and inoculated into a population that previously has never been exposed to that pathogen, whereas in conservation microbial control the impact of an already established pathogen is enhanced by manipulating crop or habitat conditions. Finally, augmentative microbial control inoculates a nonexotic pathogen at the time needed, or the crop field is inundated by microbial pesticides whose efficacy relies on the primary infection and not the secondary one as in the other techniques.

Main Insect Pathogens Used for Microbial Control

There are several kinds of microbes that cause diseases in insects and are used to control insect pests. Groups with more species and microbial control uses are: bacteria, viruses, fungi, and nematodes.

- Bacteria. The most known and used bacterium is *Bacillus thuringiensis* (Bt). This bacterium has some interesting properties that make its use very compatible with other IPM methods. Entomopathogenic action of Bt originates in some insecticidal toxins – the so-called delta endotoxins – that are produced by the bacterium during its sporulation and once ingested by the insect is activated in the digestive system and causes gut paralysis, feeding cessation, and later larvae show general paralysis. One of the most valuable characteristics of Bt toxins is their selectivity according to the Bt strain. Historically Bt has been used to control specific Lepidoptera pests and, to less extent, some Diptera and Coleoptera. Nowadays, the range of pests targeted by Bt toxins include species of some other insect groups. Also importantly, Bt can be produced in large scale and at a reasonable price by fermentation and then formulated like an insecticide to easily spray or powder the crop. Genes encoding the production of Bt toxins may be transferred by genetic engineering to crop plants which become resistant to insects that are susceptible to the Bt toxins introduced into the plant (see section "Crop Resistance"). Biopesticides based on Bt are

widely used in world agriculture, particularly in IPM programs and organic farming. Their relatively high prices and low permanence on the crop plant, mainly reduced by UV radiation when the crop is grown in the open air, are major limitations. Bt biopesticides are less than 1% of the pesticide world market in economic terms.

- Viruses. There are many types of insect pathogenic viruses but only baculoviruses are commercially available for insect pest control, mainly for Lepidoptera and Hymenoptera pests. Baculoviruses have a characteristic that gives them some advantages in comparison with other entomopathogens: they are able to create secondary inoculums and ulterior epizootics so that permanence of control efficacy may be longer than for other microbial control agents, although, like Bt, they are very sensible to deactivation by UV radiation. Furthermore, viruses are very selective and thus especially good for integrating their use in IPM programs. Genetic engineering techniques have shown a high potential to overcome some of the limitations of baculoviruses; higher knockdown effects and host range are two of the traits introduced in genetically modified baculoviruses. Probably their high price is the principal constraint for repeated field applications.
- Fungi. They comprise microorganisms that cause diseases on a variety of insect groups. In comparison with the two above, fungi are able to act in topical applications and do not need to be ingested to be active. This is why they are preferably used against sucking insects like whiteflies, scales, or aphids. Several fungal entomopathogens occur naturally and reduce insect pest incidence in agroecosystems but they are also mass produced and applied as biopesticides. Efficacy of fungal biopesticides is largely limited by low relative humidity although suitable formulations may attenuate this constraint; also UV-radiation protectors in fungal biopesticides are being tried to prolong their persistence on crop plants.
- Nematodes. Nematodes are a quite large group including many species that have several kinds of associations with insects. Facultative and obligate parasitism is among these. Some insect parasitic species have established a symbiotic relationship with entomopathogenic bacteria that confer to these kinds of nematodes a more virulent action against insect pests. In this case the bacterium is responsible for killing the host and, once dead, to preserve the host insect from being invaded by other microorganisms and therefore available for the nematode. These entomopathogenic nematodes are the most valuable for pest control, especially when part of their life cycle is in the soil, where nematodes are more effective. For pests that do not inhabit the soil, nematode desiccation at low humidity conditions is a major limitation of these agents for use in microbial control.

Use of Entomopathogens for Insect Control in IPM: Advantages, Disadvantages, and Techniques

Microbial control is one of the most promising methods to control insect pests within IPM programs. It is highly specific and selective for nontarget arthropods like natural enemies, it is harmless for vertebrates including man, it has very little risk of environmental pollution, it is easy to apply as most of them are formulated to be sprayed with conventional machinery, and there are techniques for engineered modified entomopathogens with improved performance. Disadvantages include short permanence in the environment for pest control, slow efficacy, moderate probability of generating resistance to the active ingredient in targeted pests, host production cost, and poor acceptance of biotechnology products by consumers in certain countries. In summary, microbial control is an easy method to integrate in IPM and should substantially replace chemical treatments if efficacy and cost problems are solved and biopesticides are perceived by public opinion as a safe replacement for chemicals.

Behavioral Control

Pheromones and Other Semiochemicals

It is well known that insects, and other animals, communicate within the same species and with individuals of other species by chemical signals; chemicals involved in communication are called semiochemicals. Intrapopulation semiochemicals are called pheromones and those devoted to communicating among species are allelochemicals. These are divided into two

categories depending if they benefit the chemical releaser (allomones) or to the receiver (kairomones). As more is known about insect behavior, more relevance is given to the role of semiochemicals in the communication governing crucial insect functions and more applications of semiochemicals are envisaged for insect control. Several kinds of semiochemicals have been investigated for both scientific and practical purposes but most attention has focused on pheromones.

Although chemical signals in insect communication had been observed some centuries ago, the chemical identification, synthesis, and demonstration of the sex attraction capacity of a pheromone was performed in the 1950s. A general enthusiasm on potentialities to govern insect behavior and suppress insect pests followed that pioneering work with considerable practical achievements but also with some limitations. The number of insect functions governed or mediated by pheromones is large; in addition to courtship behavior – the most studied for practical applications – social, physiology, trail, defense, finding, discriminating, and aggregating on the host plant are among insect biology features with pheromone involvement. The chemical nature of pheromones is quite diverse according to insect taxon and function. Pheromone composition usually has to meet two main requirements: be highly specific and be easily transportable by air currents. This double requirement is in part contradictory as volatile compounds need to be short molecules, with low molecular weights, but long enough to make possible several combinations of atoms for specificity. As pheromones are typically blends of several organic components synthesized by the insect or less commonly sequestered from the plant, specificity is achieved not only by chemical structure of components but also by the exact composition of the mixture.

Main Application of Pheromones for IPM

The main applications of pheromones for insect pest control may be included in one of the three following groups:

- Pheromones for *detection and monitoring* pest populations. One first application of pheromones is to know when and where a pest population is present. The pheromone is put in a trapping device and number of trapped individuals recorded. There are many kinds of traps that have been used; optimal trap design depends on pheromone composition and insect species. Generally, trap catch numbers will give relative estimates from which absolute numbers of pest population cannot be derived. Early warning, determination of timing for control intervention according to the pest population phenology, early detection for quarantine actions, and dispersal studies are some of the purposes that may be reached with pheromone trapping. However, decisions that need to know population numbers or densities rarely may be based solely on trap catch records unless a sound relationship between relative and absolute estimates had been previously established.
- Pheromones may be used to directly control insect pests by *mass trapping*, that is, by trapping a sufficient number of individuals from a pest population. Usually a huge amount of traps is needed to remove a significant number of individuals to lower pest damage. The rice stem borer – as other Lepidoptera – is controlled nowadays by mass trapping in the Mediterranean area. A first interesting variant of mass trapping with pheromone traps is practiced with *trap trees* in bark beetles. When a pioneer bark beetle recognizes a tree as a suitable host, it starts releasing an aggregation pheromone. This is soon followed by another attractant emitted by the tree that complements aggregation of many bark beetles on the same tree that may be destroyed, burnt, or sprayed with insecticides to kill many beetles. A second variant of mass trapping is when the attracted insect is not glued on the trap but killed or sterilized and in any case eliminated. Some of the most harmful fruit flies are caught in traps, sterilized, and released again to the environment for control as in the sterile technique programs (see below in the section "Genetic Control"). Principles of mass trapping are very simple but many limitations have prevented successful application. One major constraint of the method is its low efficacy when pest population densities are high; in these circumstances a reducing treatment is needed before applying mass trapping.

- Species in which orientation of one sex to the other for mating is performed by sex pheromones are sensitive to the application of *mating disruption* techniques. The principle, as in the other cases where pheromones are involved, is rather simple. The permeation of the air with synthetic pheromone components – or the whole pheromone blend – interferes with the orientation of the searching sex that usually is unable to meet the other sex; oviposition is thus prevented and population rapidly declines. Despite many studies conducted on the mechanisms underlying mating disruption techniques, much remains still unknown. A number of factors influence feasibility and efficacy of these techniques. Systems for pheromone delivery have to assure air permeation for all the period during which males and females may meet and mate, a requirement difficult to satisfy for very volatile molecules. Synthetic components to be delivered have to be produced and released at reasonable prices; otherwise, the technique is not competitive with insecticides that are cheaper and relatively easy to apply. Efficacy of mating disruption has repeatedly shown to be drastically limited by high pest population densities and this means that mating disruption has to be applied in early generations when populations are still low and forecasting whether they will reach economic thresholds is difficult. In many countries authorization to sell pheromones for mating disruption purposes has to follow hard administrative processes too close to chemical insecticide registration; this sometimes deters companies from investing money in research and development. Dozens of pests are controlled nowadays by mating disruption techniques with acceptable efficacies, especially to manage Lepidoptera in vineyards and fruit orchards.

Compatibility of Pheromones in IPM Systems

Pheromones are easy to integrate into IPM systems because they are very compatible with other control systems such as biological control. Selectivity of the method is a major advantage. As environmentally friendly substances they do not cause pollution problems, generally have no toxic effects on nontarget species, and permanence in the environment is low. When used for monitoring pheromones are easy to work with and field technicians do not need particular skills.

Pheromones are an elegant tool for IPM and used more and more profusely sometimes without sufficient rigor. On the other side, scientific research is not sensible enough for solving real problems that would lead to a faster adoption of pheromones in the field. As stated by Millar [13] more pragmatism is necessary in research on pheromones [13]. The deeper knowledge of how pheromones work acquired in the last decades should allow focusing our efforts on those systems that can be most effective. Furthermore, globalization of insect pests should push local and national efforts to more international cooperation because "my pest today may be your pest tomorrow" and vice versa.

Genetic Control

Genetic pest control comprises those techniques that use the insect pest for its own destruction. Genetic control consists of the release of sterilized, sterile, or incompatible individuals of one species into its wild population to cause a high proportion of sterile matings and hence reduce or eliminate the wild population. Three main methods causing sterility by different mechanisms are available for agricultural pests: sterile insects, incompatible insects, and hybrid sterility. Some other mechanisms have been exploited to control pests by genetic methods. Note that use of resistant/tolerant host crops is not considered within this section of genetic control.

In the sterile insect technique, insects are mass reared in controlled conditions, sterilized, and then released into the field. Insects can be sterilized by irradiation or by chemosterilization. Gamma irradiation, in which dominant lethal mutations arise as a result of chromosome break in treated cells, has been the most used technique in field programs. One of the key aspects to be determined before applying the technique in the field is the optimal irradiation dose to produce enough degree of sterility without causing somatic damages in the sterilized individual. The optimal dose is a function of several factors including insect species, its sex and physiological age, and the level of sterility required. Males are usually sterilized and released. Efficacy of the method relies on

the capacity of released males to compete with wild males to mate with wild females. Progeny of females mated with sterile males will die soon after egg oviposition. Ideally insect mass rearing procedures should target males for sterilizing and releasing because females may need a higher dose for sterilization and once released they may be harmful for the crop as a consequence of ovipositing.

With no doubt the successful eradication of the parasitic screwworm fly – the larvae of which eats the living tissue of warm-blooded animals including humans in some circumstances – in wide areas of North and Central America contributed decisively to an increase in the amount of funds devoted to the research and applications of the sterile insect technique. In agricultural pests there have also been a number of successful male sterile programs, particularly in fruit flies. The Mediterranean fruit fly, *Ceratitis capitata*, has been eradicated in some countries of Latin America and they have been declared medfly-free regions; those countries can export fruits to countries which have fruit flies as quarantine pests. In contrast, attempts to use sterile male techniques in several moth pests have failed mainly due to the difficulty or mass rearing the moth at reasonable prices.

Insect incompatibility has been also used in field conditions but less extensively than the sterile insect technique. One of the methods of insect incompatibility used is based on the effect of crossing sexes in two conspecific populations resulting in only partial embryonation and thus population decrease. Incompatibility may be caused by microorganisms that are present in one population and not in the other. For practical pest control purposes, the release of one sex of one geographic population into another location may result in nonviable progeny and therefore population suppression.

Finally, sterility also may be achieved by crossing individuals of two species that produce apparently normal but completely or partially sterile hybrids. If the hybrid mates with at least one of the parent species, it can be mass reared and released in the field for genetic control. An advantage of this system comes from the fact that it does not need a sterilization method and the quality of released individuals is better than in the case of irradiation.

Genetic control has been shown as successfully practicable in commercial conditions in several cases. However, some failures have taught us about the constraints of the method. Reinvasion of treated areas by gravid females is one of the common causes of failure. Real knowledge of the dispersal capacity of the targeted species should prevent applying the sterile male technique in too small areas. Area wide programs are particularly needed for sterile insect technique application to prevent early recolonization of the treated area from neighboring zones.

One of the key factors for successful pest control with the sterile male technique is the ability of treated individuals to compete with wild males for wild females. Lack of adequate information on how to assure competitive treated males and about mating behavior could have been the main failure cause of many genetic control programs. More attention was then devoted to developing quality control of mass-reared insects that are now routinely applied in many programs. A major conclusion after several years of applying genetic control programs is that the method by itself may be insufficiently effective to be uniquely applied and in many situations it may be useful when integrated in IPM systems to control the target and other interacting species.

Another perspective about the future of genetic control relates to potential contributions of insect molecular biology, more developed in model insects like the fly Drosophila but increasingly interesting for insect pests and pest natural enemies. As genetic control was soon seen as very limited by the lack of appropriate genetic material or by too reduced fitness in irradiated individuals, transgenesis may contribute to renewing the interest for genetic pest control. Introduction of desired transgenes into target insect species or the release of insects infected with engineered microorganisms – mainly rickettsia-like, Wolbachia – which mate with no infected individuals cause reduced fitness progeny. The reader curious about potential contributions of molecular biology to genetic control should consult the review by [14].

Cultural Control

As for any organism, insect populations – and thus pests – have a variable rate of increase depending on,

among other factors, biotic and abiotic factors (see Fig. 2). An insect population becomes a pest in the agroecosystems when the insect environment is favorable enough to enhance population increase until economic threshold is reached and control measures have to be adopted. To manipulate that environment to make it less favorable for pests is called cultural control. Many kinds of crop or habitat manipulations devoted to constrain pest population development or enhance natural enemy numbers and activity may be included within the term.

It is quite difficult to list the practices that may be applied to reduce pest populations or enhance natural enemies. Some of the practices routinely applied are apparently unrelated to pests and natural enemies and only when they are eliminated or modified their implication in pest control is discovered. In other cases growers abandoned cultural practices that had been used to control pests due to the efficacy and reliability of cheap pesticides and they had to rediscover their usefulness. (a) Crop rotation, for example, prevented populations of non-generalist herbivorous insects from building up high populations without moving from the same field; crop monoculture provoked a high resource concentration on the same place for a long time and facilitated insect development and reproduction.

In addition to crop rotation, some general cultural practices are used to lower pest populations: (b) removal of crop residues between two successive seasons may reduce insect survival in unfavorable seasons (e.g., cold winters or dry summers); (c) management of planting or harvesting can avoid pest populations peaking at particularly susceptible crop growth stages; (d) unbalanced fertilization use to favor some herbivore insects like aphids that can multiply per several units their rate of increase when fed on plants with an excess of nitrogen; (e) the same as in the previous point could be said for other agricultural inputs like water, mulching, or plant hormones that alter physical environment and crop plant physiology in favor of or detriment to pests.

Many cultural practices have an important impact on pest populations by enhancement of *natural enemies*. Weed management, beyond preventing damages to the crop should take into account their role in insect biology. Nonagricultural vegetation in margins and hedgerows may offer shelter, refuge, or food sources for predators and parasitoids, but also for herbivorous insects that colonize crops early in the season. This double role of margins has to be carefully studied before margin management practices are recommended. The behavioral manipulation of the insect pest and their natural enemies may allow making the protected resource (e.g., the crop) unattractive for the pest and attract it to an unprotected resource (e.g., nonagricultural plants). The opposite done for natural enemies in this kind of strategy is called "push and pull" [15]. *Intercropping* – the cultivation of two or more crops simultaneously on the same field – is another potential way to manipulate the environment for making it unfavorable to the pest. It is frequently observed that there are fewer pests in fields with intercropping than in monoculture; more attractiveness for natural enemies and less for herbivorous insects are two hypotheses to explain such a phenomenon.

Biotechnology and IPM

Emerging Biotechnological Techniques for IPM

Developments in plant biotechnology have contributed decisively to progress in agriculture in general and IPM in particular in the last decades of the twentieth century [16]. Biotechnology has been defined by the United Nations Convention on Biological Diversity as any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use (http://www.cbd.int/convention/ accessed on January 27, 2010). Probably, genetically engineered crops are the most socially known products of biotechnology applied to plant breeding. However, there are many other achievements and tools issued from plant biotechnology that have allowed progress in the scientific basis of IPM and multiple applications in the last decades and may allow even faster progress in the future. Following are some achievements of plant biotechnology that are relevant for IPM: (a) incorporation of insect resistance genes into commercial crop varieties; (b) the design of chemical and biological novel insecticides; (c) genetic modification of insect pests with lethal characters for genetic control or with beneficial traits to improve activity and efficacy of

biological control agents; (d) rapid and reliable detection of insecticide resistance before genes responsible of resistance are widespread in the pest population and control fails in the field; and (e) identification of arthropod species and biotypes for pest diagnostics or trophic studies.

Host-Plant Resistance: Integrating GM Crops into IPM Systems

Host-plant resistance has been used in IPM to a rather limited degree (see above). Host resistance to herbivore insects is generally a quantitative trait that is difficult to manage and long to be incorporated into crop plants in conventional plant breeding programs. Techniques of genetic engineering have allowed some of those problems to be overcome by faster identification of insect resistance sources by means of molecular markers associated to resistance traits and by speeding up gene transfer from those sources to crop varieties to produce the genetically modified (GM) crops. The capacity to produce entomopathogenic toxins and digestive enzymes inhibitors have been the most used characters to confer insect resistance to crop plants by means of genetic engineering techniques. However, a varied array of other characters has also been successfully introduced into crop plants for insect pest control purposes. Insecticidal capacity of an entomopathogenic bacterium, *B. thuringiensis* (Bt) is caused by some of the toxins that the microorganism produces when it sporulates. Truncated genes expressing Bt toxins (dozens of Bt toxins and corresponding genes have been identified) have been transferred to several crop and forest plants to give the so-called Bt crops and plants. More than 35 million ha of maize and 15 million ha of cotton with Bt-expressing genes (alone or stacked with other transgenes) were grown in the world in 2009 [17] to control mainly Lepidopteran pests [17]. In Spain – the European country with the largest surface of Bt maize, the only GM crop allowed for cultivation in Europe – a survey conducted among more than 400 growers on the economic, social, and environmental impact of Bt maize found this: a mean increase of 12% in the gross margin in Bt growers, more than a 50% decrease in the number of insecticides applied due to the cultivation of Bt maize; this reduction was not very high because only a minority of growers used to spray against the pest targeted by the GM crop due to low efficacy [18].

In spite of the potential contribution of Bt crops to the sustainability of IPM through the selective control of key pests and the important savings of insecticide sprays, the deployment of GM crops has been very controversial in some areas like the European Union. Major risks concern potential development of resistance to Bt toxins in targeted pests and negative effects that Bt crops could have on nontarget organisms (NTO). The development of resistance to Bt toxins in targeted pests would cause a significant loss of an important tool for selective control of certain pests in the framework of IPM programs and organic agriculture and could drastically reduce the lifetime of Bt crops. Most countries that grow Bt crops have specific programs to monitor targeted pest populations for the evolution of resistance and have implemented strategies to prevent resistance development. Until now, no Bt-resistance has been reported even in areas with almost 15 years of cultivation of Bt crops. More public attention has been paid to potential negative effects of GM crops on nontarget organisms. Most of the work conducted in this area has been devoted to Bt maize and practically no negative effects on biological control functions have been reported [19]. Consequently, Bt crops may prevent a substantial part of current insecticide usage and they can be integrated with biological control into more sustainable IPM programs.

Introduction of Traits into Insects

The introduction of deleterious or beneficial traits into insects has been achieved in several cases for varied purposes. Lethal gene inoculation into wild pest populations for genetic control may overcome some of the problems of conventional sterile insect techniques in which insects may be harmed when they are sterilized with irradiation or chemosterilization techniques and their competitive ability decreased. However, introduction of desirable traits for enhanced efficacy of biological control agents is a remarkable contribution of biotechnology to IPM. Unfortunately, most programs in that last respect have focused on insecticide-resistant genes that increase the possibility of using chemical insecticides and biological control in a compatible way. Despite this innovative approach,

which may contribute to increasing the effective application of biological control, it also may lead to increased use of chemical insecticides.

Detection and Monitoring of Insecticide Resistance

Insects, like any other organism, may become resistant to insecticide active ingredients if they are submitted to frequent pressure of that ingredient. More than 600 insect species are nowadays resistant to one or more insecticides (http://www.pesticideresistance.org/search/1/). As fewer insecticide active ingredients are available in world agriculture, more is needed to increase the lifetime of registered substances and to implement strategies for resistance development prevention.

Several tactics to prevent resistance development in the pest population may be implemented and may succeed at least to delay the wide spread of resistance genes in the targeted population. Commonly, insecticide resistance in a pest is first detected when typical doses fail to control it and usually it is too late. Even at low frequency, earlier detection of the presence of resistance genes in the targeted population before they become common is crucial for the successful implementation of any antiresistance strategy application. As resistance is caused by a varied set of mechanisms, there are also several methods to detect resistance genes. Biochemical, immunological, and molecular methods are available now for the most common insecticides that allow screening a large amount of individuals for resistance gene presence. Improved comprehension of resistance mechanisms should lead to developing more specific, faster, and cheaper methods in order to monitor more populations at reasonable prices.

Identification of Arthropods

IPM needs the correct identification of insect species (or even biotype) in multiple situations: to apply a specific and selective method to control a certain species, to release a biological control agent that needs particular characteristics only available at biotype level, to detect quarantine pests in border inspections, and to study predator diet in trophic ecological studies. Morphological features that classically have been used for insect identification are frequently unknown or they are difficult to observe for nonspecialists. Biotechnological tools also may be valuable for various ecological studies that need markers for distinguishing target individuals from nontarget ones. Some of the current applications of biotechnology for identifying insects and their functions include: markers for dispersal measurements or to estimate insect densities by capture, release, and recapture of marked individuals, silencing genes for investigation of the function of certain proteins, invasion and spreading processes, and phylogenetic relationships between taxonomical groups.

Novel Bioinsecticides and Tension Actives

As described in microbial control, entomopathogenic microorganisms are used to control pests although some factors linked with their costly production and narrow host range are limiting applicability. Entomopathogens may be genetically engineered to incorporate genes expressing foreign proteins with new insecticidal capacities, including larger host range, or proteins that negatively interfere with insect metabolism and physiology like insect hormones or juvenile hormone esterase that are involved in insect metamorphosis. A major concern about the bioengineered entomopathogens deals with the fate and permanence of foreign genes in the environment. Another promising line of biotechnological research in relation to IPM includes biosurfactants. A considerable amount of pest control agents is applied as sprays that need surfactants for correct application and spreading on target surfaces. Classical chemical surfactants are increasingly rejected by consumers and legislation due to their environmental impact and this stimulates the research on alternative biosurfactants. These are a group of heterogeneous secondary metabolites produced by a variety of microorganisms during their growth with significantly improved characteristics in comparison with homologous chemicals: those are biodegradable, have a lower per se toxicity, have more environment-friendly characteristics, require cheaper fermentative processes to produce them, are efficient at more variable conditions and at lower quantities, and have the potential of tailoring to suit specific applications. In addition to their activity as surface tension

reducers and other chemical functions, biosurfactants have shown considerable biological antifungal and antiviral activity although in many cases their mode of action is poorly understood.

Implementation of IPM: Incentives and Constraints

More than 20 years ago, Wearing [20] wrote an article reporting results of a survey conducted among researchers and extensionists of three regions of world agriculture (Australia–New Zealand, Europe, and USA) on the IPM implementation process [20]. Still in 2010, many of the conclusions remain valid and following is a summary of those aspects of IPM implementation that enhance or constrain the adoption of IPM systems in western agriculture. There is general agreement among scientists about the accelerated progress of research on pest knowledge and control as well as the slow adoption of the new methods in practice. Knowing the key elements in the technology transfer and implementation of IPM may accelerate its application.

Among incentives to adopt IPM systems in Wearing's work, most surveys first perceived the cost advantage, followed by the development of pesticide resistance in local pest populations, the hazard to the grower from using pesticides, and environmental issues. The surveys also examined the major constraints for IPM implementation. The importance of obstacles for faster and wider application of IPM varied from one region to another. Whereas in USA over the 50% of respondents ranked social/market obstacles first, in Australia–New Zealand organizational obstacles were the first ranked, and technical obstacles were signaled in Europe as the least important to implement IPM.

In Europe much has been done in labeling agricultural products issued from IPM technology, particularly from regional administrations: IPM labels and integrated production (IP) labels have proliferated in the second half of the twentieth century in northern and southern Europe as well. The International Organization for Biological and Integrated Control (IOBC/WPRS) soon established the bases of IP and later developed guidelines in specific crop groups (see the organization Web site www.iobc-wprs.org). Food retailers and marketing food chains more recently have developed auditing procedures, in part inspirited in IPM principles which have pressed growers to progress more rapidly to more integrated production techniques. The impact of labeling and certification initiatives on IPM adoption has been quite variable in each country and commodity but the initiatives probably have contributed to publicize IP and IPM techniques among consumers.

A common question among policy makers is how to measure IPM adoption. Most likely, data on acquisition and use of tools for implementing IPM in the field (for instance, monitoring devices, meteorological receptors, users of warning systems and significant Web sites, and varied software for decision making among others) in combination with data related to pesticide usage (amount and economic value of pesticides sold, commodity rejection because of high residue levels, inspection of pesticide residues in food trading, and significant habitat and ecosystem elements) may give an approximate idea of the progress made by the implementation of IPM. The number and size of companies (including small local firms) devoted to selling IPM products (e.g., natural enemies for biological control) may be another realistic way to monitor IPM adoption in world agriculture. Data on all these indicators are very disperse but perhaps they allow moderate optimism for faster IPM adoption in developed and emerging countries. Additionally, more strict legislation on pesticide use and the "disappearance" of most insecticides in the coming years may accelerate this process.

Future Directions

Most elements of IPM are among the factors that contribute greatly to the sustainability of agriculture. Beyond IPM, integrated control of insect pests, diseases, and weeds should progress toward the integration of all elements of agroecosystems to produce a balanced and harmonized growth of plants through true integrated production techniques. Exploitation of local natural resources and energy saving are common benefits of implementing novel integrated management systems. Additionally, most insecticides are being prohibited by western legislations. This should be the main focus of IPM progress to accelerate the transition of classical pest control based on chemicals to more integrated approaches.

Review of the vast literature on IPM confirms that success has come from a fundamental understanding of the processes acting in agroecosystems, rarely from a revolutionarily new control tactic. However, faster adoption of IPM strategies and tactics should also come from a more intense linkage among research, development, education, extension, and production. It is well documented that much of the scientific progress issued from R&D is not applied in practice or it takes too long to do it. Analysis of surveys conducted to identify the major incentives for the adoption of IPM systems by growers shows that an innovation is not adopted unless it contributes to producers' economic goals and meets the requisites for acceptance by the whole society.

Acknowledgments

Mr. Thomas Holland, MA from Stanford University in California, and now working at the Centro de Linguas at the Universidade da Coruña, proofread the English for this entry. His patience and professionalism has, I hope, helped ensure a comprehensible reading.

Bibliography

1. Oerke EC, Dehne HW (2004) Safeguarding production-losses in major crops and the role of crop protection. Crop Prot 23:275–285
2. Stern VM, Smith RF, van den Bosch R, Hagen KS (1959) The integrated control concept. Hilgardia 29:81–101
3. Kogan M (1998) Integrated pest management: historical perspectives and contemporary developments. Annu Rev Entomol 43:243–270
4. Higley LG, Pedigo LP (1996) Economic thresholds for integrated pest management. University of Nebraska Press, Lincoln
5. Klassen W (2008) Area-wide insect pest management. In: Capinera JL (ed) Encyclopaedia of entomology, vol 2. Springer, Dordrecht, pp 266–282
6. Mumford JD, Norton GA (1993) Expert systems. In: Norton GA, Mumford JD (eds) Decision tools for pest management. CAB International, Wallingford, pp 167–179
7. Southwood TRE, Henderson PA (2000) Ecological methods, 3rd edn. Blackwell Science, Oxford
8. Van Driesche RG, Bellows TS (1996) Biological control. Chapman & Hall, New York
9. DeBach P (1974) Biological control by natural enemies. Cambridge University Press, London
10. Bigler F, Babendreier D, Kuhlmann U (eds) (2006) Environmental impact of invertebrates for biological control of arthropods: methods and risk assessment. CAB International, Wallingford
11. Follett PA, Duan JJ (eds) (2000) Nontarget effects of biological control. Kluwer, Boston
12. Van Lenteren JC (ed) (2003) Quality control and production of biological control agents. CAB International, Wallingford
13. Millar JG (2007) Insect pheromones for integrated pest management: promise *versus* reality. Redia 90:51–55
14. Gould F, Schliekelman P (2004) Population genetics of autocidal control and strain replacement. Annu Rev Entomol 49:193–217
15. Cook SM, Khan ZR, Pickett JA (2007) Push and pull strategies for insect pest management. Annu Rev Entomol 52:375–400
16. Shelton AM, Bellinder RR (2007) Role of biotechnological advances in shaping the future of integrated pest management. In: Koul O, Cuperus GW (eds) Ecologically based integrated pest management. CAB International, Wallingford, pp 269–288
17. James C (2009) Global status of commercialized biotech/GM crops: 2009. ISAAA brief n. 41. Ithaca, NY
18. Gomez-Barbero M, Berbel J, Rodríguez-Cerezo E (2008) Bt corn in Spain – the performance of the EU's first GM crop. Nat Biotechnol 26:384–386
19. Romeis J et al (2008) Assessment of risk of insect-resistant transgenic crops to non target arthropods. Nat Biotechnol 26:203–208
20. Wearing CH (1988) Evaluating the IPM implementation process. Annu Rev Entomol 33:17–38

Irrigation Management for Efficient Crop Production

Elías Fereres, Margarita García-Vila
Institute for Sustainable Agriculture, IAS-CSIC and University of Cordoba, Cordoba, Spain

Article Outline

Glossary

Application efficiency Relationship between the target irrigation depth (depth of water stored in the root zone to be used by the crop) and the depth of water applied to meet this target during a single irrigation event.

Conservation agriculture (CA) An agricultural production system aimed at achieving a sustainable and profitable agriculture through the application of three principles: minimal soil disturbance, permanent organic soil cover, and diversification of crop species in rotations or associations.

Decision support systems (DSS) Interactive information systems (not limited to computerized systems) that aid decision makers to identify and solve problems, and make decisions, which may be rapidly changing and are not easily specified in advance.

Deficit irrigation (DI) An irrigation strategy based on applying irrigation depths that are less than the full crop water requirements (ET), either throughout the crop life cycle (continuous or sustained deficit irrigation) or during specific stages that are insensitive to water stress (regulated deficit irrigation).

Distribution uniformity A measure of the spatial evenness with which irrigation water is distributed across a field.

Evapotranspiration (ET) The combination of two separate evaporation processes whereby water is lost to the atmosphere, on the one hand from the soil surface and crop surfaces (canopy interception) and on the other hand, from inside the leaves and other organs through pores called stomata, a process termed "transpiration."

Irrigation return flows The combination of surface and subsurface water flows resulting from the runoff and drainage following the application of irrigation water which may be available for subsequent appropriation from either a stream or an aquifer downstream of the original use.

Leaching requirements The depth of water needed to displace the excess salt accumulation in the soil profile resulting from irrigation, and aimed to maintain the salt balance in the crop root zone.

Soil water balance The state of soil water in the crop root zone resulting from the balance between water inputs from precipitation and irrigation and the water losses to evapotranspiration, runoff, and drainage below the root zone.

Water use efficiency (WUE) The ratio between the water volume used for a specific purpose and the water volume derived from a source to accomplish that purpose.

Definition of the Subject

The anticipated population growth in the coming decades will place large worldwide demands to increase the global production of food, animal/fish protein, livestock feed, fiber, and biofuels. Such an increase must come primarily from enhancing productivity, given the constraints in further land expansion for agriculture. Irrigated agriculture currently produces more than 40% of total production on 17% of the land. It is therefore imperative that irrigated agriculture not only sustains its current rates of productivity but that they be increased in the future. Irrigation

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

expansion has taken place over the last 60 years, and is currently under pressure from other sectors to reduce its share of the freshwater resources. Efficient crop production under irrigation in the future would be essential to produce more food with less water than is used today. This goal is a challenge that will not be easy to achieve without new and innovative approaches in irrigation management and in crop productivity. These innovations would also have to address the problems created by the return flows from irrigation which will threaten its sustainability, unless solutions are found to resolve permanently the environmental impacts of irrigation.

Introduction: Background on the Sustainability of Irrigation

The practice of irrigation started soon after agriculture was discovered thousands of years ago. Near the main rivers in the arid zones, water was diverted to the fields where crops were grown in areas and times of lack of rainfall, in an attempt to obtain a stable food supply. The Sumerian civilization that inhabited the Mesopotamia plains is believed to be among the first that used irrigation for crop production. Other locations where major irrigation developments took place early in the history of modern agriculture include the Yellow River basin of China, the Indus River Valley in Pakistan, and the Nile River Valley of Egypt. Many civilizations developed successfully in the past centuries, their food security heavily dependent on irrigation. History is filled with cases, however, where irrigated agriculture failed after some time, leading to the decline and even the disappearance of civilizations (e.g., Mesopotamian civilizations). The causes for failure included technical as well as economical, social, and political factors, but all combined suggest that irrigated agriculture may not be sustainable indefinitely. On the contrary, cases where irrigation has been practiced successfully for millennia, such as the Nile River basin in Africa and many areas of Southeast Asia, prove that it is possible to sustain irrigated agriculture in the long run [1].

Nowadays, irrigation is by far the largest consumer of developed freshwater on a global basis. It is estimated that irrigated agriculture currently uses about two thirds of water diversions, while industry and urban diversions amount to around 20 and less than 10%, respectively [2]. Such a high level of consumption in agriculture is due to the fact that crop plants require a continuous supply of water to replace the water transpired from their leaves and other aerial organs. The water demand arises because the crop is exposed to strong evaporative demand (due to the fluxes of solar and thermal radiation and warm, dry air). For carbon dioxide to enter the leaves, the microscopic leaf pores (stomata) must be open. But when the pores are open, water vapor freely escapes from the interior of the leaves which are nearly saturated with water. Because of the differences in concentration of carbon dioxide and water vapor between the interior of the leaf and the air, 50–100 molecules of water are lost for every molecule of carbon dioxide taken up. Crop water consumptive use is thus an unavoidable consequence of wet crop surfaces being exposed to dry air. Nevertheless, despite the large amounts of water that are transported through the plants, if they are not capable of taking up soil water to replace the losses, water deficits develop which can be detrimental to yield [3]. Therefore, if irrigated agriculture is to be sustainable, it needs sufficient water to meet its requirements at present and in the future.

Presently, more than 260 million ha of irrigated lands exist, representing 17% of the cultivated area but more than 40% of food production worldwide [2]. Future increases in population combined with changes in dietary habits toward the consumption of more animal protein will require sustained increases in crop production in the next decades well above present levels. Additionally, there may be demands on agricultural products for uses other than food production, such as energy from biomass. The increase in production cannot come from a significant expansion of the area devoted to agriculture for at least two reasons. On one hand, the area best suited for agricultural use is already under production, and only in a number of regions in the American and African continents there is additional land that has not been put under cultivation. Furthermore, that expansion would alter further the balance between agricultural and natural ecosystems, which much of the world population would oppose, arguing for the environmental preservation of the remaining areas that are not in cultivation.

If land expansion will not be possible, the goal would then be to increase productivity, the production

per unit of cultivated land. Crop productivity of the main cereals has been increasing steadily since the 1960s, albeit at rates that are apparently declining in recent years [4]. Given that the average productivity of irrigated lands is more than twice that of rainfed areas, it appears that preserving irrigated agriculture would be essential for future food security. The issue is whether it would be possible to expand irrigated agriculture beyond its present level by transforming rainfed into irrigated areas. While some expansion may be possible in the foreseeable future, it is difficult to see how a major world expansion of irrigation would occur, given the present commitments of freshwater (supplies already overcommitted in many arid and semiarid areas), the perceived strong opposition from urban societies, and the uncertainties that climate change brings to future water supplies. It is therefore essential that the productivity of irrigated lands be increased to cope with future demands, and this will have to be done at efficiency levels higher than those achieved at present. The reduction in water used in irrigation per unit production emerges then as a critical issue in the area of crop production in the future.

Irrigated agriculture has to manage large amounts of water that must be utilized with a high level of efficiency. This is not the only requisite of good management, however. All irrigation waters contain salts, and crops transpire pure water; thus, the irrigation process concentrates the salts in the soil profile at a rate which mostly depends on the salt content of the irrigation water. In this respect, the salinity that develops under irrigation would make cropping unsustainable unless the salts are evacuated to prevent the salinization of the crop root zone. In many areas, natural rainfall is sufficient to leach out the salts from the potential root zone. However, when waters of high salinity are used for irrigation in arid areas of limited rainfall, salt leaching must be performed by applying irrigation in excess of the crop needs. The control of salinity is one important requisite of a sustainable irrigated agriculture. However, the water applied in excess of the consumptive use must be disposed of, and this creates a whole host of potential environmental problems associated with water pollution [5]. Many of the problems caused by the return flows from irrigation occur outside the farms, at the level of the irrigation district or at the basin level, and are discussed below.

The Process of Irrigation at Different Scales: From the Field to the Basin

When a field is irrigated, the water applied may be lost via surface runoff or by deep percolation, or may be stored in the crop root zone for subsequent uptake by the crop and lost as ET. However, the focus of irrigation management for efficient crop production extends well beyond a field, up to the farm, the irrigation district, and the watershed. The water balance is the unifying concept that connects the water disposition in the different scales. In any field, farm, or watershed, it is possible to quantify a water balance in which the incoming water in the form of rain or irrigation must be balanced by the water lost as evapotranspiration (ET), runoff, deep percolation, and that stored in the soil profile.

When scaling up from an irrigated field, one needs to consider the situation upstream and downstream of that field. Irrigation water originates from storage reservoirs, flowing streams, or from the groundwater. In all cases it must be conveyed and distributed among the different areas, their farms, and individual fields. The process of distribution entails a number of losses due to direct evaporation from reservoirs and open conduits, leakages from canals, and management losses in the handling of distribution networks that deliver the water to individual farms [6]. Farmers on collective networks do not always have access to water when they need it, and that may cause imbalances between the supply of irrigation water and the crop demand. Those that pump directly from the groundwater have greater flexibility at the expense of additional energy usage. At any rate, by the time the water is delivered to a particular field, there have been distribution and management losses upstream that are often substantial. The classical work of Bos and Nugteren [7] quantified the efficiency of irrigation along the path from the source of water to the field and found very low values, of the order of 40–50%. Hsiao et al. [8] have also analyzed the efficiency losses in the network, from the dam to the crop, and have highlighted the dramatic differences between good and bad situations, given the multiplicative effects of the chain of efficiencies from the source of water until it is transpired to the atmosphere [8].

At the field scale, water may be used beneficially for ET and for salinity control or may not have a beneficial

use when it is lost through runoff or drainage. However, at higher scales, water that evaporates from a watershed is considered a loss or consumption, while water running off a field can be recovered downstream and may not be lost to the system. Equally, water that percolates below the root zone may reach the groundwater from which it can be pumped and recovered. Thus, there are consumptive and nonconsumptive uses of irrigation water. Water applied as irrigation may be used consumptively in the ET process, while the network and runoff losses may be recovered downstream and used by others within the basin. Water used in one farm within a basin is not always consumed in that basin and can be used severa1 times before it leaves the basin.

The difference between water use and consumption is important to understand whether water conservation efforts will result in net water savings [9]. If *all* the water lost outside the ET can be recovered, then efficiency improvements via reducing runoff or percolation losses would not lead to net water savings. On the contrary, if the losses are partially or not recoverable at all, because either the water quality is deteriorated or the losses end in a saline sink, then the water saved at the field scale also represents (partial) savings at the basin scale. Thus, knowing the fate of water all along its path, it is critical to determine whether the water saved on the farm may be part of the recoverable or of the unrecoverable losses.

The basin perspective of water conservation could change the emphasis on where to act when irrigation improvements are sought, and whether such improvements are really needed. Nevertheless, the picture would not be complete if two other issues are not included in the overall assessment of basin irrigation management. One is the fact that field and farm losses, while they could be recovered downstream, often have negative environmental consequences. Surface runoff may carry sediments and chemicals that act as pollutants in streams, lakes, and dams. Drainage waters pick up salts, fertilizers, and other chemicals in the soil profile and may contaminate the groundwater. The other issue is the energy requirements for recovering the losses. Whether is surface runoff recovered at the end of a field or groundwater pumped from the aquifers, additional energy is always needed to recover the losses. Needless to say, the water quality of these return flows would always be worse than that of the original irrigation water.

Irrigation Management Goals for the Improvement of Sustainability

An agriculture that aims toward sustainability should be economically viable, efficient in the use of the natural resource base, socially equitable, and must have a minimal environmental impact. These requisites are essential when defining the goals of irrigation management for efficient crop production. Economic viability in irrigated agriculture is usually associated with *high production levels,* because the investments needed for irrigation development would not be justified under low levels of production. Thus, irrigation must be managed in such a way as to avoid water deficits that reduce economic yield. Not only high production should be sought but also, *high productivity* in relation to the use of production inputs, including water, should be an important goal. Contrary to the common belief, de Wit [10] demonstrated that production inputs are used at their highest efficiency when yields approach the maximum potential. Therefore, it is possible to combine both goals with judicious management.

Equity is an important goal in irrigated agriculture because, first of all, irrigation development represents a quantum leap in terms of increased income relative to rainfed agriculture. Also, sometimes water supplies are managed rigidly by water authorities or are insufficient relative to the potential demand; thus, *equitable distribution* of the available water supply is definitely an important goal of sustainable irrigation management. How this goal is pursued would depend on a number of socioeconomic, political, and cultural factors discussed below. Finally, irrigation can have a number of detrimental effects on the environment that must be minimized. Firstly, without *control of salinity* agriculture cannot be sustainable [11]. However, the leaching of salts and other chemicals from the soil profile end up as part of the return flows and contribute to the nonpoint pollution generated by irrigated agriculture. To minimize these negative effects on the environment, irrigation must be managed in such a way as to *avoid runoff* and *minimize deep percolation.* To achieve this goal, the engineering of irrigation systems (to distribute water uniformly) and the scheduling of irrigations (correct timing and application amounts) are the main instruments to be optimized when walking the fine line

of achieving high production and productivity while reducing the environmental impact of irrigation.

Management Options: Strategic, Tactical, and Operational

In this entry, we are assuming that farmers have already made a choice among the different irrigation methods, basing their decision on their access to capital, the availability of water and its distribution mode, their management skills, and the labor and energy costs. There are frequent interactions between engineering and management in irrigation that are frequently ignored but are covered here, even though the focus is on management.

The temporal scale determines the nature of irrigation management decisions. Operational decisions are those that must be made in the short term, within days; for instance, the advancing or delaying of one irrigation application. Tactical decisions are those that are taken within the irrigation season once it has started, and have a time scale of days to weeks. One example would be the adjustment of the number of irrigation applications within the season, if the water supply has been reduced after planting. Strategic decisions have a time scale of months to years, and are normally taken before the season starts. The preseason decisions pertaining to the allocation of water to the different fields and crops are examples of strategic decisions.

Crop and Cultivar Selection

Farmers' choice of crops in commercial agriculture is a complex decision that is, above all, based on factors related to production economics and marketing, while other socioeconomic and biophysical factors are considered afterward. The water-related factors such as crop water usage are most important when the supply available is less than the anticipated demand. Crop consumptive use depends primarily on the evaporative demand, the length of season, and the fraction of incoming radiation intercepted by the crop canopy. There are ample differences among the crop ET of different species. These differences are also modulated by the type of climate in which crops are grown. In that respect, there are major differences between tropical and temperate climates. In tropical climates, reference ET (the ET from a standard grass surface: ETo) is relatively constant throughout the year and irrigation is used during the dry season; here, the critical issue is the duration of the growing season, with the goal of producing multiple crops in 1 year. Production per day is the best indicator of efficiency in tropical climates, where crops must follow a sequence that uses best the land and water available.

In temperate climates, evaporative demand during winter is a small fraction than that in the summer. For instance, in Mediterranean-type climates, ETo oscillates between 1–2 mm/day in winter and 6–8 mm/day in summer. Thus, winter crops require much less water than summer crops per unit time. Additionally, seasonal rainfall occurs primarily from fall to spring in those climates, thus reducing the irrigation needs of winter and spring crops. One environmental factor that indirectly affects the crop water requirements in temperate climates is air temperature. Temperatures in winter are low in such climates and that slows down the rate of crop growth and development, thus lengthening crop duration. For instance, the season of winter cereals may last 6–7 months while maize, a summer crop, may be grown in 4 months. These differences in season length balance out some of differences in ETo between winter and summer, but still winter crops generally use less water than summer crops. As an example in some interior valleys of Mediterranean climate, wheat ET is between 350 and 400 mm while maize ET ranges between 600 and 650 mm. Perennial crops have higher ET rates, alfalfa ranging between 800 and 1,200 mm, and deciduous trees between 700 and 1,000 mm. Evergreen tree crops should have the highest ET, however, both citrus and olive have strong stomatal control of transpiration and their seasonal ET does not exceed values that range between 700 and 1,000 mm, depending on the specific climate.

Crop choice is therefore the most important factor in determining irrigation water requirements. Cultivar differences in water use are considerably smaller than the differences among crops, and are directly related to season length. Early cultivars of maize in temperate climates may use 10–20% less water than late-maturing cultivars. Similar differences have been observed in other crops. Even though such differences are relatively small, they can be important when the crop sequence is such that only short-season cultivars fit in the rotation or when the water supply available is limited. Genetic

improvement of the intrinsic transpiration efficiency (TE, g CO_2/g H_2O) [12] has not been successful until now. The limited variability encountered within crop species has not led to cultivars that have higher productivity in irrigated agriculture, although some yield advantage (of the order of 10% at around 1 t/ha yield levels) has been achieved when selecting wheat cultivars for high TE in rainfed environments [13]. The use of genetic engineering in the future [14] may offer new opportunities for enhancing other basic responses that can indirectly improve crop WUE (e.g., maintenance of harvest index under water stress).

Planting dates also have some effects on crop ET in temperate climates. Early plantings of spring or summer crops have lower ET by avoiding the times of peak ETo. One example is that of winter plantings of sunflower in Mediterrranean climates. By planting early, the growing season is displaced away from the summer and the seasonal ET is less, even though the season may be longer due to the slow growth and development in the early crop stages. The irrigation requirements may even be lower because of the higher rainfall probabilities in early spring. In general, plantings of summer crops have been moved as early as feasible to reduce irrigation requirements; this is the case of maize in many areas where the optimal planting dates have moved more than 40 days over the last 30 years. To displace the growing season any further the temperature limitation to growth and development must be overcome. Progress has already been made in crop improvement; for example, maize has expanded significantly into colder areas in the last decades, but more breeding efforts are needed to achieve higher growth rates under low temperatures in the principal irrigated crops.

Contrary to the measurable effects of varying planting dates on crop ET, the variation in planting density within commercial practices has little influence on the ET of annual crops. This is because the differences in radiation interception among different planting densities are small and restricted to the short period of early canopy development. It is important, however, in the case of perennials, as tree or vine densities have a strong influence in the intercepted radiation for several years after the initial planting, and may be carried over the entire life of the orchard or vineyard. Biomass production and thus yield of most crops is directly related to the seasonal intercepted radiation. Any agronomic practice that favors quick canopy development and complete radiation interception should increase production and will have a smaller effect on crop ET, thus increasing the efficiency of water use.

Optimal Use of Rainfall and of Stored Soil Water

The goal of irrigated crop production is to use all sources of water supply as effectively as possible. Soil water storage from rainfall or from preplanting irrigation is an effective way of using the water resource. To maximize stored soil water, infiltration should be enhanced so that surface runoff is minimized. Low infiltration rates decrease the effectiveness of rainfall to the point that only a small fraction of it may be stored in the potential crop root zone for subsequent use by the crop. Many irrigated soils have problems of slow infiltration, either inherent to their physical makeup or caused by the application of irrigation water for a long time that alters negatively the surface infiltration properties of the soil [15].

Excessive tillage of some irrigated soils and, more importantly, the traffic required in crop intensification under irrigation has exacerbated the problems related to low infiltration, which not only reduces the effectiveness of rainfall but also affects the distribution of irrigation water. The use of minimum tillage, no-tillage, surface residues, permanent beds, and of controlled traffic is all being incorporated into what is now known as conservation agriculture [16]. These soil management systems have been used successfully in many world areas for sometime now, primarily under rainfed agriculture, but they are now increasingly being adopted for irrigated agriculture as well. The benefits of conservation agriculture (CA) include soil surface protection by the crop residues, the maintenance of soil organic matter, increased infiltration, and better distribution uniformity. The limitation of CA is that it needs to be tailored to the specific situation thus requiring field experimentation before it is introduced in a new area or system. The widespread expansion of CA in the main agricultural areas of the world [17] suggests that it will be adapted to many irrigated systems in the near future.

The critical role of rainfall, while being obvious in rainfed agriculture, is nearly as important in irrigated

agriculture because it leads to a reduction in irrigation demand. The best strategy for optimal use of stored soil water is the conjunctive use of both, the applied irrigation water and the soil reserve. It is desirable that the soil is partially depleted to allow the storage of anticipated rainfall in the root zone, although such depletion has to be managed to avoid yield-reducing water deficits (See below). As the crop approaches maturity, it is recommended to rely more on the stored reserve and use as much of it as possible, thus reducing irrigation water use. Ideally, the soil reserve should be almost totally depleted when the crop is harvested, assuming it will be replenished by rainfall. Obviously, to manage the soil reserve, it must be quantified from planting to harvest. Growers need to know the level of soil water at planting and the rate of water use (ET) relative to the depths of irrigation and rainfall. Therefore, making best use of the rainfall and of the water reserve requires carrying out a seasonal water budget for each crop. It is also important to evaluate the risk of basing a limited irrigation strategy on using a large fraction of the stored rainfall, because in the event of a drought, irrigation would be insufficient to meet the crop demand and this strategy may not be sustainable.

Technical Irrigation Scheduling

Decisions on when to irrigate and how much water to apply – the irrigation scheduling process – are commonly made by irrigators around the world solely based on experience. There are, however, a collection of technical procedures and tools developed to forecast the timing and amount of irrigation applications. Some sort of irrigation on demand is a prerequisite for the application of these technical procedures, because when the delivery method of the network is on rotation, the farmers have no flexibility to vary the irrigation interval and they tend to use all the water they receive as insurance for uncertain conditions.

Among water-sensing devices, soil water sensors were perhaps the first instruments that were introduced for irrigation scheduling in the 1950s, and it is remarkable that they have enjoyed a certain degree of success until recently. There is now a new generation of soil water sensors that track soil water status continuously, rather than providing point measurements as the traditional instruments such as the tensiometer offer. Unfortunately, the new developments have not resolved the quantification of volumetric soil water content with depth, a parameter that is still most reliably measured with the neutron probe since the early 1970s. The regulatory constraints on this nuclear instrument have limited its use even for research in many countries, with the result that reliable data on soil water balance and crop ET are difficult to obtain with soil water measurement methods. The information from current sensors is treated as trends and these tendencies, often observed at more than one depth, are the basis for making decisions, rather than using a threshold value of soil water at a given depth as the indicator of irrigation timing. Protocols have been developed to automate irrigation scheduling on the basis of soil water status [18].

The use of plant water sensors for irrigation management lagged behind that of soil water sensors by about 2 decades. The pressure chamber was the first portable instrument that was rugged enough to be used under field conditions, although it is manually operated and its readings cannot be automated. It is now used commercially in some areas for irrigation scheduling of tree crops and vines. Other plant sensors have not been as successful for their use in practical scheduling, although they have been around for sometime. The most notable example is that of dendrometers, sensors that detect the variations in stem diameter, which were used since the 1950s and have today the same degree of precision they have had since the 1970s, but they became popular for research 20 years later. Protocols to be used with trunk diameter sensors have also been proposed [19] although its use, while attractive, has been mostly limited for research purposes. One of the most promising plant-based indicators is the canopy temperature as measured by infrared thermometry. Jackson and coworkers (summarized in [20]) developed several indicators based on canopy temperature, the Crop Water Stress Index (CWSI) being the most popular. Threshold values of CWSI have been found for several crops and its use as a water stress indicator is gaining acceptance. Plant-based parameters are best used as pre-visual indicators of water stress, rather than being indicative of irrigation timing and amount. Their strength resides in providing a specific, crop-based calibration for other methods

that use either soil water sensors or the water balance procedure. One important limitation of all soil- and plant-based sensors is the variability in the measurements, as discussed in Coping with Spatial Variability: Precision Irrigation.

The most robust technique for wide use in irrigation scheduling is the one using the soil water budget. Here, irrigation timing is computed by adding the crop ET losses minus effective rainfall until a soil water level termed "the allowable depletion" is reached. The basic information in this method is that of crop ET, computed as the product of a crop coefficient times ETo. After a method for computing ETo has been standarized and widely accepted [21], agrometeorological weather stations provide the information needed for calculating ETo from meteorological variables. In some developed countries, networks of weather stations now provide the ETo information routinely. The pan evaporation is a viable alternative for estimating ETo to the more sophisticated automated weather stations. Computer programs have been developed for calculating the water balance of fields, and irrigation scheduling services have been developed, mostly by public agencies and by consultants as well. These services have been around for several decades now but most farmers have been reluctant to pay for them. Nevertheless, irrigation advisory services are becoming more popular in areas of scarce or expensive water. One pattern that has been observed is that farmers subscribe to these technical procedures or services for a few years and then, they no longer use them. Perhaps they perceive that they have acquired sufficient knowledge during that time period, a reason that may also explain why the use of sensors is discontinued after some time by many growers.

Interactions Between Irrigation Methods and Their Management

Irrigation has been practiced for thousands of years by flooding the soil surface and keeping the water standing until it infiltrates. This method is named surface irrigation and it is still the most popular method worldwide. In surface irrigation, the soil intake rate determines the depth of water that infiltrates and if its properties are spatially variable, the farmer will not have good control of the amount of water applied. Pressurized irrigation methods (sprinkler and microirrigation) were invented much more recently, about 70 years ago. Since then, they have enjoyed increasing popularity in areas where farmers have access to sufficient capital to shift from surface to pressurized systems. In other, newly developed areas where topography and/or water infiltration properties made the use of surface methods impractical, pressurized methods have been preferred over surface irrigation. One advantage of pressurized systems is that the depth of applied water does not depend on soil properties but is determined directly by the run time. Farmers' preferences for pressurized systems are based on the need for better control and the greater skills needed for effective surface irrigation management. The higher capital and energy requirements are two limitations of pressurized systems relative to surface irrigation.

The key feature of irrigation systems in relation to their management is the degree of uniformity of water distribution. Regardless of the method, high distribution uniformity prevents excessive percolation losses in some areas of the field and the development of water deficits in others. To emphasize the importance of high distribution uniformity suffices here to summarize an example in which the performance of two systems with low (70%) and high (90%) distribution uniformity (DU) were compared for maize irrigation [22]. The additional depth of water needed under low uniformity to achieve maximum yields amounted to 400 mm, or 70% of the net irrigation requirements. The requisites for high DU include an appropriate design, good maintenance, and correct operations. Nowadays, efficient irrigation cannot be practiced unless high to very high DU values are achieved.

All irrigation methods have potentially high performance that can eliminate or minimize runoff and percolation losses, but they seldom achieve their potential due primarily to lack of maintenance or to mismanagement. To optimize the operation of existing surface irrigation systems the water delivery procedures often need to be changed. There are needs relating to adjusting the flow rates delivered to fields, altering the operation of delivery networks, and sometimes land consolidation is also needed. Such changes not only require capital investments but agreements among

various users as well. The introduction of pressurized systems (sprinkler and drip) in collective irrigation networks also requires changes in the physical infrastructure (e.g., reservoirs). Thus, there must be economic incentives for the farmers and access to capital to introduce the improvements needed to increase the potentia1 application efficiency and distribution uniformity at the system level.

Control of Salinity and of Return Flows

Irrigated agriculture cannot be sustainable unless salinity is properly managed. Salts that accumulate in the root zone must be leached but the amount of leaching must be kept to the minimum if the environmental impacts of drainage waters and the return flows are to be controlled. Determining the leaching fraction (proportion of the ET that must be added for salt leaching) should be based not only on the quality of irrigation and drainage waters but on the need to avoid excessive percolation. Here again, high DU is essential when the target leaching fraction is of the order of 5–10%, which is only achievable under very high DU values.

The use of salt-tolerant crops is often proposed as a means of controlling salinity. It is true that there is an ample range of salinity tolerance among crop plants and that it is possible to exploit waters of low quality for irrigation of salt-tolerant crops. However, because the process of salt concentration in the profile in the absence of leaching is inexorable, the use of salt-tolerant crops should be considered as a temporary measure until excess salts can be leached out of the potential root zone. Sometimes, tolerant crops have been used for some years in the event of a drought that limits the irrigation supply available for leaching. Their long-term use in dry areas should not be considered sustainable, given the progressive salinization of irrigated soils. After some environmental problems related to toxicity caused by the selenium content of the return flows, the drainage from a large area in California, San Joaquin Valley, was interrupted in 1989 [23]. The rainfall in the area is negligible, thus salt leaching depends on artificial drainage. As of 2010, the area is still under irrigation; irrigation systems installed have high DU values and there is an extensive monitoring program of soil salinity. Perhaps long-term control of salinity would be feasible with systems that have very high DU, and where irrigations are scheduled with precision, keeping most of the salts near the bottom of the root zone, as it was first proposed by Hoffman et al. [24].

At scales beyond that of a field or farm, the concerns shift toward the quality of return flows, its reuse, and the environmental impacts of irrigation. The decline in water quality after the water has gone through the irrigation process has an associated cost that needs to be quantified. The concept that water lost to a farm is recovered downstream needs to have associated an economic analysis of the energy costs and the quality deterioration costs of recovering the return flows. If minimum leaching is practiced at the field scale, the amount of return flows is also reduced but that increases the concentration of salts and other contaminants in the drainage waters. Eventually, it may be possible to reduce the quantity of return flows so much so that they can be disposed of in evaporation ponds that become salt sinks. It would then be possible to either accumulate or export the salts and make the agriculture of that region fully sustainable.

Coping with Spatial Variability: Precision Irrigation

The major challenge that technical farm irrigation management has faced and continues to face is how to cope with variability. Under field conditions, both spatial and temporal variabilities are the norm rather than the exception. The strong spatial heterogeneity of soil water properties even in what are considered uniform soils, combined with the variations in the distribution of irrigation water applications, and the uncertainties of rooting depth and densities, all contribute to create a heterogeneous environment that farmers have to manage as accurately as feasible. The problem has increased in magnitude over the last decades due to the increase in size of the management units, in an attempt to reduce production costs by managing uniformly larger and larger field units. The complexities involved in dealing with the variability problem are such that, until very recently, the common solution chosen by irrigators was to apply water in excess so that the risk of inducing water deficits in some parts of the field is minimized. Because of the difficulties that farmers and technicians have had in characterizing the variability,

significant uncertainty is introduced and often the irrigation management decisions may be in error.

To advance solutions for coping with the variability problem in irrigation management what is needed is to be able to characterize the variation across a field, and also to have the option of applying variable amounts of water within that field. The objective would then be to apply variable water depths under non-uniform crop growing conditions to match the requirements of every area of the field, while minimizing the environmental consequences that uniform irrigation over a variable field would have. The technologies for variable water application are already available in self-propelled sprinkler systems and can lead to significant water conservation [25]. Significant efforts in the engineering of irrigation systems have been undertaken recently to offer the flexibility of applying spatially variable amounts of water (and agrochemicals) for the different pressurized methods, including microirrigation [26]. These new capabilities should enable growers to increase productivity and minimize environmental impacts of irrigation.

While the engineering solutions for precision irrigation are underway, there is still the need, not only to characterize and monitor the variability but to interpret the causes of the variations in crop growth and development. The characterization of irrigation performance through remote sensing [27] is a promising area, as it enables performance evaluation in a fast and an inexpensive way, and can also identify the areas in need of improvement. Interpreting the underlying causes of variations among and within fields is much more difficult, however. The use of remote sensing techniques has progressed substantially in recent years by developing capabilities for detecting a number of vegetation properties with very high resolution (e.g., [28]). High-resolution imagery cannot be acquired from current satellites, and a number of initiatives to obtain them from aerial vehicles flying closer to the ground have been launched recently. As an example that is relevant for irrigation management, Berni et al. [29] applied models based on canopy temperature estimated from high-resolution airborne imagery, obtained with an unmanned aerial vehicle, to calculate tree canopy conductance and the CWSI of heterogeneous canopies, such as those of tree crops.

Use of Simulation Models and of Decision Support Systems

Decision-making in crop production has been the focus of numerous studies, mainly on the description of the decision-making process and decision outcomes [30]. The numerous decisions dealing with irrigation management include, not only the scheduling and application of the available water to different crops over the irrigation season, but also strategic decisions related to crop choices and seasonal water allocation. Irrigation is a complex operation, based on technical and agronomic knowledge, and on sociological factors which may include a negotiating process among irrigators [31]. Recent advances in information and telecommunication technologies allow farmers to acquire vast amounts of site-specific data for their farms, with the ultimate goal of *reducing uncertainty* in decision-making. However, farmers face many difficulties in efficiently managing, analyzing, and interpreting the vast amount of data collected, while considering both the costs and value of the information [30].

The tactical decisions related to irrigation scheduling have been discussed above; however, strategic decisions that must take into account the complex nature of agricultural systems and changes in environmental conditions are difficult to make without tools that assist the farmer in the decision-making process. Among the tools available in the area of water management are the Decision Support Systems (DSS), which were first used in the early 1970s as a radical alternative to large-scale management information systems [32]. There are different types of DSS; many of them are expert systems [33], which have the problem of handling multiple experts to evolve decisions and uncertainty. Linear programming, dynamic programming, and non-linear programming are the most popular modeling techniques; while recently, genetic algorithms [34] have been used to generate optimal solutions more efficiently [35] than dynamic programming (e.g., [36]). Also, DSS have been shown to help decision-making at different scales: at the field level, considering only one crop (e.g., [37]); and at the farm scale, with multiple crops (e.g., [38]).

Because irrigation decisions include many factors, the DSS combine crop simulation models with econometric models to assist farmers in optimizing irrigation

management, according to environmental, socioeconomic, and political prospects [39]. The yield response to different irrigation levels is one of the inputs of DSS that traditionally has been quantified as empirical crop-water production functions [40–42]. Even though these functions have been used profusely, they are site-specific and difficult to extrapolate without costly, empirical calibration. An alternative to crop-water production functions is the use of dynamic crop simulation models [43]. Among the simulation models usable for irrigation decision-making are CropSyst [44], EPIC [45], CROPWAT [46], APSIM [47], and CERES [48]. However, most of these models require detailed information (difficult to obtain) about parameters that describe plant behavior (APSIM, CERES), or make use of simple empirical functions (CROPWAT). The models must be calibrated, validated, and be sufficiently robust to provide reliable predictions. For this reason, detailed models may be less practical than simpler but robust models [49] such as the recently published FAO water productivity model, AquaCrop [50]. AquaCrop is a model focused on simulating attainable yield in response to the water available, and it is thought to have an optimum balance between accuracy, simplicity, and robustness [50]. In water-limited situations, these models can be helpful in determining the optimal level of irrigation water that leads to maximizing income (e.g., [51]).

To make informed decisions that will enhance the efficiency of irrigation, farmers need to be able to assess how agricultural systems respond to internal (e.g., new technology) and external changes (such as those in the economic and political context, water constraints, or climate change). Therefore, DSS may be used for scenario analyses, showing the effects of alternative scenarios on irrigation management for efficient crop production [39]. The possibility of DSS implementation to assist farmers on irrigation management creates opportunities to establish a relationship that leads to the solution of problems and research feedback, redirecting the paths of research to better solve problems. Until now DSS are not commonly used directly by farmers as they are considered complex tools, which usually lack a user-friendly interface that permits easy access by the users. Irrigation advisory services of the irrigator's communities may be the right platform for the introduction of DSS, being potentially a major breakthrough in improving the use and management of irrigation water.

Management Under Water Scarcity

At present and more so in the future, irrigated agriculture will take place under water scarcity. Insufficient water supply for irrigation will be the norm rather than the exception, and irrigation management will shift from emphasizing production per unit area toward maximizing the production per unit of water consumed, the water productivity (WP).

Water Allocation Constraints and Their Impact on Management

While irrigation is an ancient technique, its expansion is very recent. The world area under irrigation has more than doubled in the last 60 years [4], in response to the increase in food demand. This expansion has required the development of additional water supply through the construction of dams for storing surface waters and exploitation of the groundwater resource. The sustainability of supply depends on the long-term rainfall and on the rate of groundwater recharge. As the irrigated areas expanded, the pressures on the finite water resources in some areas increased and the balance between supply and demand was altered (e.g., [52]). Two issues cause the imbalances; first, periodic drought cycles reduce the availability of water supply, some times during several years. Then, the increases in the demands from other sectors of society, notably the environment that has been neglected in the past, compete with irrigation demands, which are often considered the lowest in priority. The expansion of groundwater use is also a cause of concern; it is possible that the abstraction exceeds the rate of recharge, causing a decline in the water table depth with time. In fact, groundwater may be considered a reservoir of supply that may be overexploited during drought years, when surface supplies are scarce. However, when the aquifers are depleted in the long run and do not recover after years of high rainfall, the groundwater overdraft is unsustainable and the abstraction must be reduced to sustainable levels.

Regardless of the causes for water scarcity, knowing the degree of supply reduction is essential for farmers to make rational decisions regarding how to manage

the limited supplies. Preseason decisions are centered on crop choice and/or to land abandonment. Matching demand to supply is achieved by selecting low-water-use crops or by leaving some land in fallow, if the supplies are insufficient to irrigate all the developed land. Once the season starts it is much more difficult to make adjustments, if the reduction in supply is significant. Changes in the irrigation system to reduce percolation and other operational losses, changes in scheduling to reduce the number of applications thus reducing E losses, and the use of deficit irrigation (see below) are the only options left to growers once the season has started and the crops have been planted.

Water scarcity does not occur overnight, and water authorities and farmers have conservative attitudes to avoid risks. Predictions of big cuts in supply that do not materialize reduce economic opportunities, but the reverse may be even more catastrophic. Thus, planning in advance by water authorities and by irrigation districts, and knowing precisely the expected level of reduction are the two key elements to manage successfully the anticipated scarcity. Seasonal predictions of rainfall would be very useful to anticipate droughts and consequently, irrigation supply reductions. One paradox is the enormous investment in climate change research relative to that devoted to medium range weather predictions, and the apparent lack of connections between two areas that should be closely related.

Deficit Irrigation

Deficit irrigation (DI) is defined as the application of water below the crop ET requirements. Therefore, water demand for irrigation can be decreased relative to full irrigation and the water saved can be diverted for alternative uses. Even though DI is simply a technique aimed at the optimization of economic output when water is limited [53], the reduction of irrigation supply to an area imposes many adjustments in the agricultural system. Thus, DI practices are multifaceted, inducing changes at the technical, socioeconomical, and institutional levels.

In the humid and subhumid zones, irrigation supplements the rainfall as a tactical measure during drought spells to stabilize production. This practice has been called supplemental irrigation [54] and, although it uses limited amounts of water due to the relatively high rainfall levels, the goal is to achieve maximum yields and to eliminate yield fluctuations caused by water deficits. Supplementing rainfall in arid areas with one or more irrigation applications is a form of DI as maximum yields are not sought. When irrigation is applied at rates below the ET under DI, the crop extracts water from the soil reservoir to compensate for the deficit. Two situations may then develop. In one case, if sufficient water is stored in the soil and transpiration is not limited by soil water, even though the volume of irrigation water is reduced, the consumptive use (ET) is unaffected. However, if the soil water supply is insufficient to meet the crop demand, growth and transpiration are reduced and DI induces an ET reduction below its maximum potential. The difference between the two situations has important implications at the basin scale [55]. In the first case, DI does not induce net water savings and yields should not be affected. If the stored soil water that was extracted is replenished by seasonal rainfall, the DI practice is sustainable and has the advantage of reducing irrigation water use. In the second case, both water use and consumption (ET) are reduced by DI but yields may be negatively affected in cases where yields are directly related to ET [56].

There are several strategies to impose the water deficits under DI, but basically there are two alternatives [56]. One is to impose the same level of deficit over the entire irrigation season (continuous or sustained DI), while the other concentrates the deficits in certain crop growth stages believed to be the least sensitive to water stress (Regulated DI, RDI). Deficit irrigation, by reducing irrigation water use, can aid in coping with situations where supply is constrained. In field crops, a well-designed DI regime can optimize WP over an area when full irrigation is not possible. It will reduce yield to a certain extent because of the linear relations between ET and the yield of the major field crops [40]. In many horticultural crops, such as fruit trees and vines, RDI has been shown to improve not only WP but farmers net income as well. Because of the differential responses among the different crops to water deficits it would be important to investigate the basis for the positive responses to water deficits in the cases where water deficits are not detrimental to yield. While DI can be used as a tactical measure to reduce irrigation water use when supplies are limited by droughts or other factors, it is not known whether it

can be used over long time periods, given that the reduction in applied water could lead to greater accumulation of salts in the profile. It is imperative to investigate the sustainability of DI via long-term experiments and modeling efforts to determine to what extent it can contribute to the permanent reduction of irrigation water use.

Future Directions

Farmers in irrigated agriculture are confronted, at the start of every season with a critical question: How much water would be available this season, and how should I distribute it among the different crops and fields? There are many procedures and tools to answer those questions in such a way that the water allocation will be used efficiently. In fact efficiency of water use in irrigated agriculture has been steadily increasing with the improvements in science and technology, and as pressures from other sectors of society mount. Irrigation management encompasses several scales, from the network down to the individual field. One of the primary management targets at the field level is, once the amount of water needed is precisely determined, to distribute it over the field as uniformly as possible. Elimination of surface runoff and minimal percolation losses are prerequisites for optimizing irrigation water use and for limiting the environmental impacts of irrigation.

Monitoring, evaluation, and real-time feedbacks for benchmarking and to assess irrigation performance is essential for efficient water use. In the future, performance evaluation will be done routinely and at low cost with the use of remote sensing techniques. These surveys will allow the identification of areas within irrigation networks in need of improvement, and farmers will have the information to modify practices or to change methods, thus achieving greater productivities. Incentives are needed, however, for farmers to adopt new technologies for more efficient water use when water supplies are abundant and/or inexpensive.

The recent expansion of irrigation combined with increased water supply limitations will lead to water scarcity in many areas. In those situations, efficient use of water will be critical for the sustainability of irrigated agriculture. Deficit irrigation will be used more, and other socioeconomic measures, such as water markets, will play a more important role in water scarce situations [57]. Planning ahead in water-limited situations would be critical to achieve optimal use of water, and it is envisaged that robust DSS that include economic models will be used to allocate the limited irrigation water available among different users in networks and among crops in farms.

Finally, bridging the yield gap between potential and actual yields offers another avenue for improving the efficiency of water use in irrigated agriculture. Crops that are limited only by solar radiation and temperatures have potential yields that are several times the current world average yields. For instance, wheat world averages are reaching 3 t/ha, while the potential yield approaches 14 t/ha. In the case of maize, average world yield is about 5 t/ha while the potential yield exceeds 18 t/ha. The yield gap is not only important in rainfed conditions [58], but under irrigated conditions as well. Differences that exist between actual and potential yields are caused by many factors, water being just one of them. Therefore, it is most important to optimize crop agronomy in all of its facets, from soil to crop management, and from pest and disease management to weed control. Most of the time, yield improvement by better agronomy does not increase crop ET significantly, and it has proven over and over again to be a very effective path for enhancing the efficiency of water use now and in the near future.

Bibliography

Primary Literature

1. Fahlbusch H, Schultz B, Thatte CD (2004) The Indus basin: history of irrigation, drainage and flood management. ICID, New Delhi
2. Molden D (2007) Water for food, water for life: a comprehensive assessment of water management in agriculture. Earthscan/IWMI, London
3. Hsiao TC, Acevedo E, Fereres E, Henderson DW (1976) Water stress, growth, and osmotic adjustment. Philos Trans R Soc Lond B 273:479–500
4. FAOSTAT (2010) http://faostat.fao.org/. Verified on 26 May 2010
5. Tanji KK (1990) Nature and extent of agricultural salinity. In: Tanji KK (ed) Agricultural salinity assessment and management. ASCE, New York, pp 1–17
6. Clemmens AJ (2006) Improving irrigation water performance through an understanding of the water delivery process. Irrig Drain 55:223–234

7. Bos MG, Nugteren J (1990) On irrigation efficiencies, 4th edn. International Institute for Land Reclamation and Improvement (ILRI), Publication 19, Wageningen
8. Hsiao TC, Steduto P, Fereres E (2007) A systematic and quantitative approach to improve water use efficiency in agriculture. Irrig Sci 25:209–231
9. Jensen ME (2007) Beyond irrigation efficiency. Irrig Sci 25: 233–245
10. de Wit CT (1992) Resource use efficiency in agriculture. Agric Syst 40:125–151
11. Van Schilfgaarde J (1984) Drainage design for salinity control. In: Shainberg I, Shalhevet J (eds) Soil salinity under irrigation. Springer, New York, pp 190–197
12. Steduto P, Hsiao TC, Fereres E (2007) On the conservative behavior of biomass water productivity. Irrig Sci 25:189–207
13. Richards RA (2006) Physiological traits used in the breeding of new cultivars for water-scarce environments. Agric Water Manage 80:197–211
14. Salekdeh GH, Reynolds M, Bennett J, Boyer J (2009) Conceptual framework for drought phenotyping during molecular breeding. Trends Plant Sci 14:488–496
15. Shainberg I, Levy GJ (1996) Infiltration and seal formation processes. In: Agassi M (ed) Soil erosion, conservation, and rehabilitation. Marcel Dekker, New York, pp 1–22
16. FAO (2010) http://www.fao.org/ag/ca/. Verified on 26 May 2010
17. Derpsch R, Friedrich T (2009) Global Overview of Conservation Agriculture Adoption. Proceedings: 4th World Congress on Conservation Agriculture, 4–7 February 2009, New Delhi, pp 429–438
18. Thompson RB, Gallardo M, Agüera T, Valdez LC, Fernandez MD (2006) Evaluation of the watermark sensor for use with drip irrigated vegetable crops. Irrig Sci 24:185–202
19. Goldhamer DA, Fereres E (2001) Irrigation scheduling protocols using continuously recorded trunk diameter measurements. Irrig Sci 20:115–125
20. Jackson RD (1982) Canopy temperature and crop water stress. Adv Irrig 1:43–85
21. Allen RG, Pereira LS, Raes D, Smith M (1998) Crop evapotranspiration: guidelines for computing crop water requirements, FAO irrigation and drainage paper 56. FAO (Food and Agriculture Organization), Rome
22. Mantovani EC, Orgaz F, Villalobos FJ, Fereres E (1995) Modelling the effects of sprinkler irrigation uniformity on crop yield. Agric Water Manage 27:243–257
23. Benson SM, White AF, Halfman S, Flexser S, Alavi M (1991) Groundwater contamination at the Kesterson reservoir, California 1. Hydrogeologic setting and conservative solute transport. Water Resour Res 27:1071–1084
24. Hoffman GJ, Dirksen C, Ingvalson RD, Maas EV, Oster JD, Rawlins SL, Rhoades JD, Van Schilfgaarde J (1977) Minimizing salt in drain water by irrigation management: design and initial results of Arizona field studies. Agric Water Manage 1:233–252
25. Sadler EJ, Evans RG, Stone KC, Camp CR (2005) Opportunities for conservation with precision irrigation. J Soil Water Conservat 60:371–379
26. Evans RG, Sadler EJ (2007) New technologies to improve crop water use efficiencies [CD-ROM]. S164. Lawrence Media
27. Santos C, Lorite IJ, Tasumi M, Allen RG, Fereres E (2010) Performance assessment of an irrigation scheme using indicators determined with remote sensing techniques. Irrig Sci. doi:10.1007/s00271-010-0207-7
28. Zarco-Tejada PJ, Berni JAJ, Suárez L, Sepulcre-Cantó G, Morales F, Miller JR (2009) Imaging chlorophyll fluorescence from an airborne narrow-band multispectral camera for vegetation stress detection. Rem Sens Environ 113:1262–1275
29. Berni JAJ, Zarco-Tejada PJ, Sepulcre-Cantó G, Fereres E, Villalobos F (2009) Mapping canopy conductance and CWSI in olive orchards using high resolution thermal remote sensing imagery. Rem Sens Environ 113:2380–2388
30. Fountas S, Wulfsohn D, Blackmore BS, Jacobsen HL, Pedersen SM (2006) A model of decision-making and information flows for information-intensive agriculture. Agric Syst 87:192–210
31. Maton L, Leenhardt D, Goulard M, Bergez JE (2005) Assessing the irrigation strategies over a wide geographical area from structural data about farming systems. Agric Syst 86:293–311
32. Arnott D (2006) Cognitive biases and decision support systems development: a design science approach. Inf Syst J 16:55–78
33. Mohan S, Arumugam N (1997) Expert system applications in irrigation management: an overview. Comput Electron Agric 17:263–280
34. Goldberg DE (1989) Genetic algorithms in search, optimization and machine learning. Addison-Wesley, Madison
35. Azamathulla HMd, Wu FC, Ab Ghani A, Narulkar SM, Zakaria NA, Chang CK (2008) Comparison between genetic algorithm and linear programming approach for real time operation. J Hydro Environ Res 2:172–181
36. Kipkorir EC, Raes D, Labadie J (2001) Optimal allocation of short-term irrigation supply. Irrig Drain Syst 15:247–267
37. Bergez JE, Garcia F, Lapasse L (2004) A hierarchical partitioning method for optimizing irrigation strategies. Agric Syst 80: 235–253
38. Bazzani GM (2005) An integrated decision support system for irrigation and water policy design: DSIRR. Environ Model Softw 20:153–163
39. Stoorvogel JJ, Antle JM, Crissman CC, Bowen W (2004) The tradeoff analysis model: integrated bio-physical and economic modeling of agricultural production systems. Agric Syst 80:43–66
40. Stewart JI, Hagan RM (1973) Functions to predict effects of crop water deficits. J Irrig Drain Div 99:421–439
41. Doorenbos J, Kassam AH (1979) Yield response to water, FAO irrigation and drainage paper No. 33. FAO (Food and Agriculture Organization), Rome
42. Vaux HJ, Pruitt WO (1983) Crop-water production functions. In: Hillel DI (ed) Advances in irrigation, vol II. Academic, New York, pp 61–97

43. Loomis RS, Rabbinge R, Ng E (1979) Explanatory models in crop physiology. Annu Rev Plant Physiol 30:339–367
44. Stöckle CO, Donatelli M, Nelson R (2003) CropSyst, a cropping systems simulation model. Eur J Agron 18:289–307
45. Jones CA, Dyke PT, Williams JR, Kiniry JR, Benson CA, Griggs RH (1991) EPIC: an operational model for evaluation of agricultural sustainability. Agric Syst 37:341–350
46. Smith M (1992) CROPWAT: a computer program for irrigation planning and management, FAO irrigation and drainage paper No. 46. FAO (Food and Agriculture Organization), Rome
47. McCown RL, Hammer GL, Hargreaves JNG, Holzworth DP, Freebairn DM (1996) APSIM: a novel software system for model development, model testing and simulation in agricultural systems research. Agric Syst 50:255–271
48. Ritchie JT, Godwin DC, Otter-Nacke S (1985) CERES – wheat: a simulation model of wheat growth and development. Texas A&M University Press, College Station
49. Sinclair TR, Seligman NG (1996) Crop modeling: from infancy to maturity. Agron J 88:698–704
50. Steduto P, Hsiao TC, Raes D, Fereres E (2009) AquaCrop – the FAO crop model to simulate yield response to water: I. Concepts and underlying principles. Agron J 101:426–437
51. García-Vila M, Fereres E, Mateos L, Orgaz F, Steduto P (2009) Deficit irrigation optimization of cotton with AquaCrop. Agron J 101:477–487
52. Sophocleous M (2005) Groundwater recharge and sustainability in the high plains aquifer in Kansas, USA. Hydrogeol J 13:351–365
53. English MJ (1990) Deficit irrigation. I: analytical framework. J Irrig Drain Eng 116:399–412
54. Debaeke P, Aboudrare A (2004) Adaptation of crop management to water-limited environments. Eur J Agron 21:433–446
55. Fereres E, Goldhamer DA, Parsons LR (2003) Irrigation water management of horticultural crops. Historical review compiled for the American Society of Horticultural Science's 100th Anniversary. HortScience 38:1036–1042
56. Fereres E, Soriano MA (2007) Deficit irrigation for reducing agricultural water use. J Exp Bot 58:147–159
57. Jury AJ, Vaux HJ (2007) The emerging global water crisis: managing scarcity and conflict between water users. Adv Agron 95:1–76
58. Sadras VO, Angus JF (2006) Benchmarking water use efficiency of rainfed wheat in dry environments. Aust J Agric Res 57: 847–856

Books and Reviews

Burt CM, Clemmens AJ, Strelkoff TS, Solomon KH, Bliesner RD, Howell TA, Eisenhauer DE (1997) Irrigation performance measures: efficiency and uniformity, Journal of Irrigation and Drainage Engineering 123, No. 6. ASCE (American Society of Civil Engineers), New York, pp 423–442

Evans RG, Sadler EJ (2008) Methods and technologies to improve efficiency of water use: W00E04, Water Resources Research 44, No. 7. American Geophysical Union, Washington

Fereres E, González-Dugo V (2009) Improving productivity to face water scarcity in irrigated agriculture. In: Sadras VO, Calderini DF (eds) Crop physiology: applications for genetic improvement and agronomy. Academic, New York, pp 123–143

Lamm FR, Ayars JE, Nakayama FS (2007) Microirrigation for crop production, Developments in agricultural engineering 13. Elsevier, Amsterdam

Malano H, Burton M (2001) Guidelines for benchmarking performance in the irrigation and drainage sector. IPTRID/FAO, Rome

Molden D (2007) Water for food, water for life, A comprehensive assessment of water management in agriculture. Earthscan/IWMI, London

National Research Council (1996) A new era for irrigation. National Academy, Washington

Passioura JB, Angus JF (2010) Chapter 2 – improving productivity of crops in water-limited environments, Advances in agronomy 106. Academic, San Diego, pp 37–75

Life Cycle Assessments and Their Applications to Aquaculture Production Systems

Patrik J. G. Henriksson[1,2], Nathan L. Pelletier[3], Max Troell[4,5], Peter H. Tyedmers[3]
[1]Institute of Environmental Sciences, Leiden University, Leiden, The Netherlands
[2]Department of System Ecology, Stockholm University, Stockholm, Sweden
[3]Dalhousie University, Halifax, Nova Scotia, Canada
[4]The Royal Swedish Academy of Sciences, The Beijer Institute of Ecological Economics, Stockholm, Sweden
[5]Stockholm Resilience Centre, Stockholm University, Stockholm, Sweden

Article Outline

Glossary

Aquaculture The farming of aquatic organisms, including fish, mollusks, crustaceans, and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators. Farming also implies individual or corporate ownership of the stock being cultivated.

Co-product allocation Partitioning the input or output flows of a process or a product system between the product system under study and one or more other product systems.

Functional unit The quantified function provided by the product system(s) under study, for use as a reference basis in an LCA, e.g., 1,000 h of light.

Life cycle assessment (LCA) An ISO-standardized analytical tool developed to evaluate environmental performance of products and processes. It constitutes a compilation and evaluation of the inputs, outputs, and potential environmental impacts of a product system throughout its life cycle; the term may refer to either a procedural method or a specific study.

System boundary Defines the inputs and outputs that are included in the study. System boundaries should be set depending on what will be relevant to the aim of the study.

Definition of the Subject

Aquaculture production has grown three times faster than the livestock sector since the 1970s, becoming a major source of edible seafood and other products. This rapid expansion has, however, had a combination of positive and negative environmental, social, and economic effects. A variety of tools are available to evaluate these impacts in an attempt to identify the most sustainable practices. One of the more recent tools that has been applied to the evaluation of aquaculture production is Life Cycle Assessment (LCA), an ISO-standardized biophysical accounting framework that allows for multi-criteria environmental performance assessments. This chapter reviews studies that have applied LCA to studying the environmental dimensions of aquaculture production to date. Methodological differences and alternative approaches are discussed, along with their influence on research outcomes. There is little homogeneity between the studies when it comes to the choice of functional unit, system boundaries, and basis for allocation. However, several clear trends do emerge that point toward imperatives for sustainable practices in aquaculture and considerations for sustainable development of the industry moving forward. Recommendations for further methodological development of LCA for application to seafood sustainability research are advanced.

Introduction

Society is increasingly aware of both the drivers and consequences of natural resource depletion and environmental degradation. Various analytical frameworks

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

have therefore been developed for the purpose of evaluating the environmental performance of products and processes. Finding a suitable tool for assessing sustainability in the rapidly developing aquaculture sector has gained increasing profile over the past 2 decades. What has emerged is the need for a tool that can incorporate multiple environmental performance criteria in the evaluation of diverse aquaculture production technologies. For this reason, there is increasing interest in, and application of, life cycle assessment (LCA) as a research framework to better understand environmental performance in this sector. The interest has, however, not been coming from within the aquaculture industry itself, but rather outside. LCA is a versatile methodology that is well suited to address a broad suite of resource use and emissions-related issues. Over the last decade, it has become an increasingly common tool for characterizing an important subset of environmental impacts in aquaculture and elsewhere.

Aquaculture Development

Even though global capture fisheries landings have declined since the late 1980s, total production of marine fisheries products has increased 67% between 1970 and 2007 (including brackish water fish). This has only been possible through a large increase in aquaculture production over the last 4 decades. Aquaculture currently provides half of all finfish destined for human consumption. Seafood from all sources accounts for about 20% of all animal proteins consumed by humans, and demand continues to grow [1]. The aquaculture industry is the fastest growing animal products' sector, with an average annual growth rate of 6.9%. At present, it provides almost 8 kg of seafood per capita year^{-1} globally [1]. In 2006, aquaculture accounted for more than 70% of global shrimp and prawn production, 47% of total food fish production, and 36% of total fish production. Mariculture of finfish dominates production in developed countries [2]. By mass, however, the majority of global production is accounted for by carp farmed in extensive and semi-intensive farms in Asia.

Aquaculture comprises an enormous diversity of farming technologies, culture settings, and species. From monoculture to polyculture systems operated in ponds, raceways, land-based tanks, along with cages, pens, poles, rafts, and longlines in open water settings, well over 250 species are currently in culture. Production technologies may also reflect traditional farming methodologies or more modern systems [3–5]. In turn, post-production processing yields a diverse range of products, including salted, dried, smoked, and various kinds of preserved fish. About 37% of all fish and fishery products are traded internationally, with the major importers being Japan, the USA, and Spain [1].

Aquaculture and the Environment

It has been proposed that aquaculture represents the most viable option for meeting future demands for fish, as well as providing economic and nutritional benefits to millions [1]. The recent rapid expansion of this sector has, however, been accompanied by a range of environmental and social concerns, including localized nutrient enrichment or depletion, chemical pollution, genetic pollution, introduction of non-indigenous species, habitat destruction, greenhouse gas (GHG) emissions, depletion of wild fish stocks, inefficient energy and biotic resource usage, and spread/amplification of diseases and parasites [2, 3, 6–9]. Of these, local-scale interactions have traditionally attracted the most attention. However, global scale interactions such as greenhouse gas emissions associated with intensive production strategies are of increasing interest. What has become clear is that each production strategy is characterized by a unique suite of environmental interactions at local, regional, and global scales. Informed decision making for improved environmental management in aquaculture, therefore, requires tools, which can provide multi-criteria environmental performance assessments and make clear the environmental trade-offs associated with specific aquaculture technologies and products.

Sustainability Tools in Aquaculture

Increasing aquaculture production to meet future demands is clearly attractive from a policy and development perspective. However, a number of critical questions related to growth in this sector must be addressed. These questions encompass complex issues associated with sustainability objectives at local, regional, and international scales. For example, a spectrum of negative ecological and social

externalities associated with aquaculture and other food production systems bear careful scrutiny and must be weighed against anticipated benefits [10]. Such comparisons need to extend beyond short-term gains and localized impacts and incorporate a long-term social-ecological resilience perspective. This requires tools to identify the most sustainable aquaculture practices, drawing knowledge from both new and traditional culture systems [5].

A wide range of tools/frameworks for assessing various aspects of environmental performance have been advanced [11], some focusing especially on food production. These include techniques such as Risk Assessment, Ecological Footprint, and Energy Analysis. Frameworks more specific to assessing seafood production systems are Fishprint and the Global Aquaculture Performance Index (GAPI) [10, 12, 13]. Most of these, however, encompass a limited range of the environmental concerns associated with aquaculture and some suffer from a lack of methodological standardization [10]. Moreover, the degree of scientific rigor in both the methods and their application is also variable. Data limitations and analytical scope have, therefore, often led to misrepresentation of the environmental consequences of specific management decisions.

LCA – The Method and Its Applicability in Aquaculture

History of LCA

LCA has, since its emergence in the 1970s, evolved from a tool whose primary application was waste management and energy efficiency management to a more general eco-efficiency measurement framework. It has close links to energy analysis, but is unique among biophysical accountancy-type tools in that it has been internationally standardized (ISO 14040-14044) [14, 15]. An LCA typically begins at the "cradle" of a product or service life cycle (i.e., at the point of primary resource extraction), and extends along the supply chain to encompass all life cycle stages of interest to a particular analysis. Single or multiple impact assessment methods may be applied. Estimation of the cumulative environmental impacts along supply chains permits attention to spatially and temporally discrete impacts not typically considered in more traditional environmental impact analyses. Impact categories range from highly quantifiable effects, such as greenhouse gas emissions or energy use, to (less frequently) more diverse social consequences, such as human health effects [16].

The outcome of a LCA is highly influenced by the ambition, skill, and objectives of the practitioner. Modern software with built-in inventory databases and impact assessment methods has simplified the LCA process, to the extent that an aquaculture system may be modeled in hours. However, the rigor of such models is to a large extent dependent on data quality. While use of generic data available in many public and commercial life cycle inventory databases may provide a starting point for scoping analyses, more context-specific data is required for robust modeling of specific production systems and technologies. Unfortunately, the former (simplified analyses) are increasingly common in the peer-reviewed literature, providing what may be misleading signals and eroding the credibility of the research framework, generally.

Software Tools and ISO

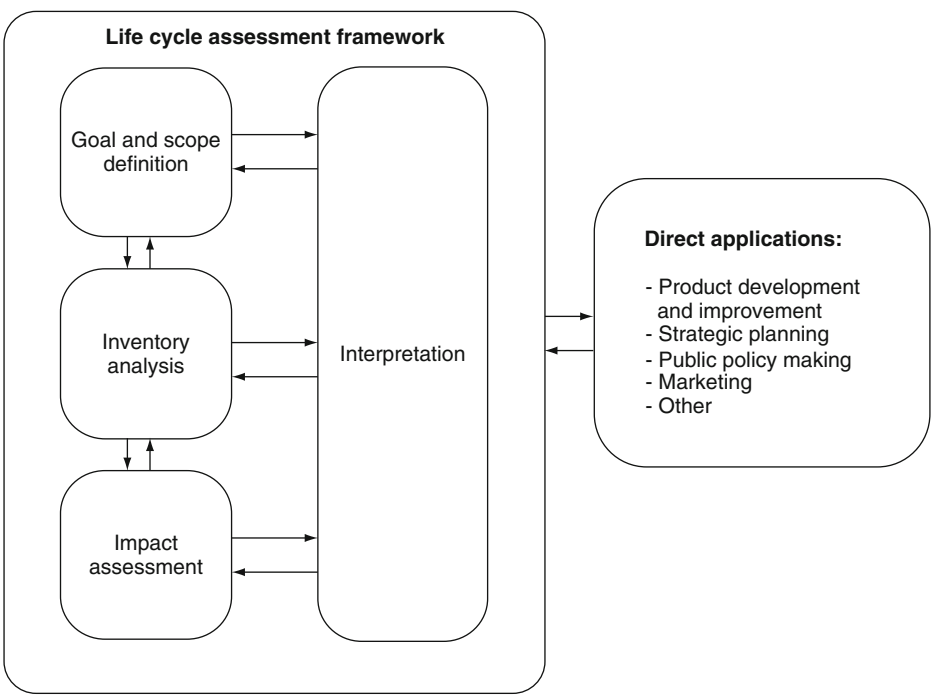
The rapid evolution and adoption of LCA have been accompanied by the creation of a variety of guidelines, manuals, and dedicated software [17]. The most commonly used LCA software platforms are GaBi and SimaPro, which are commercial products. Others, such as CMLCA and openLCA, are available free of charge. There are also several life cycle inventory databases, which have been developed, with the most extensive being the EcoInvent database (www.ecoinvent.ch). Such databases provide inventory data for materials and processes common to most product systems – for example, the production of materials such as concrete, steel, and plastic; the provision of energy carriers such as diesel or regionally specific electricity mixes; or transportation modes like air freight, rail freight, or private automobile transport. As these "background" data are often premised on different methods, assumptions, and rigor, sourcing data should be done with care as different databases apply different methodologies. It is therefore recommended to be consistent when choosing sources of background systems data and also to be aware of the methodology used before making comparisons between studies. ISO compliance does not require that studies are comparable, only that

they follow the same requirements. Individual studies also often differ in functional unit (the unit of output against which impacts are quantified), system boundaries, and allocation criteria.

A LCA study is, in accordance to ISO standards, carried out methodically through four phases (Fig. 1). As an initial phase, the goal and scope definition will specify the main characteristics of the study. Goal is specified as the application, audience, and reason for the study, while scope outlines the product system to be studied, functional unit, system boundaries, allocation procedures, impact categories, data requirements, assumptions, limitations, data quality, and format of the report. System boundaries specify the processes that are to be included in the product system and are in turn set by the cutoff criteria. This is followed by a Life Cycle Inventory Analysis (LCI), which involves the collection and calculation of data as well as allocation of burdens, in cases where input or output flows involve more products or processes than the defined unit. The impacts of these results are then, in the third phase (Life cycle impact assessment, LCIA), assessed according to their contribution to the impact categories defined in the goal and scope phase. Finally, the three previous phases are interpreted and communicated to the anticipated audience.

Functional Unit

Seafood commodities are farmed for different purposes, which complicates the choice of functional unit. Most LCA studies to date have used live weight mass at the farmgate or mass of processed product as the functional unit. Variable protein contents and edible portions between aquatic animals therefore may complicate direct comparisons. For example, the edible portion of an oyster is only about 18% of its wet weight, a number that in turn is subject to local variation [18] while the edible yield from an Atlantic salmon (*Salmo salar*) typically exceeds 50%. Local customs may further confound the decision as certain parts of the fish that may be discarded as inedible in one region are considered good for human consumption in another region. Two such examples are herring roe and catfish



Life Cycle Assessments and Their Applications to Aquaculture Production Systems. Figure 1
The general methodological framework for LCA studies according to ISO 14040 (2006) and its four comprised phases

stomachs; both of which are considered offal in the western world but are prized in parts of Asia [19, 20]. Ideally, the functional unit should reflect the function of the product system. For aquaculture products intended for consumption as food, such functions might include the provision of caloric energy, protein, or omega fatty acids, etc.

Setting System Boundaries

System boundaries delineate those processes formally included in an analysis from those that are excluded. There are only general requirements on how to set these boundaries, though they should be decided relative to the research objectives and include all elements having a non-trivial influence on research results. Most LCA studies in aquaculture limit their system boundary to the farmgate, whereas some include processing, packaging, marketing, and consumption phases [21–23]. Thrane [24] evaluated the effects of post-harvest stages of the life cycle of seafood products and concluded that they have a significant impact on the overall environmental performance of most seafood products. For example, inclusion of the processing phase contributed an additional 10% to life cycle energy use, while inclusion of the consumption phase resulted in an average 25% increase in cumulative energy demand [24].

Allocation

A common issue faced by many LCA practitioners is how to allocate environmental burdens to products and materials that are co-produced with other products [25]. Examples in aquaculture include the allocation of environmental burdens between targeted catch and bycatch used in fishmeal production, the multiple products derived from corn and other commodity crops used in feeds, and alternative uses of fish by-products or aquaculture wastewater used to fertilize other crops. The ISO standards for LCA do provide guidelines for an allocation decision hierarchy, but leave considerable room for interpretation. According to the standard, environmental burdens are primarily to be allocated according to an underlying physical relationship, if subdivision is unavoidable. The standard further states that where such relationships cannot be established, the allocation should reflect other relationships between the input system and output system. In reality, the final choice of allocation basis will likely reflect the goals of the study, as well as the worldview of the practitioner. Some of the more common bases for allocation in aquaculture and their characteristics are summarized in Table 1. Accessibility of the different allocation factors differs depending on location and situation, where regional differences will play a large role in making generalizations. Time and spatial scales will also have a great influence on the allocation factors that do not represent physical relationships, as these will not remain static.

There are several things to keep in mind when choosing a basis for allocation. The first is that nothing limits a study to only one allocation factor; each allocation scenario may be treated differently depending on the circumstances. Results may also be presented using several of the allocation factors, thus enabling readers to interpret results according to their own perspective or worldview. No matter how the allocation problem is approached, it is very important to be clear in the supporting text about which methods have been used. It is, however, important to keep in mind

Life Cycle Assessments and Their Applications to Aquaculture Production Systems. Table 1 The most commonly used bases for allocation and their characteristics

Allocation factor	Accessibility	Physical relationship	Static	Market oriented
Mass	Good	Yes	Yes	No
Value	Average	No	No	Yes
Nutritional energy content	Average	Yes	Yes	No
System expansion	Poor	Sometimes	Sometimes	Sometimes

that the basis of allocation can influence the allocated burden by as an order of magnitude [26].

Impact Categories and Impact Assessment

Any quantifiable performance measure can be included within the LCA methodology, from emissions to the well-being of workers, as long as: (a) a causal relationship between the variable of interest and the provision of the functional unit can be established and (b) a defensible impact assessment methodology is available [27]. A wide variety of impact assessment methods are available for use in LCA, most of which have been applied to assessments of seafood production systems [27]. These methods may describe environmental interactions that have relevance at local (e.g., eutrophication), regional (e.g., acidification), or global (e.g., greenhouse gas emissions) scales. Generally, impact assessment methods are based on peer-reviewed, internationally accepted environmental accounting protocols. Some of these continue to evolve, and novel methods emerge in response to newly identified issues [27, 28].

Labor

Labor is rarely accounted for in LCA due to the difficulty of establishing defensible system boundaries and quantifying associated environmental impacts. It can also be debatable if the number of employees should be considered as a negative or positive input. It is, however, important to keep labor in mind when comparing traditional and modern production systems. Labor can be presented in a number of different ways, either separately or incorporated into the individual impact categories [28, 29]. Several methods have been suggested on how to quantify the environmental impacts of labor. Suggested reference units include metabolic energy, calorific content of food consumed, national fuel share, or other more complex equations [29].

LCA in Food Production

Working with non-Static Systems

The adaptation of LCA from the characterization of static industrial systems to food production systems typified by significant variability has brought with it new challenges. Annual fluctuations occur both on the farm, as productivity will depend on water temperature, extreme weather events, algae blooms, etc., as well as on indirect variables such as oil prices and public demand. Fisheries providing fishmeal and fish oil for use in aquafeeds offer a good example of such variability, as fuel inputs per tonne of fish landed will fluctuate with season, stock status, gear type, and skipper [30, 31]. The aquaculture sector is particularly dependant on annual production of the anchoveta fishery off South America for both of these commodities, which in turn is strongly influenced by El Niño-Southern Oscillation (ENSO) events [32]. Increased use of compound feeds and higher oil prices have also boosted prices of both fishmeal and oil over the last decade [1]. Such fluctuations not only affect economic allocation but also catch per unit effort. It can therefore be hard to set average fuel consumption for fishing fleets, especially since the species and status of the stocks used for fishmeal production often are unknown [33]. The situation is further complicated in certain parts of the world where low value fish are used directly as fish feed [33].

Agricultural crops – the other major source of aquafeed inputs – may also experience significant annual fluctuations, with larger variability in developed countries, for crops such as maize and wheat [1, 34]. Farmgate prices will further affect the LCA as many feed formulators and aquaculturists quickly adjust to price trends in their choice of feed inputs or cultured species in efforts to maximize profits [20, 35]. One such example is the constant push toward higher stocking densities of shrimp in SE Asian polyculture systems, where the large profits that are to be made often outweigh the risk of white-spot disease [36]. Farming practices, as for feed composition, are also under constant change, which emphasizes the importance of considering the time scales used in LCA studies. Ultimately, a balance must be struck between feasibility and the goals of the study in pursuing representative data and models.

The relevance of such variability will, in part, be determined by the scope of the analysis and specific research questions of interest. Variability might be accommodated when modeling at regional scales by applying average data over specified spatial and temporal horizons. It is increasingly common to account for and report such variability in published outcomes. In other cases, quantifying variability might comprise

research foci – for example, understanding the influence of variable field-level nitrous oxide emissions on the overall greenhouse gas intensity of crop production at local scales.

LCA in Aquaculture

The first LCA studies focusing on aquaculture systems were conducted in the beginning of the new millennium, with an increasing application of the methodology to aquaculture issues toward the end of its first decade [37–39]. As the number of studies increased, so too did the seeming detail of analysis and possibly also the accuracy of the results. Most have focused on production systems in developed countries (see Table 2). The methodological detail used between studies does, however, vary widely and makes broad comparisons between studies difficult. This includes differences in functional unit, system boundaries, data sources and quality, and choice of allocation criteria.

A common theme that has emerged from LCA research of intensive, finfish aquaculture production systems is the importance of feed provision in supply chain environmental impacts [21, 22, 42–44]. For example, Pelletier et al. (2009) found that feed provision accounted for, on average, 92% of quantified impacts in global salmon farming systems. Of particular importance are fisheries and livestock products, which typically have higher impacts per unit mass relative to crop-derived feed inputs (Fig. 2). Also of note is that on-site processes have only made a substantial contribution in highly mechanized systems, where industrial energy inputs are required to maintain water quality [3, 20, 21, 40, 44].

Although animal-derived feed ingredients usually have a higher impact per unit mass compared to crop-derived inputs [22, 43, 45, 46], their inclusion may support more rapid growth of the cultured organisms and, in some cases, result in a higher quality product [47]. By including a larger portion of agriculturally sourced materials in feeds, environmental burdens may be reduced. In this light, it might be anticipated that rearing herbivorous or omnivorous species is environmentally preferable. However, both feed composition and feed conversion efficiency must be considered in determining and comparing impacts between cultured organisms [44].

The choice of impact categories in aquaculture LCAs varies widely (Table 2). These include resource depletion and emissions-related environmental concerns, as well as toxicological potentials. The only categories almost consistently applied are global warming potential, acidification, and eutrophication, while cumulative energy demand is also very commonly evaluated. There is an expected but imperfect correlation of cumulative energy demand and global warming potential (Table 3), since much of the feed-related emissions for agricultural inputs do not arise from fossil fuel combustion. Rather emissions of nitrous oxide and methane are typically as or more important. It is only in systems where ecosystem services have been replaced to a large extent by anthropogenic processes that on-farm energy demand has a significant impact on the total energy consumption.

Guiding the Way for More Sustainable Aquaculture and Alternative Farming Methods

Feed Production

Improvements of feed conversion ratios (FCRs) for piscivorous fish in addition to an increased inclusion of non-fish ingredients has led to great environmental improvements over the last decades [46]. Additional improvements are to be made by identifying and sourcing for the least-environmental-cost feed formulations; especially for sources of fishmeal and oil [22]. In reduction fisheries, effective management will play a major role in reducing fuel consumption along with associated environmental impacts while sustaining output from the industry [30, 50]. In many fisheries, boats today have to travel further to find productive fishing grounds and invest more fishing effort to maintain catches. This has resulted in a sixfold increase in energy consumption for some capture fisheries over the last two decades [51]. A collapse of one of the large reduction fisheries, of the scale that occurred to the anchoveta fishery in the 1970s, would further drive up the energy intensity of aquafeeds and culture products as well as have devastating effects on the aquaculture industry as supply of fishmeal and oil supplies already are outpaced by demand [1, 52].

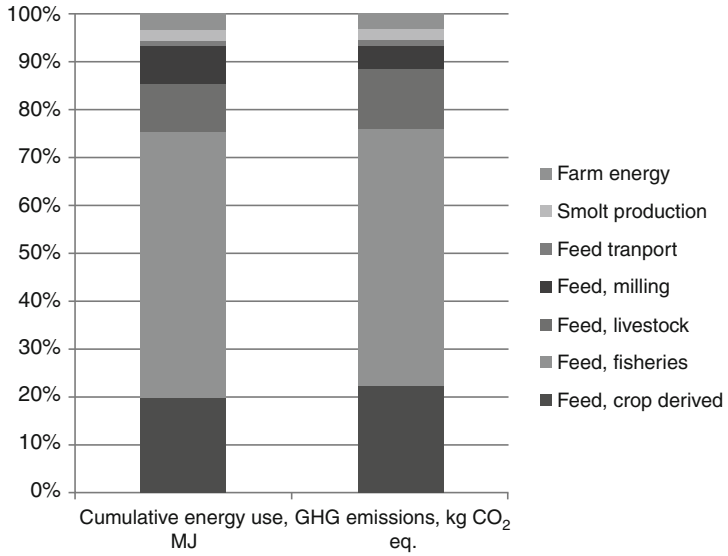
Decreasing fishmeal inclusion has, to a large extent, been driven by increasing public awareness about "fish-in to fish-out" ratios, as a result of labeling and

Life Cycle Assessments and Their Applications to Aquaculture Production Systems. Table 2 Overview of species, functional unit, system boundary, allocation factor, and impact categories applied for a selection of published LCA studies on aquaculture systems. One ton at farmgate is the most prominent functional unit while global warming potential, acidification, and eutrophication are the only impact categories that are included in all studies

Species	Reference	Functional unit	System boundary	Allocation factor	Cumulative energy demand	Fossil fuel use	Biotic resource use	Abiotic depletion potential	Water dependence	Surface use	Global warming potential	Ozone depletion potential	Acidification	Eutrophication	Phtotochemical oxidant formation	Freshwater aquatic ecotoxicity	Marine aquatic ecotoxicity	Terrestrial ecotoxicity	Human toxicity	Respiratory impacts from inorganics	Carcinogens
Blue mussels	Iribarren et al. [23]	1 kg of dry edible mussel flesh	Post consumption waste	System expansion	✖			✖			✖		✖	✖	✖	✖	✖	✖	✖		
Shrimps	Mungkung [70]	1.8 kg block of frozen shrimp	Post consumption waste	Monetary				✖			✖	✖	✖	✖	✖				✖		
Rainbow trout, sea-bass, and turbot	Aubin et al. [21]	1 t live weight	Farmgate	Monetary	✖		✖		✖		✖		✖	✖							
Salmon, different farming methods	Ayer and Tyedmers [40]	1 t live weight	Farmgate	Gross nutritional energy content	✖			✖			✖	✖	✖	✖			✖		✖		
Arctic char	Ayer and Tyedmers [40]	1 t live weight	Farmgate	Gross nutritional energy content	✖			✖			✖	✖	✖	✖			✖		✖		

Life Cycle Assessments and Their Applications to Aquaculture Production Systems. Table 2 (Continued)

Species	Reference	Functional unit	System boundary	Allocation factor	Cumulative energy demand	Fossil fuel use	Biotic resource use	Abiotic depletion potential	Water dependence	Surface use	Global warming potential	Ozone depletion potential	Acidification	Eutrophication	Photochemical oxidant formation	Freshwater aquatic ecotoxicity	Marine aquatic ecotoxicity	Terrestrial ecotoxicity	Human toxicity	Respiratory impacts from inorganics	Carcinogens
Atlantic salmon, different feeds	Pelletier and Tyedmers 2007 [43]	1 t live weight	Farmgate	Gross nutritional energy content	✖		✖				✖		✖	✖			✖				
Trout, flow through/ recirculating system	d'Orbcaster et al. [41]	1 t live weight	Farmgate	Monetary	✖		✖		✖	✖	✖		✖	✖							
Trout	Papatryphon et al. [37]	1 tonne live weight	Farmgate	Monetary	✖		✖				✖		✖	✖							
Atlantic salmon	Ellingsen and Aanondsen [39]	200 g fillet	Processed fillets	Mass/ Monetary		✖					✖	✖	✖	✖		✖				✖	✖
Atlantic salmon	Pelletier et al. [22]	1 tonne live weight	Farmgate	Gross nutritional energy content	✖		✖				✖		✖	✖							



Life Cycle Assessments and Their Applications to Aquaculture Production Systems. Figure 2
Cumulative Energy Use and Greenhouse gas emissions from salmon production. Feed production is by far the largest contributor to environmental concerns, constituting 93% of the energy use and 94% of the GHG emissions. The feed represents an average from farms in Norway, UK, Canada, and Chile with 41.8% of the ingredients derived from crops, 49.4% from fish, and 8.8% from livestock. Data from: Pelletier et al. [22]

certification initiatives [9, 46]. It has also been strongly influenced by rising prices for these commodities, due to increased competition from the aquaculture sector [1]. The overall demand for fishmeal and oil in aquaculture has, despite this, increased due to a larger share of farmers using compound feeds and increasing aquaculture production over time [46, 53]. At present, in most aquaculture LCAs, the amount of fishmeal and oil used is only reported as life cycle inventory data. Beyond the standard impact categories, methods to account for the ecological impacts of producing these products are underdeveloped. To date, only a few researchers have, e.g., applied a measure of biotic resource use in life cycle assessment, following the methods originally advanced by Pauly and Christensen [54]. This method quantifies the net primary productivity, as measured in carbon, required to support the provision of a specified amount of fish-derived material, taking into account trophic level and species-specific meal and oil yield rates. Biotic resources used can differ by as much as an order of magnitude between different sources of fishmeal and oil [22]. Alternative sources of proteins that could in part replace fishmeal include soy meal, wheat gluten, bone, feathers, blood, livestock co-product meal, and seafood processing materials [46, 55]. A variety of vegetables oils may also be partially substitutable for fish oils. The environmental performance of systems using, e.g., agriculture alternatives for fishmeal and oil needs, however, to be carefully analyzed as such substitution does not guarantee improved performance. Switching to the culture of low-trophic species is often described as a solution for more sustainable aquaculture [6]. While this would allow for great reductions in fish inclusion rates, the higher FCRs associated with lower quality feeds may result in only marginal improvements in GHG and related life cycle impacts [44]. In contrast, a switch to more energy and climate-friendly fertilizer production either through efficiency improvements in existing fertilizer plants or the use of biological nitrogen fixation in place of conventional N fertilizers could, however, offset some of the impacts associated with crop production [56, 57].

Improvements of feeding practices on farms can both reduce costs, emissions, and FCR [58]. The amount of feed added is often calculated according to feeding charts or as a percentage of the fish biomass. These generalizations often result in inefficient feed

Life Cycle Assessments and Their Applications to Aquaculture Production Systems. Table 3 Summary of the different results for 1 t of seafood product at farmgate

Species	Country	Source	CED (MJ) t^{-1}	GWP kg CO_2-e t^{-1}
Turbot, recirculating	France	Aubin et al. [21]	290,986	6,017
Sea-bass, cages	Greece	Aubin et al. [21]	54,656	3,601
Rainbow trout, flow through	France	Aubin et al. [21]	78,229	2,753
Atlantic salmon, net-pen	Canada	Ayer and Tyedmers [40]	26,900	2,073
Atlantic salmon, Land base	Canada	Ayer and Tyedmers [40]	97,900	2,770
Atlantic salmon, Bag	Canada	Ayer and Tyedmers [40]	32,800	1,900
Atlantic char, land-based recirculating	Canada	Ayer and Tyedmers [40]	353,000	28,200
Trout, recirculating system	Denmark	d'Orbcastel et al. [41]	63,202	2,043
Trout, flow through	Denmark	d'Orbcastel et al. [41]	34,869	2,015
Atlantic salmon	Norway	Ellingsen and Aanondsen [39]	65,000[a]	N.A.
Blue mussels, fresh	Spain	Iribarren et al. [23]	N.A.	472
White-legged shrimps	Thailand	Zimmo et al. [49] and Mungkung [70]	45,600	N.A.
Trout, portion sized	France	Papatryphon et al. [37]	37,842	1,851
Trout, large sized	France	Papatryphon et al. [37]	62,774	2,499
Atlantic salmon, organic crop/25% soy meal and 100% canola oil substitute	Canada	Pelletier and Tyedmers [43]	9,860	690
Atlantic salmon, organic crop ingredients/ fisheries by-product meals and oils	Canada	Pelletier and Tyedmers [43]	26,900	1,810
Atlantic salmon, organic crop/conventional animal meals and oils	Canada	Pelletier and Tyedmers [43]	17,100	1,250
Atlanti salmon, conventional	Canada	Pelletier and Tyedmers [43]	18,100	1,400
Atlantic salmon	Worldwide	Pelletier et al. [22]	31,100	2,160
Tilapia, Lake	Indonesia	Pelletier and Tyedmers [44]	18,200	1,520
Tilapia, Pond	Indonesia	Pelletier and Tyedmers [44]	26,500	2,100

Energy use in Norwegian salmon farming reported by Ellingsen and Aanondsen [39] was calculated by assuming that 15% of total reported energy use was used in the processing and distribution phases

utilization as it does not take factors such as species, genetic stock, feed composition, water temperature, or growth rate into account [58]. In addition, feeding efficiency is also influenced by the type of farming facility as confined bodies of water, such as raceways or bags, allow for more efficient feeding practices and effluent management [59].

Replacing Ecosystem Services with Anthropogenic Processes at Farm Site

Traditional extensive aquaculture systems depend, to a large extent, on labor and natural energy inputs [3]. Solar energy inputs promote in-situ production utilizable by some farmed animals while tidal energy or other natural watercourse flows provide means for water exchange.

As for energy consumed in highly mechanized production systems, such as most intensive land-based systems, the energy for farm-site activities often originates from fuel as farm-sites typically lack access to alternative sources of energy. It is therefore recommended to consider the location characteristics of the farm-site before implementing artificial services. Farms situated in areas with high water turnover, i.e., in streams, tidal zones, or exposed coasts, may cause little or no impact on the local ecosystem. Farms situated in areas without such hydrodynamic conditions need to treat their wastewater to avoid negative impact on the environment or run as closed systems. It is, however, important to acknowledge that environmental impacts from farm release need to be analyzed from an ecosystem perspective, which implies considering additional pollution sources and more regional scale effects and thresholds.

LCA has limited capacity to predict the actual consequences of many of the estimated impact potentials [60]. It may be justifiable to have such an approach for the impact categories that are operational on a global scale, such as global warming potential or ozone depletion. For more regional consequences, however, it can be highly misleading to make comparisons of the impacts between two localities. To address this in a more justifiable manner, several country-specific factors, such as RECIPE, TRACI, EDIP2003, and LUCAS [61], have been developed and arguments have been raised for similar factors on regional scales [62].

Transmission of disease and parasites between wild and farmed stock and introduction of non-indigenous species are both major concerns associated with aquaculture that have yet have not been addressed by LCA methodology [63, 64]. Nor is the framework necessarily conducive to accommodating such interactions since it is necessary to be able to link the impact to the production of a functional unit following a clear and quantifiable cause-effect pathway. Both of these types of impacts have attracted much public attention and have been major incentives for closed farming facilities. Transmission of sea lice has, apart from having potentially detrimental effects on wild fish stocks, been estimated to account for 6% of the product cost in salmon farming [64]. Floating cages and net cultures allow for free movement of pathogens from farmed to wild stocks [64]. They also run an increased risk of large escape events of domesticated fish due to their vulnerability to extreme weather events, marine mammal interactions, failing infrastructure, and management errors. Such events can lead to the introduction of non-indigenous species as well as undermining the genetic fitness of wild stocks. It has been estimated that aquaculture is responsible for 16% of all introductions of non-indigenous species to European coastal waters, and further introductions are to be expected with changing climate [63].

The use of chemicals and antibiotics on farm sites is still only generally covered by existing impact categories. These practices can result in long-term effects such as antibiotic-resistant bacteria or other public health risks [10]. They may also lead to contaminated water sources and loss of biodiversity [10]. Shrimp farms are implicated most frequently for using large amounts of antibiotics to reduce stock losses. On several occasions, this has resulted in product recalls and import bans by the EU, Canada, and the USA [10]. The social consequences of this might be direct (e.g., lower water quality) or indirect, as import bans can seriously affect the financial viability of many farmers [65]. Even if LCA has the potential to account for such social and economic consequences, there is a lack of metrics to describe how to include socioeconomic indicators [28]. Overcoming the hurdles associated with the development of such impact categories would allow for better estimations of overall sustainability, including environmental, social, and economic variables [28]. However, it should be recognized that LCA is not necessarily conducive to accounting for the full spectrum of sustainability concerns. As such, it should be considered a complement to, rather than a replacement for, other metrics.

Discussion

As the number of LCA studies describing aquaculture systems increases, so too does our understanding of a broader suite of the environmental costs of aquaculture production. In some cases, it would appear that aquaculture may indeed provide an inexpensive and sustainable source of food and other products; however, this will depend on numerous factors including cultured species, production technology, and socioeconomic characteristics. To date, LCA research has helped to identify those aspects of the life cycle that contribute disproportionately to environmental degradation, allowing for the

identification of improvements opportunities. There is, however, always the danger of oversimplification where results get misinterpreted as a result of inaccurate data or where results are not put into their relevant context. LCA should therefore not be seen as an all-encompassing tool, but rather as a screening tool, which allows for the mapping of good practices. Additional environmental and socioeconomic analyses can thereafter be applied to strengthen assessment of sustainability.

As for advancements that can be made within the sector, the major challenge will be to find good sources of low impact feed inputs for fed aquaculture systems, especially for fish oil that currently drives the demand for wild marine resources [46]. This would, of course, ideally be combined with further advances in feed utilization by fish in culture. A shift toward organically produced crop inputs may also reduce the impacts of fed aquaculture, while bringing other benefits such as biodiversity improvements and superior soil quality [57, 66]. However, the choice of some resource intensive "organic" inputs can negate much of the life cycle environmental benefits associated with organic crop production [57]. Another alternative protein source is offal meal from fish processing. Tilapia, for example, has an offal yield of about 67% of live weight [59]. This does, however, increase the risk of disease transmission and/or recycling of environmental contaminants [53]. Moreover, the environmental costs of producing these materials from a life cycle perspective may be high [22]. Recently, there has been increased interest in the use of fish processing co-products for biofuel feedstock [67]. Conversion of high-quality protein into biofuels appears rather wasteful when the environmental burdens associated with producing certain high protein feed inputs are taken into account. It is clearly desirable to identify and implement optimal uses of high-quality protein toward the overarching sustainability objective. This must include, among other things, attention to the environmental dimensions of alternative protein production and use strategies. Since our ability to make informed decisions will be strongly influenced by the robustness of our models and the extent to which they actually reflect the environmental impacts associated with the products and systems of interest, methodological decisions such as choice of allocation criterion should be clearly communicated and defended in the context of each analysis.

Increasing trade flows of aquatic products from developing to developed countries highlights the need for more LCA work beyond production systems in the developed world. It also indicates that there are substantial opportunities for expansion of aquaculture in developed countries. Tyedmers et al. [2] points out that the USA only accounts for 1% of global aquaculture production, half of which is made up by channel catfish. Future developments may also to be expected within mariculture, as much recent effort has focused on the development of marine fin-fish hatcheries and offshore cages.

Impacts involved with on-site activities can more easily be avoided by selecting for better farm locations, use of renewable energy, improved utilization of ecosystem services, and farming of more tolerant species. One example of such a species is Pangasius catfish, which has reached high production levels in Vietnam over the last decade. This fish does not require aeration as it can utilize aerial respiration when the oxygen level drops. The farms also often utilize tidal floods for water exchange [20]. As for tilapia, the tolerance toward hypoxia and changes in pH is much higher than other species normally found in western aquaculture. This would, again, lower the inputs needed for maintaining water quality. Wastewater quality can also be improved by the use of settlement ponds and/or plant production to remove nutrients. Cultivation of plants such as *Azolla* spp. and duckweed within ponds can also enhance carbon fixation and be used as feed inputs [48, 68]. Duckweed may further reduce GHG emissions as it will shade the pond and thereby limit the ammonia volatilization rate [49]. Some of these feed inputs may, however, negatively influence the color and the taste of the final product, which makes them less suitable as feed inputs [48].

Converting to energy conserving practices will not only have environmental benefits, it can also improve the economic profitability of farms and reduce vulnerability to peaks in the price of oil. Lower intensity systems are also less sensitive to mechanical or infrastructure failures, such a black outs, which otherwise can cause mass mortality. This is especially important in developing countries where there is less access to spare parts and power failures are recurring events. Open systems are, on the other hand, more vulnerable to other events such as algae blooms, pollution, and

extreme weather events, which may cause the loss of an entire crop. Areas ravaged by extreme storm events, such as typhoons, may suffer from weeks of poor water quality as large quantities of sediments may lead to high turbidity for long periods of time.

More knowledge on the true carbon emissions associated with aquaculture is needed as there is increasing interest in its potential as a climate-friendly source for food and biofuels [68, 69]. LCA can play an essential part in the screening for sustainable farming practices and also provide information for the implementation of carbon credit schemes. This need is especially critical for developing countries, as this is where the majority of production occurs and exports are increasing. This will, however, pose a challenge as farming methods are highly diverse and data on farm practices are usually limited.

Also, some important environmental impacts from aquaculture are at present not quantifiable using the LCA framework, such as spread of diseases and parasites. These impacts have been attracting widespread public concerns and have influenced development of farming methods in many countries. The associated consequences could, for example, be accounted for as biotic resource use. Concerns associated with deforestation to produce agricultural land should also be further discussed, as it is only partially covered in current LCA practice and literature.

Future Directions

LCA provides a robust tool for dealing with an important subset of sustainability concerns, many of which have historically been overlooked in discussions of environmental management in aquaculture. However, it must be kept firmly in mind that decisions made during the analytical process strongly influence research outcomes.

It is, therefore, important to continue the discussion about such methodological decisions, including the kind of LCA framework applied (i.e., attributional versus consequential LCA), systems boundaries, and choice of allocation methods for LCAs of aquaculture production. In the least, it would be constructive for all practitioners to clearly communicate and defend all methodological choices, as they may ultimately send different signals to the industry and policy makers working with sustainability issues.

A more standardized methodology within the sector would certainly facilitate the advancement of the field. This could be achieved by better communications between major practitioners or by the development of a manual to guide the community toward one common framework. Even still, there will always be deviations from common practice as each study serves a unique purpose, stressing the need for more transparency.

Even though ongoing initiatives for developing the LCA framework exist, it is important to acknowledge that the present framework has limited ability to accommodate the other two pillars of sustainability, namely, social and economic impacts [28]. Thus, even if LCA provides a tool with great potential to guide the aquaculture industry toward more sustainable production, its framework needs to be reinforced by other analytical tools to capture a wider range of sustainability concerns. The need to include other tools alongside LCA has already been recognized and recent projects, such as the SEAT project (www.seatglobal.eu), include complementary tools to give a more holistic measurement of sustainability. Limitations aside, however, the rapid development of this sector, coupled with the diverse range of possible culture species and technologies, demands careful attention to environmental effects at all relevant scales. LCA can and should play an important role in guiding decisions oriented toward sustainability in aquaculture.

Bibliography

Primary Literature

1. FAO (2009) State of world fisheries and aquaculture 2008. FAO, Rome
2. Tyedmers P, Pelletier N, Ayer N (2007) Biophysical sustainability and approaches to marine aquaculture development policy in the United States, A Report to the Marine Aquaculture Task Force
3. Troell M, Tyedmers P, Kautsky N, Rönnbäck P (2004) Aquaculture and energy use. Encyclopedia Energy 1:97–108
4. Troell M, Joyce A, Chopin T, Neori A, Buschmann A, Fang J-G (2009) Ecological engineering in aquaculture – potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. Aquaculture 297:1–9
5. Troell M (2009b) Integrated marine and brackish water aquaculture in tropical regions: research, implementation and prospects. In: Soto D (ed) Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper, 529. FAO, Rome, pp 47–131

6. Naylor R, Goldburg R, Primavera J, Kautsky N, Beveridge M, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405:1017–1024
7. Naylor R, Williams S, Strong D (2001) Aquaculture – a gateway for exotic species. Science 294:1655–1656
8. Naylor R (2005) Aquaculture and ocean resources: raising tigers of the seat. Annu Rev Environ Resources 30:185–218
9. Pelletier N, Tyedmers P (2008) Life cycle considerations for improving sustainability assessments in seafood awareness campaigns. Environ Manage 42:918–941
10. Bartley D, Brugére C, Soto D, Gerber P, Harvey B (2007) Comparative assessment of the environmental costs of aquaculture and other food production sectors, Methods for meaningful comparisons, FAO/WFT Expert Workshop, 24–28 April 2006, Vancouver, Canada. FAO Fisheries Proceedings, no. 10, Rome
11. Finnveden G, Moberg Å (2005) Environmental systems analysis tools – an overview. J Cleaner Prod 13:1165–1173
12. Folke C, Kautsky N, Berg H, Jansson Å, Troell M (1998) The ecological footprint concept for sustainable seafood production: a review. Ecol Soc Am 8(1):S63–S71
13. Volpe JP, Beck M, Ethier V, Gee J, Wilson A (2010) Global aquaculture performance index. University of Victoria, Victoria, British Columbia, Canada
14. International Organization for Standardization (2006) Environmental management – life cycle assessment – principles and framework (ISO 14040:2006). International Organization for Standardization, Geneva, Switzerland
15. International Organization for Standardization (2006) Environmental management – life cycle assessment – requirements and guidelines (ISO 14044:2006), Geneva, Switzerland
16. Cordella M, Tugnoli A, Spandoni G, Santarelli F, Zangrando T (2008) LCA of an Italian Beer. Int J LCA 13(2):133–139
17. Baumann H, Tillman A-M (2004) The Hitch Hicker's Guide to LCA. Studentlitteratur, Lund
18. Wright DA, Hetzel EW (1985) Use of RNA: DNA ratios as an indicator of nutritional stress in the American oyster Crassostrea virginica. Mar Ecol Prog Ser 25:199–206
19. Thrane M, Nielsen E, Christensen P (2009) Cleaner production in Danish fish processing – experiences status and possible future strategies. J Cleaner Prod 17:380–390
20. Henriksson P (2009) Energy Intensity in Tropical Aquaculture, MSc thesis, Stockholm University
21. Aubin J, Papatryphon E, van der Werf H, Chatzifotis S (2009) Assessment of the environmental impact of carnivorous finfish production systems using life cycle assessment. J Cleaner Prod 17(3):354–361
22. Pelletier N, Tyedmers P, Sonesson U, Scholz A, Zeigler F, Flysjo A, Kruse S, Cancino B, Silverman H (2009) Not all salmon are create equal: life cycle assessment (LCA) of global salmon farming systems. Environ Sci Technol 43(23):8730–8736
23. Iribarren D, Moreira M, Gumersindo F (2010) Revisiting the life cycle assessment of mussels from a sectorial perspective. J Cleaner Prod 18(2):101–111
24. Thrane M (2004) Environmental impacts from Danish fish products – hot spots and environmental policies, PhD thesis, Ålborg University, Ålborg, Denmark
25. Reap J, Roman F, Duncan S, Bras B (2008) A survey of unresolved problems in life cycle assessment. Int J Life Cycle Assess 13:290–300
26. Thrane M (2006) LCA of Danish fish products. Int J Life Cycle Assess 11(1):66–74
27. Pelletier N, Ayer N, Tyedmers P, Kruse S, Flysjo A, Robillard G, Ziegler F, Scholz A, Sonesson U (2007) Impact categories for life cycle assessment research of seafood production: Review and prospectus. Int J Life Cycle Assess 12(6):414–421
28. Kruse S, Flygsjö A, Kasperczyk N, Scholz A (2009) Socioeconomic indicators as a complement to life cycle assessment – an application to salmon production systems. Int J Life Cycle Assess 14:8–18
29. Zhang T, Dornfeld D (2007) Energy use per worker-hour, evaluating the contribution of labor to manufacturing energy use, part 3:B1, pp 189–193. In: Takata S, Umeda Y (eds) Advances in life cycle engineering for sustainable manufacturing businesses, Proceedings of the 14th CIRP conference on life cycle engineering, Waseda University, Tokyo
30. Tyedmers P, Watson R, Pauly D (2005) Fueling global fishing fleets. Ambio 34(8):635–638
31. Ruttan L, Tyedmers P (2007) Skippers, spotters and seiners: analysis of the "skipper effect" in US menhaden (Brevoortia spp.) purse-seine fisheries. Fish Res 83(1):73–80
32. Sandweiss D, Maasch K, Chai F, Andrus C, Reitz E (2004) Geoarchaeological evidence for multidecadal natural climatic variability and ancient Peruvian fisheries. Quatern Res 61: 330–334
33. Tacon A, Metian M (2009) Fishing for aquaculture: non-food use of small pelagic forage fish – a global perspective. Rev Fish Sci 17(3):305–317
34. Naylor R, Falcon W, Zavaleta E (1997) Variability and growth in grain yields, 1950-94: does the record point to greater instability? Populat Dev Rev 23(1):41–58
35. Tacon A, Silva S (1997) Feed preparation and feed management strategies within semi-intensive fish farming systems in the tropics. Aquaculture 151:379–404
36. Kautsky N, Rönnbäck P, Tedengren M, Troell M (2000) Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture 191:145–161
37. Papatryphon E, Petit J, Kaushik S, van der Werf H, Kaushik S (6–8 October 2003) Life cycle assessment of trout farming in France: a farm level approach. In: Halberg N (ed) Life Cycle Assessment in the Agri-food sector, Proceedings from the 4th International Conference. Bygholm, Denmark
38. Aubin J, Papatryphon E, van der Werf H, Petit J, Morvan Y (2006) Characterisation of the environmental impact of a turbot (Scophtalmus maximus) re-circulating production systems using Life Cycle Assessment. Aquaculture 261:1259–1268
39. Ellingsen H, Aanondsen S (2006) Environmental impacts of wild caught cod and farmed salmon – a comparison with chicken. Int J Life Cycle Assess 1:60–65

40. Ayer N, Tyedmers P (2009) Assessing alternative aquaculture technologies: life cycle assessment of salmonid culture systems in Canada. J Cleaner Prod 17:362–373
41. d'Orbcaster RE, Blancheton J-E, Belaud A (2009) Water quality and rainbow trout performance in a Danish Model Farm reciculating system: comparison with a flow through system. Aquacult Eng 40:135–143
42. d'Orbcaster RE, Blancheton J-E, Aubin J (2009) Towards environmentally sustainable aquaculture: comparison between two trout farming systems using Life Cycle Assessment. Aquacult Eng 40:113–119
43. Pelletier N, Tyedmers P (2007) Feeding farmed salmon: Is organic better? Aquaculture 272:399–416
44. Pelletier N, Tyedmers P (2010) A life cycle assessment of frozen Indonesian tilapia fillets from lake and pond-based production systems. J Ind Ecol (in press)
45. Alder J, Campbell B, Karpouzi V, Kaschner K, Pauly D (2008) Forage fish: from ecosystems to markets. Annu Rev Environ Res 33:7.1–7.14
46. Naylor R, Hardy R, Bureau D, Chiu A, Elliott M, Farrell A, Forster I, Gatlin D, Goldburg R, Hua K, Nichols P (2009) Feeding aquaculture in an era of finite resources. Proc Natl Acad Sci 106(36):15103–15110
47. de Francescoa M, Parisia G, Médaleb F, Lupia P, Kaushikb S, Poli B (2004) Effect of long-term feeding with a plant protein mixture based diet on growth and body/fillet quality traits of large rainbow trout (*Oncorhynchus mykiss*). Aquaculture 236(1–4):413–429
48. Edwards P, Anh Tuan L, Allan G (2004) A survey of marine trash fish and fishmeal as aquaculture feed ingredients in Vietnam. ACIAR Working Paper No. 57
49. Zimmo O, van der Steer N, Gijzen J (2003) Comparison of ammonia volatilization rates in algae and duckweed-based waste stabilization ponds treating domestic wastewater. Water Res 37:4587–4594
50. Driscoll J, Tyedmers P (2010) Fuel use and greenhouse gas emission implications of fisheries management: the case of the New England Atlantic herring fishery. Marine Policy 34(3):353–359
51. Tlusty M, Lagueux K (2009) Isolines as a new tool to assess the energy costs of the production and distribution of multiple sources of seafood. J Cleaner Prod 17:408–415
52. Pauly D, Christensen V, Guénette S, Pitcher T, Sumaila U, Walters C, Watson R, Zeller D (2002) Towards sustainability in world fisheries. Nature 418:689–695
53. Tacon A, Metian M (2008) Global overview on the use of fishmeal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 285:146–158
54. Pauly D, Christensen V (1995) Primary production required to sustain global fisheries. Nature 374:255–257
55. Tacon A, Hasan M, Subasinghe R (2006) Use of fishery resources as feed inputs for aquaculture development: trends and policy, implications. FAO Fisheries Circular. No. 1018. Rome, FAO, 99 p
56. Rafiqul I, Weber C, Lehmann B, Voss A (2005) Energy efficiency improvements in ammonia production-perspectives and uncertainties. Energy 30:2487–2504
57. Pelletier N, Arsenault N, Tyedmers P (2008) Scenario-modeling potential eco-efficiency gains from a transition to organic agriculture: life cycle perspectives on Canadian canola, corn, soy and wheat production. Environ Manage 42:989–1001
58. Cho C, Bureau D (2001) A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. Aquac Res 32:349–360
59. Boyd C, Tucker C, McNevin A, Bostick K, Clay J (2007) Indicators of resource use efficiency and environmental performance in fish and Crustacean aquaculture. Rev Fish Sci 15:327–360
60. Potting J, Hauschild M (1997) Spatial differentiation in life-cycle assessment via the site-dependent characterisation of environmental impact from emissions. Int J Life Cycle Assess 2(4):209–216
61. ILCD handbook (International Reference Life Cycle Data System) (2010) Analysis of existing environmental impact assessment methodologies for use in life cycle assessment, JRC background document, 1st edn, European Union
62. Gallego A, Rodríguez L, Hospido A, Moreira1 M, Feijoo G (2010) Development of regional characterization factors for aquatic eutrophication. J Life Cycle Assess 15(1):32–43
63. Occhipinti-Ambrogi A (2007) Global change and marine communities: alien species and climate change. Mar Pollut Bull 55:342–352
64. Costello M (2009) How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. Proc R Soc 276: 3385–3394
65. Cato J, Lima dos Santos C (1998) European Union 1997 seafood-safety ban: the economic impact on Bangladesh shrimp processing. Mar Resource Econ 13(3):215–227
66. Reijnders L, Soret S (2003) Quantifications of the environmental impacts of different dietary protein choices. Am J Clin Nutr 78:664S–668S
67. Wiggers V, Wisniewski A, Madureira L, Chivanga Barros A, Meier H (2009) Biofuels from waste fish oil pyrolysis: continuous production in a pilot plant. Fuel 88(11):2135–2141
68. Bunting S, Pretty J (2007) Global carbon budgets and aquaculture – emissions, sequestration and management options. Centre for Environment and Society Occasional Paper 2007-1. University of Essex, Essex
69. Hossain S, Salleh A, Boyce A, Chowdhury P, Naqiuddin M (2008) Biodiesel fuel production from algae as renewable energy. Am J Biochem biotechnol 4(3):250–254
70. Mungkung R (2005) Shrimp aquaculture in Thailand: application of life cycle assessment to support sustainable development, PhD thesis, Center for Environmental Strategy, School of Engineering, University of Surrey, Surrey, UK

Books and Reviews

Mungkung R, Gheewala SH (2006) Use of life cycle assessment (LCA) to compare the environmental impacts of aquaculture and agri-food products. In: Barley DM, Brugére C, Soto D, Gerber P, Harvey B (eds) Comparative assessment of the environmental costs of aquaculture and other food production sectors: methods for meaningful comparisons, FAO/WFT Expert Workshop, 24–28 April 2006, Vancouver, Canada. FAO Fisheries Proceedings, no. 10, Rome, FAO, pp 87–96, 207

Ayer N, Tyedmers P, Pelletier N, Sonesson U, Scholz A (2007) Co-product allocation in life cycle assessments of seafood production systems: review of problems and strategies. Int J Life Cycle Assess 12(7):480–487

Baumann H, Tillman A-M (2004) The hitchhiker's guide to LCA: an orientation in life cycle assessment methodology and application. ISBN 91-44-02364-2

Guinée J (ed), Gorrée M, Heijungs R, Huppes G, Kleijn R, de Koning A, van Oers L, Wegener Sleeswijk A, Suh S, Udo de Haes H, de Bruijn J, van Duin R, Huijbregts M (2002) Handbook on life cycle assessment: operational guide to the ISO Standards. Series: Eco-efficiency in industry and science. Springer, Dordrecht

Livestock Somatic Cell Nuclear Transfer

Sergio D. German, Keith H. S. Campbell
School of Biosciences, University of Nottingham, Sutton Bonington, Loughborough, Leicestershire, UK

Article Outline

Glossary

Aneuploid An unbalanced number of chromosomes.

Blastocyst A stage of early development where the embryo contains a fluid-filled cavity and two cell populations: an inner cell mass and an outer layer of trophectoderm cells.

Cell cycle The period between the birth of a cell and its division. During a single cell cycle, the cell must duplicate all of its components, including DNA, to form two equal daughter cells.

Chromatin The combination of DNA and proteins, mostly histones.

Cytoplast An enucleated cell used as a recipient for a donor nucleus. Generally in SCNT, the recipient cytoplast is an enucleated oocyte.

Diploid The cell or nucleus contains two complete copies of the genome or a complete complement of chromosomes (2n), in general, a single maternal and a single paternal.

Donor cell The cell that provides the genetic material (nucleus) for SCNT. The resulting animal will be a genomic copy of the animal from which this cell was collected.

Haploid The cell or nucleus contains only a single copy of the genome or half of the chromosome complement (n). Pronuclei are, in general, haploid, containing either a single maternal genome or a single paternal genome.

Karyoplast A cell or a membrane-bound portion of a cell containing the donor nucleus enclosed. In livestock SCNT, the karyoplast is generally an intact cell.

Meiosis The process of reduction in the number of chromosomes that occurs during germ cell formation. Following a round of DNA synthesis, a single cell undergoes two rounds of division resulting in four cells, each containing a haploid genome. During division, independent assortment of parental chromosomes and homologous recombination generate a unique haploid genotype in each of the germ cells.

Metaphase II (MII) Stage during the second meiotic division where the chromosomes are aligned at the metaphase plate prior to segregation of sister chromatids to opposite poles. In most mammalian species, mature oocytes arrest at MII and meiosis is reinitiated and completed upon fertilization.

Parthenote An unfertilized zygote produced by activation of an oocyte. A parthenote may be haploid or diploid for maternal DNA, a gynogenote, or following enucleation and replacement with paternal DNA, an androgenote.

Tetraploid The cell or nucleus contains four haploid copies of the genome (4n).

Zygote The 1-cell stage of development of a fertilized embryo. During most of the first cell cycle, the zygote will contain two pronuclei containing the maternal and paternal DNA.

Definition of Cloning and SCNT

Cloning is the production of genetically identical individuals by the process of asexual reproduction. In animals, the term has been applied to offspring that are produced by the technique of nuclear transfer (NT). The process of nuclear transfer involves the production of an embryo by transferring nuclear genetic material from a donor cell (karyoplast) into a recipient cell from which the genetic material has been removed (cytoplast). Two factors determine the clonality of the resultant offspring. One is the recipient cell, generally an oocyte or unfertilized egg obtained from an unrelated animal.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

The second is the donor cell, which can be obtained from a variety of sources including embryos, germ line, and somatic tissues of fetuses and adult organisms. When cells from somatic tissues are used, the procedure is termed somatic cell nuclear transfer (SCNT). In most species, transfer of the donor nucleus is primarily carried out by cell fusion with fewer reports of direct injection. In the case of fusion, the complete contents of the donor cell are introduced into the recipient cell, while in the case of injection, the donor nucleus might be accompanied with a proportion of the donor cytoplasm.

While SCNT became publicly known with the creation of Dolly the sheep in 1997, NT was already in the realm of science since 1928, with the first successful SCNT experiments carried out in frogs in the late 1950s. By creating a genetic copy of a known animal, SCNT opens many opportunities in several fields including biomedical research and agriculture.

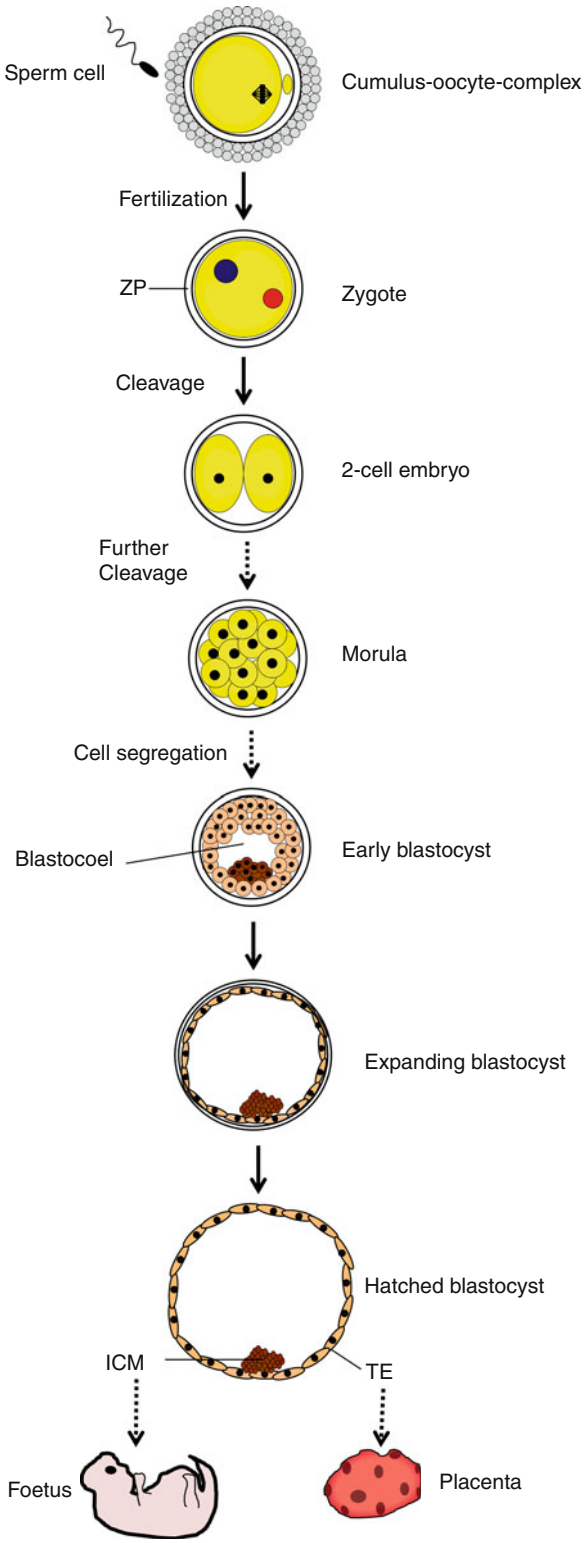
Introduction: Embryo Development and Highlights of NT

In animals, reproduction occurs primarily by sexual means, resulting in the creation of a new individual that contains genetic material (DNA) from two contributing parents of different sexes, each parent contributing half of the genetic material. Conception of a new individual occurs by the fusion of specialized cells or gametes, sperm in males and ova in females. Sperm are haploid (n) and contain a single copy of the genome, meanwhile ova, in most species, at the time of fertilization are arrested in metaphase of the second meiotic division and are diploid (2n). After fusion, however, the ovum completes meiosis to become haploid, and the resultant zygote is diploid (2n), containing a full set of chromosomes derived from both parents. The ovum is a large cell that contains sufficient maternally derived proteins and mRNAs to control early development. Following fertilization, during the early stages of development, no net growth occurs (Fig. 1); transcription from the embryonic genome is low but subsequently increases rapidly at a particular stage termed maternal to zygotic transition (MZT) [1]. The timing of the MZT is species dependent, for example, in mice, it occurs at the 1–2-cell stage; in pigs and humans at the 4–8-cell stage; in cattle and sheep at the 8–16-cell stage; while in the amphibian *Xenopus laevis*, it occurs approximately at the 4,000-cell stage [2]. After this transition, the embryonic genome orchestrates its own development. The embryo continues to increase in cell number within the confines of the zona pellucida (ZP), which acts as a protective shell. By the blastocyst stage, a fluid-filled cavity, called blastocoel, has formed and two distinct cell populations have emerged: the inner cell mass (ICM) and the trophectoderm (TE). As the blastocyst expands, build-up pressure together with the secretion of enzymes causes the ZP to break, enabling the blastocyst to hatch. Now the blastocyst is ready to interact with the endometrium and eventually to implant. By cell proliferation and differentiation, the ICM will form the fetus and some extraembryonic tissues while the TE will give rise to most of the placenta (Fig. 1).

The zygote is a totipotent cell, having the capacity to produce the fetus and the placenta. In contrast, sperm and ova are specialized cells. How do they acquire totipotency? Following fertilization, unknown factors present in the ovum's cytoplasm reprogram the paternal and maternal genomes so that their specialized gamete functions are erased to give way to an embryonic totipotent genome. This capacity to reset a specialized genome into a less specialized or totipotent genome is termed nuclear reprogramming. It is this nuclear reprogramming ability of the ovum that is harnessed in NT technology.

The first rudimentary NT experiments were performed by Hans Spemann in 1928. He used a donor nucleus from a 16-cell embryo and a presumptive zygotic cytoplast, resulting in a normal salamander [3]. Interestingly, Spemann had already predicted the possibility of cloning an adult animal by doing NT with an adult donor nucleus in what he termed the "fantastical experiment" [3].

In 1952, Briggs and King used NT to address the question of nuclear equivalence, that is, whether or not the different cells of a multicellular organism have the same genome. They developed the NT procedure by transplanting nuclei from frog blastula cells into enucleated oocytes [4]. Later on, Briggs and King tested the nuclear equivalence of more developmentally advanced frog cells and found that the developmental potential of reconstructed embryos gradually declined with increased donor cell differentiation [5]. However, John Gurdon pushed the field further by generating



Livestock Somatic Cell Nuclear Transfer. Figure 1 (Continued)

cloned adult frogs from differentiated epithelial somatic cells at the tadpole stage [6, 7], providing strong evidence for nuclear equivalence. Thus, the first successful SCNT had been achieved, but attempts to clone adult frogs failed [8].

Nuclear transfer in mammals lagged behind, with the first attempt in 1975 using morula-stage blastomere donors and unfertilized rabbit eggs. Even though the extent of reprogramming required for a blastomere, compared with a fully differentiated cell, is quite reduced, the reconstructed cloned embryos did not develop beyond the early cleavage stages [9]. In 1983, McGrath and Solter transplanted zygotic nuclei into enucleated zygotes resulting in mice developing to term, showing that the technique of nuclear transfer in mammals was feasible [10]. However, such a zygotic nuclei-replacement experiment did not involve any nuclear reprogramming, and when the same approach used more developmentally advanced blastomere nuclei as donors, it failed [11]. This failure in mice NT was followed by several breakthroughs in which enucleated oocytes were used as recipients of blastomere nuclei, leading to the production of cloned animals in sheep [12], cattle [13], and pigs [14]. In contrast to McGrath and Solter's failure in cloning mice, these results suggested that the enucleated oocyte is a better recipient than the enucleated zygote for reprogramming the donor blastomere nucleus.

Ten years passed until another breakthrough advanced the field of mammalian nuclear transfer: Keith Campbell and colleagues at the Roslin Institute succeeded in cloning a lamb by NT using donor nuclei from an established cell line derived from embryos [15]. In this study, cell cycle coordination between the donor cell and recipient oocyte was carried out

Livestock Somatic Cell Nuclear Transfer. Figure 1
Flow diagram illustrating mammalian development. Following fertilization of the MII oocyte by sperm, a zygote with the two parental pronuclei is formed. By cleavage, the embryo develops sequentially through the 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst stages. By the blastocyst stage, the first differentiation event has formed the inner cell mass (ICM) and the trophectoderm (TE). These two cell populations will form the foetus and placenta, respectively

prior to NT in order to improve the development of reconstructed embryos as previously determined by this group [16, 17]. One year later, this same group, led by Ian Wilmut, used a similar approach and shook the world's news with the creation of "Dolly" the sheep, the first mammal to be cloned from an adult somatic cell [18]. This study showed that an adult somatic cell retains all the information necessary to generate a new organism. After the creation of Dolly, much research has been done in SCNT and several species have been cloned from adult cells. Despite advances in the field, the frequency of development of embryos produced by SCNT remains low, with only 1–10% reaching term, depending on the species. Moreover, many cloned animals are born with abnormalities.

Applications of Livestock SCNT

Soon after the arrival of Dolly came the realization that SCNT had several potential applications. For instance, derivation of embryonic stem (ES) cells from human blastocysts was eagerly sought for regenerative medicine research. The rationale was that cells from a patient could be used for SCNT to derive ES cells, which in turn could be induced to differentiate into a specific tissue. Since such a tissue would be genetically identical to the patient, it could replace a damaged tissue in the same patient without the risk of immune rejection. Due to the ethical concerns of destroying human embryos, SCNT for regenerative medicine has been fiercely opposed. Fortunately, this problem seems mostly circumvented with the revolutionary creation of induced pluripotent stem (iPS) cells [19]. These cells have similar properties to ES cells and can also be derived from the somatic cells of a patient without the use of human oocytes or embryos. Since then, much of the field of regenerative medicine research has shifted from SCNT to iPS cell technology. However, recent studies have found genetic and epigenetic abnormalities in iPS cells [20–22], making them potentially unsafe for medical uses. Whether or not iPS cells are inferior to ES cells is currently the focus of heated debate. Since some of the problems found with iPS cells have not been reported with ES cells derived from SCNT embryos [23, 24], the process of nuclear reprogramming occurring in the oocyte appears to be of better quality than the one taking place during the derivation of iPS cells. Very little is known about the mechanism of nuclear reprogramming and the factors present in the oocyte responsible for triggering or carrying out such process. If these factors are found, they could potentially contribute to generate safer iPS cells for regenerative medicine. Thus, further research in the mechanism of nuclear reprogramming following SCNT is needed.

Apart from being a useful tool for basic research with potential indirect applications in regenerative medicine, SCNT has wide applications in agriculture. For instance, an animal of exceptional productivity can be multiplied by SCNT to increase commercial output or to reduce environmental impact. However, caution must be taken with such approach. First, consumption of products derived from cloned animals is not yet widely accepted by the public, and in some countries, there are regulations forbidding their entry into the human food chain. Second, the SCNT technology is so inefficient at present that its application for livestock multiplication might not be economically justified. Finally, it is important to keep in mind that clones are not necessarily true copies of their donor because of epigenetic differences, which result from faulty nuclear reprogramming following SCNT (discussed later). Slight epigenetic differences can affect the physiology of the cloned animal and thus its productivity. Such consideration should especially warn breeders of race-winner horses, as a cloned horse is unlikely to be a winner again.

A more realistic agricultural application of SCNT is to reproduce animals of high genetic value for breeding purposes. For instance, a prizewinning bull could be cloned and subsequently produce thousands of offspring by artificial insemination. To a lower extent, the genetic value of a cow could also be disseminated by SCNT followed by artificial reproductive technologies. While there might be risks associated with consumption of products from cloned animals due to potential epigenetic errors originated during nuclear reprogramming, their offspring should be safe since epigenetic and imprinting errors are normally erased during gametogenesis.

More importantly, SCNT has represented a great technical advance in the production of transgenic animals (this is discussed in detail in the entry ► Nuclear Transfer to Produce Transgenic Mammals,

this volume). In turn, transgenic animals have a myriad of other applications, which are discussed throughout this volume. Furthermore, transgenic animals producing pharmaceutical or novel proteins are good candidates for multiplication by SCNT since the cost of using this technology would be relatively small compared to the product harvested from the cloned animals.

SCNT can also be used for cloning individuals from an endangered species population. Examples of this application include the cloning of wild cattle (Gaur and Banteng) and wild sheep (European Mouflon) [25]. These examples resorted to interspecies SCNT in which a close-related species is used as cytoplasmic recipients and surrogates for the donor. Therefore, the resultant offspring will be a hybrid containing a genomic copy of the endangered donor and most mitochondrial DNA (mtDNA) from the close-related recipient. While the physiological consequences for this are unclear, it certainly raises ethical issues. Besides, other efforts such as restoration of the natural habitat and assisted breeding programs are likely to be more effective at helping an endangered population.

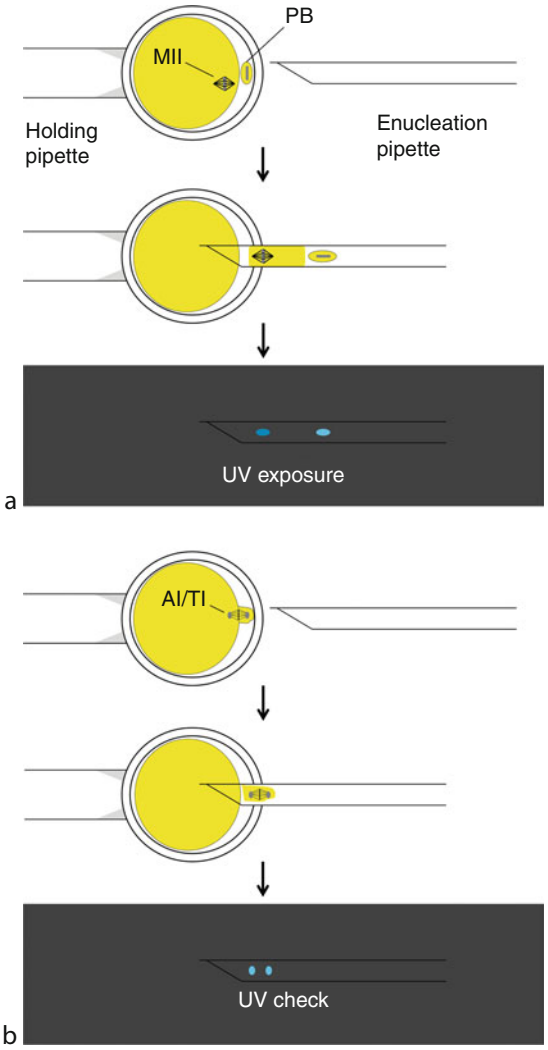
Taken together, SCNT has some interesting applications, but the ethical implications surrounding this technology must be fully considered before rushing into implementing SCNT in the field. Rather than an end, SCNT is especially useful when used as a tool in research, production of transgenic animals, and in cloning of prizewinning animals for breeding purposes in agriculture.

NT Techniques

NT is a complex multistep procedure, with several alternatives at some steps, which include oocyte maturation, cumulus cell removal, enucleation, nuclear transfer, activation, and embryo culture. Culture of the donor cells is important and usually accompanied with cell-cycle synchronization prior to NT. Enucleation and nuclear transfer are the most important and difficult steps. To carry out these steps, most laboratories use micromanipulators. A micromanipulator is a relatively complex instrument composed of a microscope, micromanipulators at both sides to hold the holding and injection micropipettes, micromanipulator joysticks to precisely maneuver the micropipettes in three dimensions, and microinjectors to precisely aspirate and expel fluid and cellular material from the micropipettes. For mice cloning, microinjectors are usually equipped with piezoelectric devices to prevent lysis of the fragile mouse oocyte during enucleation and nuclear transfer. An alternative to micromanipulators is a technique called handmade cloning (HMC) and seems equally efficient as micromanipulator-based NT (for a review of HMC, see [26]). Following is a brief discussion of the different steps involved in the entire NT procedure with focus on livestock species unless stated otherwise.

Oocyte maturation is usually required in livestock cloning as immature oocytes are commonly obtained from ovaries collected from slaughterhouses. Alternatively, in vivo–matured oocytes can be obtained by stimulating superovulation, which is followed by oviduct flushing after slaughtering the animal. In vitro maturation (IVM) entails mimicking to some extent the in vivo environmental conditions found in the ovary at the time of the LH (luteinizing hormone) surge. Thus, three key hormones, LH, FSH (follicular stimulating hormone), and estradiol, are added to the IVM medium to stimulate maturation. Oocytes are surrounded by cumulus cells, forming a cumulus-oocyte complex (COC); cumulus cells aid oocyte maturation. COCs are incubated at about 39°C in livestock species and for 24 h (e.g., cow and sheep). Following the required maturation period, oocytes are denuded from the cumulus cells using hyaluronidase to digest the extracellular matrix that holds cumulus cells together. Removal of cumulus cells is necessary to allow visualization of the oocyte's chromatin during enucleation and to prevent inserting a cumulus cell accidentally inside the oocyte during enucleation and cell/nuclear transfer procedures. Now the denuded oocytes are ready for enucleation.

A few enucleation methods exist. The most commonly employed method uses micromanipulators to aspirate the chromatin. In this method, the oocyte is held steady at one side with the holding pipette while the enucleation pipette is inserted from the opposite side to remove, depending on the maturation state, the extruding spindle or first polar body together with the metaphase plate (Fig. 2). In order to confirm enucleation, it is desirable to visualize the chromosomal material. In mice and human oocytes, which have a clear cytoplasm, chromatin can be readily observed under bright



Livestock Somatic Cell Nuclear Transfer. Figure 2
Diagram illustrating enucleation of livestock oocytes. (**a**) When enucleation is carried out at metaphase II (*M*II), the polar body (*PB*) and a portion of cytoplasm is blindly aspirated to remove the underlying MII plate. Chromatin cannot be observed under bright field illumination, thus UV light exposure (*dark field*) is essential to confirm enucleation of the pre-stained chromosomes. (**b**) When enucleation is carried out earlier at anaphase I/telophase I (AI/TI), the extruding spindle is aspirated. In contrast to MII enucleation, the AI/TI spindle can often be observed directly inside the enucleation pipette. However, enucleation of pre-stained DNA can be checked with UV light. In both enucleation methods, the oocyte is removed from the field of view to avoid damage by direct UV irradiation when exposing the enucleation pipette

field illumination. However, the oocytes of livestock species have high lipid contents that hamper chromatin visualization, and therefore, enucleations must be made "blind" (Fig. 2a). Generally, to aid enucleation of livestock oocytes, they are briefly preincubated with the vital DNA stain Hoechst. The stained karyoplast is confirmed in the enucleation pipette by UV light following enucleation. Although this procedure does not expose the oocyte to direct UV irradiation, conflicting evidence exist whether Hoechst diminishes the developmental competence of the oocyte.

Alternatively, a blind-enucleation method without DNA staining uses the first polar body as a point of reference to subsequently remove the underlying metaphase II (MII) plate. Unfortunately, the MII plate can shift away from the polar body, rendering its removal inaccurate. However, when blind enucleation is performed earlier at anaphase I/telophase I (AI/TI) stages of the cell cycle, removal of the complete spindle is close to 100% accurate (Fig. 2b) [27]. A newer microscopy technology based on birefringence, the PolScope, allows noninvasive visualization of spindles not only in human oocytes but in other species as well. Probably due to the high cost of the PolScope, this enucleation method has not gained much popularity.

Direct enucleation by aspiration can be chemically assisted with cytoskeleton-relaxing agents, resulting in a protrusion cone containing the oocyte's chromosomes. Drugs used for this purpose include demecolcine and nocodazole, both interfering with microtubule polymerization. Other enucleation methods that have been attempted are centrifugation and laser ablation. In HMC, the zona pellucida is digested, and one third of the oocyte containing the extruding polar body and metaphase plate is manually bisected with a microblade under a stereomicroscope.

Donor cells are usually cultured in vitro and synchronized in a specific phase of the cell cycle prior to NT in order to ensure a known ploidy content. In livestock species, donor cells are preferentially used in G_0 and, to a lower extent, in G_1. Quiescent G_0 cells are arrested in the G_1 phase of cell cycle by either serum starvation or cell-contact inhibition. The advantage of these two methods is that they are drug independent, while methods to arrest cells at G_1 usually require the use of pharmacological agents. Alternatively, actively dividing presumptive G_1 cells can be selected by

collecting small-sized cells. A more accurate drug-free method to select for G_1 cells from actively dividing cultures involves using the eukaryotic "baby machine", which briefly consists of an effluent that collects newly divided and floating daughter G_1 cells from the culture medium (this methodology is described in [28]). Such a methodology would be especially useful when it is desirable to clone ES cells in G_1 (discussed further in the section Coordination of Donor and Recipient Cell Cycles). In mice cloning, ES cells are often arrested in metaphase by nocodazole or colcemid treatments.

A few alternatives exist for inserting the donor nuclear material into the enucleated oocyte (Fig. 3). In livestock species, the most common method is to insert the whole donor cell (cell transfer) into the perivitelline space to achieve contact between the membranes of donor and recipient cells. The donor DNA is then incorporated into the enucleated oocyte by cell fusion (Fig. 3a) (discussed below). Another method is to directly insert the nucleus (nuclear microinjection) into the enucleated oocyte, a common practice in mice cloning (Fig. 3b). The advantage of this method is that it bypasses the need for fusion. Nuclei are isolated using a micropipette with an inner diameter smaller than the donor cell. Pipetting the cell in and out breaks the membrane and releases the nucleus. Interestingly, it is also possible to microinject the whole cell into the enucleated oocyte cytoplasm, thus bypassing the need for nuclear isolation or fusion (Fig. 3c). Although it is unclear how the donor plasma membrane is degraded, high blastocyst development has been reported with this technique [29]. Another alternative method is to directly microinject a broken cell containing mitotic chromatin and cytoplasm, known as chromosome transfer (Fig. 3d). In HMC, phytohemagglutinin is used to glue the donor cell to the enucleated oocyte, followed by fusion.

When cell transfer is employed, the recipient-donor couplet must be fused to deliver the donor chromatin into the recipient oocyte. This is commonly achieved by electrofusion. In this method, the couplet is placed between two electrodes and short electric pulses are applied to fuse the recipient-donor membranes together. A second method involves the use of inactivated sendai virus (SV). Donor cells are briefly incubated in a solution containing SV prior to cell transfer, achieving oocyte-cell fusion by viral particles. SV-mediated fusion is less laborious and might be less stressful to the reconstructed embryo than electrofusion [30].

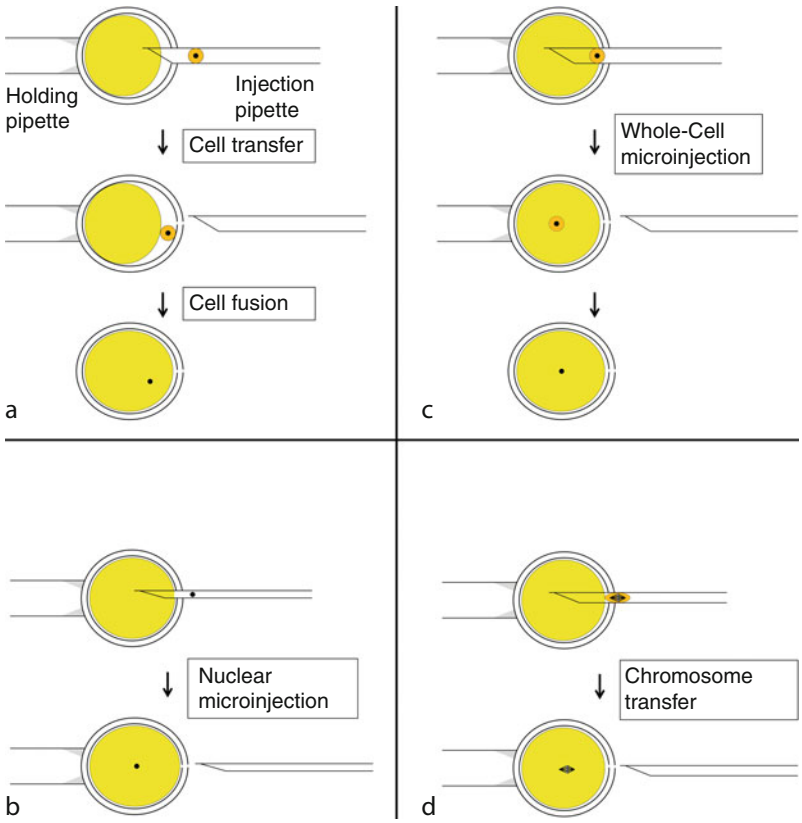
Once the donor chromatin has been incorporated into the enucleated oocyte, the reconstructed embryo is activated to begin development. The reconstructed embryo is then ready for embryo culture. Different methods of activation and embryo culture are discussed later in this entry in relation to how they affect NT outcomes. When cloned embryos reach the blastocyst stage in vitro, they are transferred to surrogate females to carry the pregnancy.

Hormonal treatments must be administered to the surrogate so that the "uterine cycle" is synchronized with the developmental stage of the transferred embryo. If this is not properly planned, the uterus will be "out of phase" compared to the blastocyst and implantation will likely fail. To achieve synchrony, in farm animals, the surrogate must be induced to enter estrus on the day of NT. Following transfer to surrogates, care of the embryos is shifted to the hands of the veterinarian. Management of pregnancy includes ultrasonography, to check pregnancy status, as well as measuring maternal levels of pregnancy proteins to assess placental development.

Problems of Cloning

Despite advances in the field, the efficiency of SCNT technology remains very low, with only 1–10% of transferred embryos reaching term, depending on the species. More than 50% of pregnancies are lost in the first trimester, and in contrast to IVF pregnancies, losses of clone pregnancies continue throughout gestation (e.g., [31]). Additionally, developmental abnormalities are commonly observed in cloned fetuses and in their placentas.

During mammalian embryogenesis, the fetus is derived exclusively from the inner cell mass (ICM) while extraembryonic tissues, including the placenta, are mostly derived from the trophectoderm (TE). It is thought that the extraembryonic membranes are the most negatively affected by SCNT, including the placentas of full-term clones. Although cloned fetuses and offspring can present abnormalities as well as large offspring syndrome (LOS), these seem to be secondary to placental dysfunction, which leads

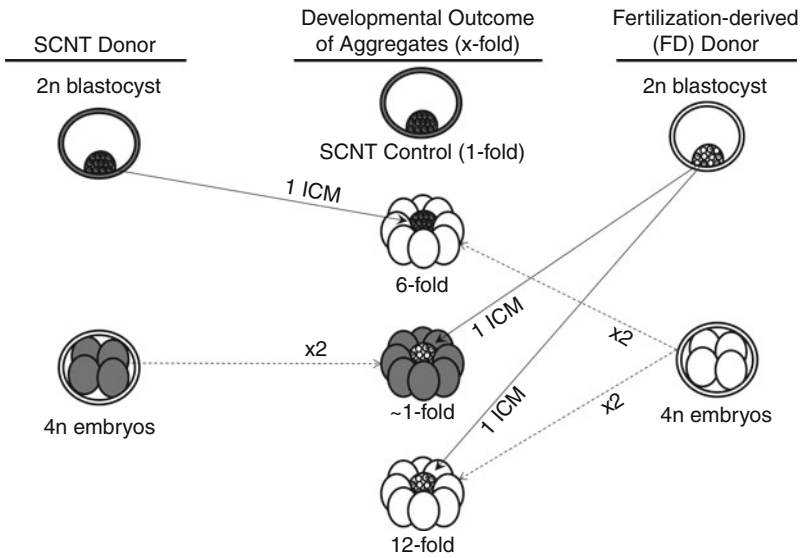


Livestock Somatic Cell Nuclear Transfer. Figure 3

Diagram illustrating different methods for donor genome transfer into recipient enucleated oocytes. (**a**) In cell transfer, an intact donor cell is transferred into the perivitelline space and the genome is then incorporated into the recipient by cell fusion. (**b**) In nuclear microinjection, nuclei are previously isolated and a single nucleus is then microinjected into the recipient. (**c**) In whole-cell microinjection, the whole cell is injected into the recipient. Eventually, incorporation of the genome into the recipient occurs by spontaneous degradation of the donor plasma membrane. (**d**) In chromosome transfer, a broken mitotic cell is microinjected into the recipient to transfer the mitotic spindle

to imbalances in placental fluid and in kidney function [32]. In sheep, placental abnormalities include a hypotrophic trophoblastic epithelium, reduced vascularization, and thickening of the trophoblast basement membrane [33]. Placentomegaly (enlarged placenta) is a common occurrence in mice and cattle cloning. Other placental abnormalities in bovine cloning include edema and hydroallantois. Developmental failure can account for fetal losses, stillbirth, and even postnatal mortality of mice, cattle, and sheep. Postnatal mortality is particularly pronounced in cloned sheep [34].

A recent study showed that indeed defects in the trophectoderm lineages rather than in the ICM are the main cause of low cloning efficiencies, at least in mice [35]. This experiment used chimeric aggregates of diploid and tetraploid embryos (Fig. 4). Tetraploid embryos are obtained by fusing the two blastomeres of a two-cell embryo. NT-ICMs were aggregated with tetraploid fertilization-derived (4nFD) early-cleavage-stage embryos. In this diploid/tetraploid "chimera," the 2n NT-ICM cells can only develop into the fetus while the 4n cells can only give rise to the extraembryonic tissues (although minimal cross-contribution was observed). By this approach, a sixfold increase in development to term was obtained compared with the nonaggregated NT control. Moreover, when ICMs from normally fertilized embryos were



Livestock Somatic Cell Nuclear Transfer. Figure 4

Experimental evidence shows that the trophectoderm lineage is severely affected in mice cloning. A diploid (2n) ICM and two tetraploid (4n) 4-cell embryos were aggregated from cloned and fertilized embryos in different combinations. The ICM forms the embryo while tetraploid blastomeres can only give rise to extraembryonic tissues. Development to term demonstrated that replacing the trophectoderm lineage in cloned embryos dramatically improves development

aggregated with tetraploid NT embryos, the birth rate was similar to that of the NT control, indicating that a normal ICM does not rescue the developmental potential of the reconstructed embryo. However, FD-ICM/4nFD aggregates showed twice the developmental rate than NT-ICM/4nFD. Thus, while trophectoderm defects are mainly responsible for low cloning efficiencies in mice, the ICM has a minor contribution as well.

It is generally thought that the low cloning efficiencies and developmental problems arise from an incomplete reprogramming of the donor nucleus.

Nuclear Reprogramming

Successful NT experiments demonstrated that differentiated cells (with a few exceptions) retain the same intact genome (i.e., nuclear equivalence) within an organism. In other words, these experiments supported that cell differentiation is not achieved through genetic changes or deletions but rather through epigenetic changes. Epigenetics regulates cell identity through chromatin modifications that are heritable through cell divisions, without altering the DNA sequence. Thus, chromatin modifications establish an epigenetic "code" that results in different gene expression programs between cell types.

Chromatin is formed by DNA associated with histone and nonhistone proteins. The chromatin template is epigenetically modified at the DNA and histone levels. DNA modification is limited to methylation (or demethylation), while histone modifications are numerous, including methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, biotinylation, and sumoylation. In turn, such modifications affect gene expression and the overall chromatin structure, which can be described as open or compact. During normal development, global changes in DNA methylation and histone modifications take place; much effort is being dedicated in understanding how these changes are altered or preserved in cloned embryos.

For SCNT to be successful, it is essential that the chromatin of the donor somatic nucleus is remodeled so that it becomes compatible not only with the totipotent gene expression program of development but

also with the rapid cell-cycle dynamics of early embryo cleavage.

Nuclear reprogramming, either following fertilization or SCNT, is the result of active and passive processes. For instance, in most mammals after fertilization, the sperm compact chromatin undergoes active remodeling in which protamines are exchanged by ooplasmic histones followed by global loss of DNA methylation. The sperm pronucleus also acquires distinct histone marks such as histone hyperacetylation. However, it is not clear whether such modification is acquired actively through the action of enzymes or it is the result of passive acquisition from the ooplasmic histone pool already rich in acetylation marks [36].

The timing required for completing nuclear reprogramming is unclear. Some researchers have suggested that the donor nucleus should be reprogrammed upon reaching embryonic genome activation. Such a restrictive reprogramming timing would be consistent with the lower cloning efficiencies usually observed in mice compared to bovine, two of the most cloned animals, the latter having more time for reprogramming the differentiated donor nucleus before genome activation. However, it has also been suggested that reprogramming continues up to the blastocyst stage.

To better understand the nuclear reprogramming process, the dynamics of the most important epigenetic marks and of gene expression during early embryogenesis in fertilized and cloned embryos are discussed next.

Reprogramming of DNA Methylation

DNA methylation is a reversible modification of the chromatin template at the DNA level in which a methyl group is predominantly added to cytosine of the dinucleotide sequence CpG. DNA methylation is a widely used mechanism of epigenetic regulation commonly associated with stable heritable gene silencing, heterochromatin, chromosome stability, genomic imprinting, X-chromosome inactivation, and inactivation of retroviral sequences. It has been shown that DNA methylation is essential for embryonic development, especially at postimplantation stages [37]. DNA methylation is carried out by DNA methyltransferases (DNMTs). DNMT1 maintains DNA methylation patterns after DNA replication by using the old strand as a template to methylate the new strand. De novo DNA methylation is carried out by DNMT3a and DNMT3b.

DNA methylation is dynamically regulated. Removal of methyl groups can be an active process when specific DNA demethylases are involved to remove the methyl groups, or it can be a passive process when failure to copy the methylation marks on the newly synthesized DNA leads to erasure of those marks during cell divisions. In contrast to DNA demethylation of the sperm pronucleus, which is thought to be an active process [38], passive DNA demethylation of the embryonic genome takes place following the zygotic stage.

After SCNT, DNA methylation reprogramming is usually inefficient. Mice and bovine cloned embryos have aberrantly hypermethylated DNA [39]. In sheep, SCNT embryos also tend to be significantly hypermethylated compared to fertilized embryos [40]. Such abnormal hypermethylation was observed from the 1-cell to the 8-cell stage and then at the blastocyst stage, in which the trophectoderm was hypermethylated relative to the fertilized control.

Interestingly, the rate of demethylation is similar in both SCNT and fertilized embryos, suggesting that the hypermethylated DNA pattern of SCNT embryos is probably inherited from the hypermethylated donor cell [38]. Alternatively, it has been suggested that cloned embryos undergo precocious DNA methylation due to the presence of DNMT1 associated with the donor nucleus, thus preventing passive demethylation [39].

DNA methylation reprogramming appears to be an indicator of developmental potential. In sheep, the proportion of SCNT embryos that were normally methylated at the 2-cell stage coincided with the proportion of surviving embryos reaching the 16-cell stage [38]. Similarly in mice, aberrant methylation of early-cleavage cloned embryos was proportionally associated to developmental failure to the blastocyst stage [41].

Reprogramming of Histone Modifications

The tails of the histone subunits H3 and H4 are targets for chromatin modifier enzymes, with histone methylation and acetylation modifications the most extensively studied. These modifications set epigenetic "marks" associated with either gene silencing or gene activity.

Histone acetylation is associated with open chromatin and gene activity. This modification neutralizes positive charges on histone tails and thus increases the overall repulsive negative charge of DNA on the chromatin structure. In turn, an open chromatin favors the binding of transcription factors to DNA.

In contrast, histone methylation can be either repressive or activating, depending in the lysine residue modified. These modifications act as signals that are "read" by proteins, which in turn can trigger downstream chromatin-modulating events. Together with methylated DNA, methylation of lysine 9 of histone 3 (H3K9), H3K27, and H4K20 has been implicated with heterochromatin and gene repression, while methylation of H3K4, H3K36, and H3K79 is associated with active gene expression.

In addition to abnormal DNA methylation, embryos produced by SCNT show aberrant reprogramming of histone modifications. Asymmetry of H3K9me3 fails to be established between ICM and TE in bovine cloned blastocysts compared with fertilized controls [42]. When histone acetylation marks are compared, however, cloned and normal blastocysts look similar [43, 44], suggesting that these marks are well reprogrammed. Yet, at earlier embryonic stages, the same histone acetylation marks show significant differences, with cloned embryos being hyperacetylated [42, 43].

Deregulation of DNA methylation and H3K9 methylation might be major problems during nuclear reprogramming [38, 42]. Their aberrant reprogramming leads to hypermethylated trophectoderm and extraembryonic tissues. As these epigenetic marks promote inactive chromatin, they are likely to cause downregulation of a substantial number of placental genes, explaining the placental abnormalities frequently reported in cloned embryos.

Reprogramming of Gene Expression

After fertilization or SCNT, the ooplasm induces global remodeling of chromatin, genome silencing, erasure of permissive epigenetic marks of differentiation-associated genes, and resetting the genome to an embryonic state [45–47]. Four classes of genes play an essential role to ensure normal embryogenesis following NT: (1) pluripotency-associated genes, (2) trophectoderm genes, (3) imprinted genes, and (4) differentiation-associated genes. Reactivation of pluripotent and trophectoderm genes must take place, whereas differentiation-associated genes must be silenced. Imprinted genes should be reactivated without altering the normal imprinting of somatic cells.

Pluripotent Genes Pluripotency is the capacity to differentiate into all embryonic cells. The cells of the ICM are pluripotent because they form the embryo. To recognize the state of pluripotency, landmarks of the ICM have been sought, such as stage-specific gene expression or antigens. These landmarks should be associated only with those stages and tissues known to have pluripotent potential such as the cleavage-stage embryo and ICM of the blastocyst. They should not be expressed in differentiated tissues of later development. Key genes associated with the ICM vary with species.

In mice, Oct4 is highly expressed and restricted to the ICM. It plays an essential role in early development as Oct4-depleted mouse embryos fail to form the ICM and are developmentally incompetent [48, 49]. Inducing overexpression of Oct4 is key to revert the gene expression profile of an adult differentiated somatic cell to one of embryonic and pluripotent characteristics [19]. Thus, in the mouse, Oct4 appears to be an ICM-specific marker that can be used to assess pluripotency.

Also in mice, other important pluripotent factors restricted to the ICM are Nanog and Fgf4 [50, 51]. Nanog is involved in maintenance of the pluripotent state of ES cells, for instance, by repressing differentiation into primitive endoderm [50]. Fgf4, a target gene of Oct4 [52], is necessary for proliferation and differentiation of both the ICM into embryonic tissues [53] and the TE into placental tissues [54].

In mouse SCNT blastocysts, Oct4 and Oct4-related genes often fail to be reactivated [55, 56]. Frequently, when Oct4 is expressed, aberrant spatial expression in the trophectoderm indicates faulty reprogramming [55]. Interestingly, when ES cells are used as donors, instead of adult somatic cells, Oct4 and related genes were always expressed in cloned blastocysts [56]. Since pluripotent genes are already active in ES cells, these genes do not need to be reactivated in ES-cloned embryos.

In human and large ungulate mammals, however, it is less clear what master regulatory factors are involved in the segregation of the ICM. For instance, Oct4 is expressed in both ICM and TE of bovine, goat, and human blastocysts [57–60]. Thus, for nonmurine species, we are left with less information to assess the extent of ICM-lineage reprogramming. Yet, Nanog does seem to be restricted to the ICM in bovine [61] and goats [59]. Similarly, Fgf4 is restricted to the ICM of bovine blastocysts [57]. Therefore, Nanog and Fgf4 are preferred ICM markers to Oct4 depending on the species.

To assess reprogramming of ICM, expression of Nanog and Fgf4 has been measured in bovine cloned blastocysts. Differences between studies were found, with similar [62, 63] and lower levels [57, 64] compared with fertilized controls. The disparity of results may reflect differences in nuclear transfer procedures leading to variable success in reprogramming.

Trophectoderm Genes In mice, the transition from morula to blastocyst that results in the segregation of ICM and TE is regulated by the antagonistic activity of Oct4 and Cdx2, respectively [65]. Together with Cdx2, Taed4, Eomes, and Gata3 are required for specification and development of the TE lineage [66, 67]. Tead4 appears to be a master factor in TE-lineage induction since it activates Cdx2 and Gata3 [67, 68].

Less is known about the mechanism of segregation of the trophectoderm lineage in other mammals. Although CDX2 protein seems to be a conserved TE-restricted marker, as observed in bovine and porcine blastocysts [61], transcripts levels of Cdx2 were also found in the ICM [57]. It is possible that a posttranscriptional regulation mechanism accounts for the difference between gene expression and gene product levels. Thus, to assess reprogramming of Cdx2, it might be more appropriate to look at the protein level. In contrast to mice, Cdx2 does not repress Oct4 expression in bovine [69], explaining why OCT4 is detected in the bovine TE [58]. Another difference between these two species is the expression of Tead4 and Gata3 transcripts, which are found in both ICM and TE of bovine blastocysts, and thus, their role in TE lineage segregation is not clear [57]. Further evidence that bovine TE formation is quite different to mice is the observation that Eomes is not detected in trophoblast tissue [70]. Surprisingly, although bovine TE cells will only contribute to the trophectoderm lineage, they are not committed to it before pregastrulation stages since they were observed to contribute to ICM in aggregation assays [69]. Taken together, these observations suggest that differences in expression of lineage markers between species might be the result of different implantation requirements. For instance, while mouse blastocysts implant readily at this stage, domestic blastocysts undergo elongation and have delayed implantations. Indeed, while OCT4 is expressed in both the ICM and TE of the bovine blastocyst, it is restricted to the ICM lineage at a later elongated stage [71].

Few studies have assessed reprogramming of the TE lineage in cloned embryos. In mice, CDX2 protein was frequently expressed normally at the blastocyst stage [72]. A similar result was found in the bovine [57]. Although reprogramming of Taed4 and Gata3 was faulty in bovine cloned blastocysts [57], these are not specific markers of the TE lineage in the bovine. Therefore, in nonmurine species, assessing reprogramming outcome of the TE lineage is currently limited to measuring CDX2 protein.

Imprinted Genes Imprinted genes are genes expressed either from the paternal or maternal chromosomes, but not both. Gene imprinting plays an essential role in development. When androgenetic (two paternal pronuclei) or gynogenetic (two maternal pronuclei) embryos are produced, embryogenesis is arrested shortly after implantation [73]. Thus, the paternal and maternal genomes have different contributions and must complement each other at fertilization for normal development to occur. Imprinting marks originate during gametogenesis and are generally protected from genome-wide reprogramming, such as DNA demethylation/methylation, during embryonic development [36].

Many imprinted genes regulate development, growth, and function of embryonic and extraembryonic tissues. Following SCNT, many imprinting errors can occur during reprogramming of the somatic chromatin. For an imprinted gene, simultaneous expression from the paternal and maternal alleles or lack of expression is the result of aberrant reprogramming. Because

of their regulatory growth function, deregulation of imprinted genes might explain several placental abnormalities and "large offspring syndrome" often observed in cloned animals.

Some researchers have proposed that long-term culture of donor cells, the usual scenario for embryonic stem (ES) cells, is an additional source for imprinting errors, and thus, it has been suggested that ES cells are a poor choice for NT [74]. However, using microarray technologies, Humpherys et al. [75] found no significant difference between ES-NT and noncultured cumulus cell-NT clones in terms of gene expression abnormalities including imprinted genes. Therefore, it is not clear whether extensive in vitro culture is a major problem for NT in terms of imprinting deregulation. Moreover, mammals seem rather tolerant to imprinting errors [76].

Differentiation-Associated Genes Following SCNT, silencing of differentiation-associated genes is often incomplete. For instance, cloned embryos produced with myoblast nuclei still expressed the myoblast marker GLUT4 [77]. Similar somatic epigenetic memory was also observed in Xenopus cloned embryos, which overexpressed endoderm or ectoderm markers according to the origin of the donor cells [78]. While insufficient silencing of differentiation-associated genes may alter metabolic demands, the functional consequences of retaining expression of somatic genes on the developmental potential of cloned embryos are unclear [46].

Several studies have compared global gene expression profiles between cloned and fertilized embryos. At the 1-cell stage of murine embryos, most transcripts (~98%) were similarly abundant between clones and IVF groups [45], although such similarity might be the result of maternal transcripts abundantly present in the oocyte. Nonetheless, similar expression profiles were obtained between cloned and fertilized blastocysts in bovine (e.g., [63, 79]). Thus, overall gene expression of cloned embryos appears normal. However, it is possible that small alterations in gene expression are amplified during postimplantation stages, which would contribute to the low developmental potential of cloned embryos [79].

In summary, perfect gene expression reprogramming may be unlikely following SCNT. Cell-type-specific genes may be incompletely silenced while imprinted genes are subject to deregulation. Reactivation of ICM- or TE-lineage genes might be problematic depending on the species. The mechanism of nuclear reprogramming is still a black box, and much further research is needed to understand where the bottleneck of NT is.

Factors Affecting Development of Embryos Produced by SCNT

Frequently, reports have shown that there is ample room for optimization in NT technology. Major investigated factors affecting NT success include quality of recipient oocyte, time of enucleation, nuclear donor source, cell-cycle coordination of donor and recipient cells, alterations in the timing and inducer of oocyte activation, and culture of reconstructed embryos.

Sources of Recipient Oocytes

Mammal females produce mature oocytes (oogenesis) during the ovarian cycle. The earliest stage of oogenesis is the primary oocyte within the primordial follicle. Primary oocytes are arrested at the first meiotic prophase in a quiescent state. Some of these are regularly recruited for further growth since puberty and thereafter. As primary oocytes resume growth, they increase in size and undergo cytoplasmic maturation. An oocyte must undergo cytoplasmic and nuclear maturation to be developmentally competent. Cytoplasmic maturation involves mRNA synthesis, translation into protein, and posttranslational modifications. Many mRNA and protein molecules are stored in the cytoplasm to function later during early embryo cleavage, before embryonic-genome activation. Some of the proteins produced, such as maturation promoting factor (MPF), are necessary for meiotic progression during nuclear maturation. Within the follicle, oocyte growth is accompanied with follicular growth. Indeed, oocytes obtained from medium- and large-sized follicles are developmentally better than those obtained from small-sized follicles. It seems that oocytes from the latter group have not completed cytoplasmic maturation [80]. Oocytes are surrounded by cumulus cells, and mutual communication is required for complete oocyte maturation.

Oocytes can be matured in vivo or in vitro. In vivo maturation involves collecting oocytes that have

naturally matured within the ovary of a live animal. In contrast, in vitro maturation involves aspirating immature oocytes from the ovaries of slaughtered animals and placing them on favorable culture conditions to complete the maturation process in the incubator. Although much understanding has been gained in oocyte maturation, in vitro maturation is still suboptimal at best. In vitro–matured oocytes have altered energy metabolism [80], higher chromosomal abnormalities [81], and lower developmental competence than in vivo–matured oocytes [82, 83].

For convenience, in livestock cloning, in vitro maturation is widely used as very large numbers of immature oocytes can be obtained from slaughterhouses at low cost. However, the poorer quality of these oocytes is likely to contribute to the low frequency of development usually observed in cloned embryos.

Enucleation of Recipient Oocytes

Nuclear maturation is resumed at the time of the LH surge in vivo or during in vitro conditions. The oocyte continues meiosis to progress from the arrested prophase I to metaphase I, anaphase I, telophase I, and cytokinesis with unequal cytoplasmic distribution, when half of the chromosomes are discarded in the first polar body. The oocyte progresses to meiosis II and is arrested at metaphase II. Following fertilization or artificial activation, the metaphase II–arrested oocyte resumes meiotic progression once more to complete metaphase II, anaphase II, and telophase II, discarding again half the number of chromosomes in a second polar body, achieving a haploid number of chromosomes. Enucleation can be carried out at any of these meiotic stages, although with possible different consequences (discussed later). Table 1 provides some

Livestock Somatic Cell Nuclear Transfer. Table 1 Examples of cloning experiments using recipients at different meiotic and mitotic stages of the cell cycle

Recipient cytoplast	Donor cell type	Species	Offspring	References
Enucleated GV oocytes (subsequently matured)	ES cells	Mouse	No	[84]
Enucleated Pro-MI oocyte (subsequently matured)	ES cells	Mouse	Nd	[84]
Enucleated AI/TI oocyte (subsequently matured)	Fetal	Sheep	Yes	[85]
	Adult	Sheep	Yes	
Enucleated MII oocyte	Embryonic, fetal, adult	Sheep	Yes	[18]
Unenucleated MII oocyte (subsequent enucleation)	Cumulus cells	Mouse	Yes	[86]
Enucleated activated MII oocyte	Blastomeres	Sheep	Yes	[17]
Enucleated PN Zygote	PN karyoplast from zygote	Mouse	Yes	[10]
	PN Karyoplast from first NT embryo	Pig	Yes	[87]
	Blastomeres	Mouse	No	[11]
	Cumulus cells	Mouse	No	[88]
TII enucleated oocyte	Blastomeres	Goat	Yes	[89]
M-phase arrested zygote	ES cells	Mouse	Yes	[90]
	Fibroblast	Mouse	ND	
2-cell embryo	Lymphocyte	Mouse	Yes	[91]

ND not determined

examples of cloning experiments carried out using recipients enucleated at different stages of meiosis or mitosis.

For NT, it is most important that the oocyte undergoes cytoplasmic maturation to construct a developmentally competent embryo, while nuclear maturation is less important because the oocyte DNA is eliminated at enucleation and does not form part of the embryo. Enucleation of prophase I oocytes, also known as germinal vesicle (GV) stage, has been attempted in the mouse, and the resultant recipients were capable of reprogramming donor nuclei [84, 92]. However, the reconstructed embryos are developmentally incompetent even when recipients are further matured in vitro prior to NT [84]. It is possible that GV oocytes are poor recipients because removal of their transcriptionally active chromatin [93] prevents synthesis of new mRNA necessary for completing cytoplasmic maturation. Similarly, enucleation of pro-metaphase I (pro-MI) oocytes also results in poor recipients [84]. Beyond interfering with mRNA synthesis, it is also likely that enucleated GV and pro-MI oocytes are poor recipients because enucleation disaggregates the cumulus-oocyte complex, thus depriving the oocyte of the beneficial communication with cumulus cells for completing cytoplasmic maturation during in vitro maturation.

Enucleation of metaphase II–arrested oocytes is the most common practice in cloning experiments. The rationale of using such recipients is evident as the COC is left undisturbed during the entire in vitro maturation period, thus maximizing the probability of cytoplasmic maturation. However, a practical alternative is to enucleate at anaphase I/telophase I (AI/TI). By doing so, the spindle is more efficiently removed along with less cytoplasm compared to MII enucleation [27]. AI/TI cytoplasts are further cultured to complete the maturation period and have produced lambs after SCNT [85].

Other recipients have been used for NT. Activation of MII oocytes with subsequent enucleation at anaphase II/telophase II has been done for practical reasons similar to those of AI/TI enucleation [89, 94]. Zygotes and two-cell embryos have also been enucleated and used as recipients for NT. These will be better discussed below in relation to the "potential loss of reprogramming factors" during enucleation and later in relation to the "coordination of donor and recipient cell cycles."

Potential Loss of Ooplasmic Reprogramming Factors

Enucleation involves removing the DNA material from the unfertilized or fertilized oocyte plus an unavoidable volume of ooplasm. It has been speculated that loss of ooplasmic volume is accompanied with a reduction of developmental potential, possibly due to the removal of cytoplasmic reprogramming factors. However, fusion of two or more cytoplasts prior to NT did not improve the frequency of development in mice [95]. Apparently, the amount of cytoplasmic factors in the oocyte is not critical for cloning success.

However, the above discussion does not take into account that the content of reprogramming factors in the cytoplast is dependent on the cell-cycle phase. The genome can be removed as interphase pronuclei in zygotes or with the spindle if recipients are mitotic zygotes or meiotic oocytes. In the latter case, the components of the nucleoplasm stay in the recipient as they are diluted in the cytoplasm following nuclear envelope breakdown. Recipient zygotes enucleated at interphase can only support development of nuclei obtained from 1-cell or 2-cell embryos [11] but fail consistently when more differentiated donor nuclei are transferred [11, 96, 97]. Kevin Eggan and colleagues have shown that zygotes and 2-cell embryos at the mitotic phase regain the reprogramming ability [90, 91]. These authors have proposed that reprogramming factors are sequestered in the nucleus during interphase but then redistributed throughout the cytoplasm during M phase [98]. Therefore, reprogramming factors would be lost when enucleation is carried out during interphase [99]. Indeed, Brg1, a component of the Swi/SNF chromatin remodeling complex necessary for embryonic genome activation, was absent in 2-cell cloned embryos when using enucleated interphase zygotes as recipients [99]. Further proof of this idea is supported by the observation that oocyte recipients at the germinal vesicle (GV) stage fail to develop after NT [84]. A possible contributing factor for the low developmental potential of enucleated GV oocytes and interphase zygotes is the low levels of maturation promoting factor (MPF) in these recipients (discussed below). Taken together, while the mechanism of

reprogramming remains largely unknown, essential reprogramming factors are present in the recipient's cytoplasm during M phase, but absent during interphase, at least in the mouse model [99].

It is still possible that some unknown factors beneficial for reprogramming might be stably associated with chromatin and lost during enucleation. It is well known that spindle factors are associated with the meiotic chromosomes, and therefore, enucleation could impair spindle function. Indeed, in human SCNT, it is apparently necessary to leave the recipient genome to achieve development to the blastocyst stage, at the cost of producing a triploid embryo [100]. How spindle function is affected by SCNT in livestock species will be discussed later in this entry.

Coordination of Donor and Recipient Cell Cycles

During a single cell cycle, a cell must double all of its components, segregate its genetic material equally to the two daughter cells, and undergo cell division. One exception occurs during the first few cell cycles of early embryo development where no net growth occurs. However, cell-cycle events associated with duplication and segregation of the nuclear DNA still occur. Another exception to the mitotic cell cycle is meiosis, which results in halving the chromosome number. Only germ cells undergo meiosis, while somatic cells only mitosis.

The mitotic cell cycle has four sequential phases: G_1, S, G_2, and M. During S phase (S for DNA synthesis), replication of DNA takes place. During M phase (M for *mitosis*), the cell divides all its components equally into two daughter cells. Cell growth occurs only during interphase, which constitutes G_1, S, and G_2. In a typical in vitro–cultured mammalian cell, interphase lasts about 23 hours (of a 24-hour cell cycle) while M phase is very short, lasting about one hour. S phase takes about half of the cell-cycle time (reviewed in [101]). The gap phases G_1 and G_2 are designed to provide extra time for the cell to grow and checkpoints for favorable growth conditions as well as for DNA damage.

At fertilization, the oocyte is arrested at MII, in a diploid state, while the sperm cell has completed meiosis and is haploid. Following oocyte activation by the sperm cell, the oocyte resumes meiosis to become haploid, thus matching the chromosome content and forming a viable diploid embryo. In NT, of course, it is not important that the donor chromosome content matches the recipient's since the oocyte chromatin is removed. Rather, the donor's cell-cycle phase should be compatible with the recipient cytoplasmic reprogramming content, which in turn depends on the recipient's cell-cycle phase. An important cytoplasmic reprogramming factor that has been extensively studied and has a profound effect on the donor nucleus is maturation promoting factor (MPF).

Campbell and colleagues, at the Roslin Institute (Scotland), carried out pioneer work in cell-cycle coordination between the donor cell (karyoplast) and recipient cytoplast prior to NT [16, 17, 102]. Their work suggests that cell-cycle coordination is a very important factor that should be taken into account prior to NT experiments to avoid damage of the donor chromatin.

For NT, mature MII cytoplasts are the recipients most frequently used. These contain high levels of MPF (also known as M-Cdk). MPF is a complex of two factors, protein kinase $p34^{cdc2}$ and cyclin B. MPF activity is regulated through synthesis and degradation of cyclin B and through phosphorylation of $p34^{cdc2}$. MPF activity is responsible for nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) of the donor nucleus following activation of reconstructed embryos. MPF declines progressively after activation or fertilization and remains low during interphase. Declining MPF levels are followed by nuclear membrane reformation and DNA synthesis. NEBD and PCC occur independent of the cell-cycle stage of the donor nucleus in MII-arrested oocytes. In contrast, when activation precedes NT for a few hours, MPF activity is low and nuclear envelope integrity is maintained because NEBD and PCC do not occur.

An intact nuclear membrane prevents replication of previously replicated DNA [16, 103]. Transferring donor nuclei in S or G_2 phases into MII cytoplasts followed by activation leads to NEBD and eventually to DNA rereplication, likely resulting in ploidy abnormalities. In addition, PCC could also cause chromosomal damage of S-phase nuclear donors. These early observations suggested that only G_1 embryonic donor nuclei should be transferred into unactivated MII cytoplasts. In contrast to unactivated MII oocytes, activated oocytes serve as "universal recipients" since MPF activity is low and NEBD/PCC do not occur, allowing

the transfer of any nuclear donor without the risk of DNA rereplication. In these reconstructed embryos, coordinated replication of nuclear DNA should occur. Indeed, unsynchronized sheep blastomere donors developed to the blastocyst stage more frequently when using activated cytoplasts (low MPF) compared with cytoplasts activated following NT (high MPF) [17].

For differentiated cells, however, activated oocytes might be poor recipients because NEBD and PCC are thought to be beneficial for reprogramming the donor nucleus. In bovine, for instance, when activated enucleated MII oocytes were used as recipients for somatic donors at different stages of the cell cycle (except S phase), development did not proceed to the 8-cell stage. However, unactivated MII oocyte recipients resulted in successful in vitro development to blastocyst [104]. Similarly, activated oocytes were shown in mice to be ineffective for development to term when using cumulus cells as donors [105]. These results suggest that activated oocytes, with low levels of MPF, fail to reprogram somatic donor nuclei. Blastomeres, on the other hand, probably require little or no reprogramming and thus can be cloned successfully with activated recipients as in Campbell et al. [17]. Not surprisingly, for somatic nuclei donors, the most common recipient is the unactivated enucleated MII oocyte.

Later research showed that donor genomes at G_0, G_1, and M phases of the cell cycle can be transferred into unactivated oocytes and produce offspring. For instance, embryonic stem (ES) cells arrested at metaphase produced live mice [106]. Metaphase-arrested chromatin donors are ready to segregate and form a pseudo-second polar body, thus eliminating the extra set of chromatin. However, G_2 donors were observed to fail at extruding the pseudo-second polar body resulting in tetraploidy [104], probably because they are "out of phase" with the meiotic cytoplast. This, combined with the rereplication event, could explain the consistent failure to produce viable offspring with G_2 donors (reviewed in [107]). For G_0 and G_1 donors, spindle function inhibitors are required to prevent pseudo-polar body formation and loss of chromosomes.

There is no apparent advantage between nuclei at G_0, G_1, or M phases for NT. Somatic cell donors are routinely synchronized at the G_0 phase of the cell cycle prior to NT. However, synchronized G_0 or G_1 fetal fibroblasts were compared and no clear superiority for either donor was found. Their relative cloning efficiencies were cell-line dependent in cattle [107]. Synchronization of ES cells at G_0 or G_1 might be problematic since they have a very short G_1 phase, and if induced to become quiescent (G_0), they likely differentiate. The most successful studies in cloning mice with ES cells used unsynchronized cell populations [108, 109], but others were less successful [110, 111]. ES cells at presumptive G_1 (by selecting small cells) were used with cloning success [112] and without success [113]. ES cells were also synchronized at M phase with moderate success in several occasions [106, 114, 115].

Taken together, enucleated MII oocytes seem to be the best recipients. However, the cell cycle of the donor nuclei might be less important since cloning success is achieved with G_0, G_1, and M donors, adapting the NT protocol accordingly.

Sources of Nuclear Donor Cells

Donor cells of different ages including embryonic, ES, fetal, and adult cells have been used for NT. It has been suggested that the age of an adult somatic donor cell could be transmitted to the clone. Such belief originated from a study showing that Dolly and other cloned sheep had shorter telomeres compared to age-matched controls [116]. The belief was later emphasized by the premature death of Dolly. However, several studies in cattle and mice showed that telomere length was normal in clones; telomerase activity was found to be reactivated in cloned bovine preimplantation embryos at similar levels to fertilized controls. Yet, further studies in sheep showed that indeed clones have shorter telomeres [117], suggesting that the mechanism of telomerase reactivation differs in this species. Nonetheless, studies have not observed premature aging in clones produced by SCNT.

Beyond age, a more interesting discussion relies on the epigenetic differences between donor cells. Differentiated somatic cell types diverge in their gene expression programs but have similar global chromatin configurations and modifications, or epigenomes. However, the epigenome of differentiated cells contrasts with that of undifferentiated pluripotent cells.

It is probably the epigenome features that render one cell more amenable to reprogramming following NT rather than the cell's specific gene expression program.

Epigenomes Differentiated cells are different to pluripotent cells in several epigenetic features. Compared to pluripotent cells, differentiated cells show expansion of repressive domains marked by H3K9me3 and H3K27me3 [118]. Similarly, differentiated cells show hypermethylated DNA compared to ES cells [119] and have lower levels of histone acetylation, consistent with heterochromatin configurations [120]. In agreement, electron microscopy showed that ES cells have dispersed global chromatin architecture, while the chromatin of the differentiated cells is more compact [121].

The epigenome of a differentiated cell can be reverted to that of an ES cell by overexpression of master-regulatory genes of pluripotency, thus forming iPS cells [19]. It has been shown that iPS and ES cells have similar global DNA methylation [20, 119] and similar global levels of the histone modifications H3K4me3, H3K9me3, and H3K27me3 [118, 119, 122].

Differentiated Versus Undifferentiated Donors It is generally postulated that an inverse relationship exists between the differentiated state of a donor cell and its "reprogrammability." This hypothesis is based on NT efficiencies obtained with different types of donor nuclei ranging in their differentiation status from the totipotent zygote to the terminally differentiated cell.

It has been suggested that a small proportion of somatic stem cells are present in a tissue sample, and these cells, rather than differentiated cells, account for the successful cases of SCNT. Studies were performed to test whether terminally differentiated cells are "clonable." First attempts to clone mice with donor lymphocyte cells failed [123]. Thus, a two-step NT procedure, in which ES cells are derived from cloned embryos and used for embryo tetraploid complementation, was later adopted for lymphocyte and olfactory neurons, resulting in viable mice offspring [124, 125]. Later on, cloned mice were produced by direct NT of T lymphocyte cells, albeit with a very low efficiency [126]. These results show that terminally differentiated cells can be cloned, but does not reject the possibility that less differentiated cells might be better donors.

In contrast to terminally differentiated cells, blastomeres give high frequencies of development after NT. Yet, the developmental potential of donor blastomeres decreases as the cleavage stage of the embryo increases. In mice, a gradual decline is observed from the 1- to the 4-cell-stage embryo followed by a steep decline from the 4- to the 8-cell stage, remaining low thereafter (reviewed in [127]). In cattle, such a significant decline in developmental potential is observed after the 8-cell stage (e.g., [13]). Apparently, the greatest decline in developmental potential after blastomere NT occurs following embryonic genome activation.

Literature reviews on NT generally support that ES cells are better than somatic cells in terms of the developmental potential of the reconstructed embryos. This view is based on experimental evidence gathered from a few early studies in mice, where good outcomes were achieved using ES cells as donors [108, 109, 112]. In these studies, the cloning efficiency (here defined as development to term after blastocyst transfer to surrogate females) ranges from 8% to 21%. These values are much higher than those obtained previously with SCNT by Wakayama and colleagues, where cloning efficiencies ranged from 0.4% to 1.6% using cumulus cells [128] and fibroblasts [129], respectively. Other studies also found similarly low mice cloning efficiencies (0–2.5%) with fibroblast cells (e.g., [130]).

However, lower efficiencies (0–5.4%) were also reported with ES cell donors (e.g., [111, 113–115]). These studies question whether ES cells have indeed better developmental potential than somatic cells following NT and suggest that major reviews are relying too heavily on positive results while overlooking the negative ones. In a critical review, Oback and Wells [127] argue that postimplantation sample sizes are generally small in animal cloning and thus insufficient to draw robust conclusions. Furthermore, they claim that many confounding factors exist in nuclear transfer experiments, rendering comparisons difficult. These factors include cell line and sex, confluency of donor cell culture, cell-cycle, passage number, and nuclear transfer procedure. Oback and Wells conclude that to determine whether ES cells have a better developmental potential than differentiated cells, it is necessary to carry out large NT experiments in which ES cells and the same cells induced to differentiate prior to NT are directly compared while keeping other parameters

constant. Taken together, the published data are inconclusive but suggest that ES cells give better cloning efficiencies than somatic cells. Indeed, only one study directly compared the developmental potential of ES cells to somatic cells, resulting in a cloning efficiency of about four times greater with ES cells [112].

According to Jaenisch's group, the cloning efficiencies with ES cells are 10- to 20-fold higher than with somatic cells after embryo transfer [76]. These authors have proposed that ES cells have better developmental potential following NT because they are undifferentiated and thus require less reprogramming than differentiated cells [76]. For instance, ES cells already express pluripotency-associated genes such as Oct4, Nanog, and Sox2, needed for early embryogenesis. In contrast, these genes need to be reactivated in differentiated cells. A second hypothesis could be that ES cells can be more easily reprogrammed as they have overall open chromatin configurations compared to differentiated cells, which typically have compact heterochromatin. The open and accessible chromatin of ES cells might facilitate the binding of nuclear remodeling factors found in the oocyte's cytoplasm.

Unfortunately, ES cells cannot be stably derived in livestock species yet. However, it is probably a matter of time until the mechanism and factors that govern pluripotency are discovered in these species. When derivation of ES cells in farm animals becomes possible, it would be of interest to further test their developmental potential compared to that of somatic cells following NT.

Differential Organelle Contribution

During fertilization, the oocyte and sperm each contribute a haploid set of chromosomes to form a diploid zygote. However, most of the cytoplasm and organelles are inherited from the oocyte. Among these are mitochondria, with their own genetic material. An exception to this is the centrosome, which is partly contributed by sperm. The differential contribution of the centrosome and mitochondria can affect NT outcomes. These contributions can be altered by the method used to transfer the donor chromatin.

Centrosome Inheritance The centrosome is the main microtubule-organizing center (MTOC) of animal cells involved, among other functions, in assembly of the mitotic spindle for cell division. This organelle is composed of two centrioles surrounded by abundant centrosomal proteins, which are often referred to as pericentriolar material (PCM). The centrosome is replicated during the S phase of the cell cycle from material stored in the cytoplasm. Mammalian oocytes have no centrioles but contain PCM that is recruited by the sperm centriole after fertilization, restoring a functional centrosome. Mice, however, are acentriolar until late preimplantation stages, achieving embryo cleavage by using maternal MTOCs.

In SCNT, the MTOCs associated with the oocyte meiotic spindle are removed while the centrosome from the donor cell is introduced [131]. It is not well understood the composition of the donor centrosome once within the recipient. Also, it is unclear whether the donor centrosome recruits leftover PCM from the recipient oocyte [131]. Nevertheless, it is thought that the donor centrosome needs to be remodeled into a zygotic centrosome for normal embryonic cleavage [131].

Centrosomes from donor cells at S phase might be reduplicated following oocyte activation in the subsequent S phase [132]. This would lead to the formation of extra spindles followed by aberrant segregation of chromosomes, a phenomenon frequently observed with S-phase donors. Even when using G_0 donors, 35% of bovine reconstructed embryos had abnormal cleavage correlated with abnormal centrosome number and distribution [133]. This suggests that providing only one centrosome does not guarantee normal centrosome function following NT.

An early NT study in monkeys suggested that removing the MII meiotic spindle depletes the oocyte of spindle proteins NuMA and HSET [134]. These proteins closely associate with maternal chromosomes and are required for mitotic spindle function. This study showed disorganized spindles with misaligned chromosomes in all reconstructed embryos, which were unable to produce pregnancies. However, in a later study using a different nuclear transfer procedure, NuMA location and spindle formation were normal in most reconstructed monkey embryos [135]. Taken together, these results suggest that different parameters of the NT procedure can affect centrosome function.

Mitochondrial Inheritance The mitochondrion is a special organelle, having a double membrane and containing its own genome (mtDNA). The main function of mitochondria is to produce energy in the form of ATP. Other functions include production of reactive oxygen species (ROS), calcium signaling, and apoptosis. mtDNA encodes for 13 mitochondrial proteins involved in ATP synthesis, for 22 tRNAs, and for 2 rRNA molecules involved in translating mitochondrial proteins [136]. Replication and expression of mtDNA are regulated by the nuclear genome through the expression of transcription and replication factors that translocate into mitochondria.

Mitochondria are maternally inherited because sperm mitochondria are targeted for destruction upon fertilization [137]. In SCNT, the donor cell mitochondria can persist and contribute to the reconstructed embryo, thus resulting in heteroplasmy (two different populations of mtDNA). Donor mitochondrial contribution is quite variable, ranging from 0% to 59% (reviewed in [138]). A bovine oocyte contains about 250,000 mitochondria, a 100-fold compared to a somatic cell [139]. Therefore, high heteroplasmy probably results from preferential amplification of donor mitochondria.

Compatibility between nuclear and the recipient's mtDNA seems to play an important role in SCNT. It has been shown that autologous SCNT (a female donor cell is transferred to its own enucleated oocyte) improves the frequency of pre- and postimplantation development when compared with heterologous SCNT [140]. Likewise, bovine reconstructed embryos developed more frequently as the similarity between the mtDNA haplotype of the donor and of the recipient cell increased [141]. Moreover, it is thought that the developmental block commonly observed in interspecies-SCNT embryos is due to genomic-mitochondrial incompatibilities [142].

Transfer of Donor Nuclei From the above discussion, it is apparent that the method chosen to deliver the donor chromatin into the cytoplast can have implications in terms of organelle inheritance of the reconstructed embryo. By whole-cell fusion, the donor cell contributes the nuclear and cytoplasmic material including mitochondria and centrosome, while nuclear microinjection does not. This would not seem to be problematic in mice since maternal MTOCs suffice to form the spindle-chromosome complex during cell division. In livestock species, however, centrioles function in spindle formation during early embryo cleavage. Nonetheless, pigs have been cloned by nuclear microinjection (e.g., [83]), suggesting that the presence of a centriole is not necessary for embryo cleavage in pigs. While cell fusion contributes mitochondria and cytoplasm, there is no evidence that either heteroplasmy or the amount of somatic cytoplasm contributed by a single donor cell is enough to reduce the developmental potential of the reconstructed embryo.

Cell fusion has been directly compared to nuclear microinjection in cloning experiments. In pigs and cattle, greater preimplantation development with the cell fusion method has been reported [143, 144]. Unless centrioles are often transferred along with residual cytoplasm during nuclear microinjection, these results suggest that centrioles are not strictly necessary for embryo cleavage, although their presence enhance preimplantation development. In mice, conflicting data have been reported when comparing the two methods. These mixed results are consistent with the exception that centrioles are dispensable in mice during preimplantation stages. Interestingly, it has been reported that the quality of blastocyst produced by cell fusion was better; analysis showed that piezo-assisted microinjection can cause DNA damage by shear forces [145].

Activation of Reconstructed Embryos

In mammals, egg activation is achieved when summation of intracellular Ca^{2+} oscillations reach a minimum threshold. Egg activation leading to Ca^{2+} oscillations is thought to be triggered by a sperm-specific isoform of PLC, known as PLC zeta (PLCζ), introduced into the oocyte following fertilization [146]. It has been suggested that summation of these Ca^{2+} oscillations encodes information for directing later development. For instance, few Ca^{2+} oscillations lead to decreased implantation, while excess Ca^{2+} oscillations, to increased postimplantation failure in mice [147].

Maturation promoting factor (MPF) also plays an important role in MII arrest and in regulating Ca^{2+} oscillations (reviewed in [148]). Inhibiting MPF activity enhances oocyte activation. Inhibition of protein synthesis (by cyclohexamide) or inhibition of

phosphatases (by 6-dimethylaminopurine [6-DMAP]) can decrease the activity of MPF and induce meiotic progression. These inhibitors are usually combined with stimulation of Ca^{2+} oscillations to efficiently activate reconstructed embryos. However, it is important to bear in mind that these chemicals have broad spectrum actions and can interfere with other cellular processes [148].

In the absence of the natural inducer of egg activation, several artificial methods exist to trigger activation of reconstructed SCNT embryos, including electrical pulses, ethanol, calcium ionophore A23187, ionomycin, strontium, and thimerosal (Thi)/dithiothreitol (DTT). With the exceptions of strontium and Thi/DTT, the other treatments induce a single Ca^{2+} oscillation [149]. Comparisons of several activation methods in mice cloning could not found a preferred method [95].

To mimic more physiological activation events, sperm-mediated activation methods have been used. Fertilized oocytes were used as recipients, but such approach has not produced any significant improvement in developmental potential to term in mice or bovine cloning [150, 151]. To avoid the added complication of removing the sperm chromatin, an alternative activation protocol involved directly injecting the sperm activating factor PLCζ, in the form of mRNA, resulting in long-term Ca^{2+} oscillations [152]. This approach improved gene expression patterns of several genes and reprogramming of the repressive histone mark H3K27me3 [153]. Therefore, mimmicking sperm-activation events might improve reprogramming of the donor nucleus.

Another factor that can be controlled in activation protocols is the timing of activation following embryo reconstruction. It is common practice to delay activation for 1–3 h to extend the time for nuclear remodeling after nuclear envelope breakdown. This "nuclear exposure" has been shown to be beneficial in bovine [104]. However, the effectiveness of this approach likely varies between species since in monkeys, it was shown that development improves by immediate activation [154].

Culture of Reconstructed Embryos

For animal cloning, the reconstructed embryos can be directly transferred to a surrogate female or cultured in vitro to the blastocyst stage followed by transfer. Since in vitro culture functions as a first filter to select growing embryos with exclusion of the developmentally arrested ones, it allows transferring fewer embryos to surrogates and is, therefore, widely used. Many factors can be manipulated in an in vitro culture system to affect the developmental outcome of cloned embryos. These factors include incubation temperature, media composition and osmolarity, oxygen tension, culture substrate, communal or individual culture, embryo concentration, cocultures, medium renewal, and embryo stress. Analyzing each of these factors is outside the scope of this entry, but the reader is encouraged to read Vajta et al. [155] for a comprehensive review on embryo culture. Instead, an eccentric culture preference of cloned embryos will be highlighted.

Usually, NT experiments culture cloned embryos in conditions designed for normal embryos. However, as discussed during *nuclear reprogramming*, a cloned embryo is usually not a normal one. Several reports support that reconstructed embryos have altered metabolism and culture requirements compared to normal embryos (e.g., [77, 156]). For instance, due to incomplete reprogramming of the donor chromatin, mice cloned embryos produced with muscle nuclei overexpress the glucose transporter GLUT4 and thus exhibit enhanced rates of glucose uptake and benefit from somatic cell culture media instead of standard embryo culture media [77]. The benefits of using somatic cell culture media included improved blastocyst formation rate and increased total cell numbers in the resultant blastocysts. These results suggest that normal embryo culture conditions might subject cloned embryos to a harsh selection process, while somatic-like culture media seem to maintain the viability of reconstructed embryos, allowing them more time to complete nuclear reprogramming. Nonetheless, sequential use of different embryo culture media has been shown to dramatically improve blastocyst development of mice reconstructed embryos [156]. Taken together, culture media for reconstructed embryos should be optimized to match the donor cell preferences.

Perinatal/Neonatal Care

Once cloned blastocysts have been transferred to surrogate animals, work is usually limited to monitoring

the progress of pregnant surrogates of livestock species. However, it is important to bear in mind that cloned animals are prone to suffer health problems arising from epigenetic errors caused by incomplete nuclear reprogramming of the donor chromatin. Respiratory difficulties seem to be the main problem in cloned neonates. Other health problems include myoarthroskeletal malformations and metabolic abnormalities. It is advisable that cloned neonates are regarded as being at high risk [157], and thus, intensive care should be provided to increase survival. Some studies suggest that veterinary intervention during the perinatal and neonatal periods can improve the survival rates of cloned livestock animals (for a review, see [158]).

Perinatal care involves monitoring readiness for birth, induced parturition, and induction of final pulmonary maturation. For yet unclear reasons, pregnancies of cloned fetuses often extend beyond the normal gestation period [157]. While this might indicate that cloned fetuses require more time in utero to complete maturation, prolonged gestations are associated with increased birth weight, dystocia, and increased morbidity and mortality [157]. To prevent these problems, induction of parturition is commonly carried out. In parallel, to aid pulmonary maturation, pharmaceutical drugs are often administered to promote production of lung surfactant necessary for alveoli inflation. Due to respiratory deficiencies, cloned neonates can quickly become hypoxic and acidotic. Lack of vigor and weak suckling reflexes are other common symptoms of cloned neonates. Thus, intensive care of the cloned neonate is crucial, even for preventative measures. Good care practice includes providing oxygen for at least one hour, heat, mechanical ventilation for more severe cases, and monitoring blood parameters [158].

Improving Development

From the above discussion, it is clear that veterinary science can play an important role in the management of pregnancy and neonatal care to improve the survival rate of cloned animals. In the laboratory, researchers have attempted many things to improve cloning efficiencies. The two most promising areas that have yielded the best results are chromatin remodeling treatments and embryo aggregation.

Chromatin Remodeling Treatments

Relaxation of the donor chromatin could enhance the reprogramming of the donor nucleus. To this end, a few different approaches have been implemented to treat donor nuclei or SCNT embryos. Well-defined chemical treatments include trichostatin A (TSA) and 5-azacytidine (5-Aza).

Early in the mouse zygote, both parental genomes are rich in histone acetylations, suggesting that these epigenetic marks are important for reprogramming. Treatment of donor cells with TSA, a histone deacetylase inhibitor, has been used to increase histone acetylation and promote opening of the chromatin. Such treatment led to increased development to blastocyst stage in bovine [159]. Later on, optimized TSA treatments resulted in significant increases (up to tenfold) in development to term compared to untreated groups in mice cloning (e.g., [160]). Remodeling of the donor chromatin with TSA has been the single most important innovation for improving consistently the efficiency of SCNT [95, 161].

DNA methylation patterns have been observed to be abnormally high in cloned embryos [36], and therefore, some researchers have attempted to correct this epigenetic abnormality by treating the donor cells with 5-Aza, a DNA demethylating drug. Such treatment, however, has led to poor blastocyst development [159]. A similar decrease in developmental potential was also observed when using 5-Aza in cloned embryos at the 2-cell stage [162]. It is thought that such failure is due to the effects of 5-Aza on massive DNA demethylation and subsequent DNA rearrangements and formation of pronuclei [163]. Chromosomal abnormalities resulting from 5-Aza treatment would be consistent with the regulatory function of DNA methylation on chromosome stability. These unsuccessful results also suggest that following fertilization, active global demethylation of the sperm chromatin must be well regulated to prevent chromosomal damage. Indeed, the sperm genome retains some methylated regions including centromeres, which contribute to chromosomal stability. When 5-Aza and TSA are used together, however, a synergistic effect has been observed in cloned bovine preimplantation development compared to TSA treatment alone [164].

Another approach to induce chromatin relaxation involves using cell extracts from Xenopus eggs to treat

differentiated donor cells prior to NT. The rationale is that many of the reprogramming factors present in the mammalian oocyte might also be present in the frog egg. This approach showed a significant increase in development to term in sheep [165]. Treated cells had lower global levels of the heterochromatic epigenetic mark H3K9me3, thus probably contributing to more relaxed chromatin configurations and to improved cloning efficiencies.

Notoriously, somatic cells have even been preheated at nonphysiological temperatures prior to NT in order to relax higher order chromatin; however, development to term was not significantly higher than nontreated control [166].

Overall, when the "right" treatment is used (such as TSA), remodeling of donor chromatin appears to improve development of cloned embryos. A better understanding of chromatin remodeling following fertilization or NT might help design a "cocktail" of drugs to efficiently remodel the differentiated chromatin of somatic donor cells.

Embryo Aggregation

Two hypotheses exist supporting the rationale of embryo aggregation. One involves the *community effect* in which the ability of a cell to take a specific differentiation pathway is enhanced when more neighboring cells are differentiating in the same way [167]. Since cloned embryos tend to have lower cell numbers than fertilized controls, at least in mice [168], the *community effect* obtained by aggregation might enhance the formation of the ICM and/or TE lineage [169]. The second hypothesis involves *epigenetic embryo complementation* [168, 170]. While the aggregated embryos are genetically identical, reprogramming defects of one embryo can be compensated by another embryo, and vice versa. Although embryo complementation is largely unknown, it is thought that cell-cell communication between blastomeres, by permeable gap junctions or by autocrine and paracrine factors, compensates for deficiencies between blastomeres [170]. It is possible that both hypotheses work together since greater cell numbers will increase the opportunities for epigenetic embryo complementation.

Aggregation of four-cell cloned embryos improved developmental potential and gene expression. In mice, expression of Oct4 increased to normal levels, the number of cells was higher at the blastocyst stage, and development to term was increased eightfold compared to single-clone embryos [168]. In bovine, embryo aggregation resulted in blastocysts with double the number of cells and in upregulation of a subset of differentially expressed genes involved in transcription, biosynthesis, and signaling compared with single-clone embryos [170]. Overall, embryo aggregation is an interesting approach to improve the quality of a cloned blastocyst.

RNA interference

While this entry was in production, an article was published in which mice cloning efficiencies were increased tenfold and the gene expression of the resultant offspring was similar to IVF controls [171]. Such impressive outcome was simply achieved by knockdown of a single gene, Xist, by RNA interference. This study showed that cloned mouse embryos usually undergo permature overexpression of Xist as well as aberrant X chromosome inactivation. Although regulation of X chromosome inactivation differs between mammals [172], cloned bovine embryos were also observed to overexpress Xist [173]. Therefore, the next logical step would be to try the same Xist knockdown approach in livestock species.

Summary Points

- SCNT involves using a somatic donor nucleus and an enucleated oocyte to produce an animal genetically identical to the donor.
- The first NT experiments were carried out in 1928, the first successful SCNTs in the late 1950s, and the first cloned adult mammal (Dolly) in 1996.
- Basic research in the nuclear reprogramming mechanism following SCNT could yield transferable knowledge to produce iPS cells safer for regenerative medicine. Realistic pharmaceutical/agricultural applications of SCNT include production of transgenic animals and cloning prizewinning animals for breeding purposes.
- NT is commonly carried out with micromanipulators, although micromanipulator-free NT is possible and effective. NT steps include oocyte maturation, enucleation, nuclear transfer, fusion, activation, embryo culture, and embryo transfer.

- Common problems in SCNT include low cloning efficiencies and developmental abnormalities. The placenta is mostly affected by the technique.
- Problems in SCNT probably originate from incomplete reprogramming of the donor chromatin. Faulty chromatin reprogramming in cloned embryos includes hypermethylated DNA and increased H3K9me3, especially in the TE.
- Gene expression reprogramming is usually aberrant in cloned embryos, with differences between species. Pluripotent genes often fail to be reactivated in mice, although reactivation is normal when ES cells are used as donors. Fewer markers exist to assess reprogramming of pluripotency and TE lineage in livestock species, and the existing data are rather conflicting. Imprinted genes are often deregulated and differentiation-associated genes incompletely silenced.
- MII oocytes are the best recipients as they have high MPF levels. Reprogramming factors are lost when enucleating interphase recipients, which also have low MPF levels.
- Donor nuclei can be in G_0, G_1, or M phase of the cell cycle. No specific cell type has been found to be advantageous for NT; however, inconclusive data suggest that ES cells are superior donors than somatic cells.
- Centrosomal structures are recovered by introduction of centrioles along with donor cell in livestock species, although normal spindle function is not always observed in reconstructed embryos.
- Increased compatibility between genomic DNA and mtDNA is beneficial for SCNT.
- In livestock species, cell fusion appears to result in better development than nuclear microinjection, consistent with centrosomal function recovery.
- There is no preferred method for oocyte activation.
- Normal embryo culture media are not optimal for reconstructed embryos.
- Cloned neonates are at high risk, and thus, intensive care should be provided to improve survival.
- Remodeling of the donor chromatin with TSA has been the single most successful innovation for improving consistently the efficiency of SCNT.
- Aggregation of cloned embryos seems to improve embryo quality by two working models: community effect and epigenetic embryo complementation.
- X chromosome inactivation appears to be deregulated in cloned embryos and correcting this by Xist knockdown can dramatically improve development.

Future Directions

Although efforts are being made toward dissecting the mechanism and the factors that bring about nuclear reprogramming of the donor chromatin, scientists are still quite far away from gaining a clear understanding. Even if there is general consensus that the low efficiency of SCNT originates from faulty nuclear reprogramming, there is no common agreement in where the bottleneck is. Does it lie in the reprogramming of the trophectodermal lineage or is the pluripotent lineage to blame? Is deregulation of imprinted genes the culprit of low cloning efficiencies or is it the incomplete silencing of differentiation-associated genes? Is incomplete remodeling of the overall chromatin structure preventing the SCNT technology from thriving? Does the bottleneck lie in the misregulation of X chromosome inactivation? Perhaps all these are contributing factors responsible for the low SCNT outcomes. Unless much further efforts are dedicated toward understanding the mechanism of nuclear reprogramming inside the oocyte, scientists will not have clear answers to such questions. The great improvement in cloning efficiencies observed with TSA treatment and Xist knockdown gives much hope and emphasizes that innovations can radically improve the technology.

In basic science, SCNT is not limited to nuclear reprogramming. For instance, SCNT interferes with centrosomal function in livestock species, and more research is needed to understand donor centrosome behavior, spindle formation, and embryo cleavage following NT in these species. SCNT can also provide insight into genomic-mitochondrial interactions, the effects of heteroplasmy, and preferential mitochondrial amplification. Such studies might warrant a change in technique such as the method by which the donor chromatin is delivered into the cytoplast.

As the science behind SCNT will be better understood through years of research, it is likely that the outcomes of this technology will closely match those

of IVF. When that day comes, widespread use of SCNT technology in livestock species would be feasible. However, being an artificial reproductive technology, SCNT is surrounded by ethical issues and regulations that should be fully contemplated to ensure good use of the technology for the benefit of society.

Bibliography

1. Baroux C et al (2008) The maternal to zygotic transition in animals and plants. Cold Spring Harb Symp Quant Biol 73:89–100
2. Newport J, Kirschner M (1982) A major developmental transition in early xenopus-embryos. 1. Characterization and timing of cellular-changes at the midblastula stage. Cell 30(3):675–686
3. Spemann H (1938) Embryonic development and induction, vol 7. Yale University Press, New Heaven, Mrs. Hepsa Ely Silliman Memorial Lectures. Yale University Press, New Haven, London; H. Milford, Oxford University Press, 401 p
4. Briggs R, King TJ (1952) Transplantation of living nuclei from blastula cells into enucleated frogs eggs. Proc Natl Acad Sci USA 38(5):455–463
5. King TJ, Briggs R (1955) Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. Proc Natl Acad Sci USA 41(5):321–325
6. Gurdon JB (1962) Adult frogs derived from the nuclei of single somatic cells. Dev Biol 4:256–273
7. Gurdon JB, Elsdale TR, Fischberg M (1958) Sexually mature individuals of *Xenopus laevis* from the transplantation of single somatic nuclei. Nature 182(4627):64–65
8. Gurdon JB, Laskey RA, Reeves OR (1975) The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. J Embryol Exp Morphol 34(1):93–112
9. Bromhall JD (1975) Nuclear transplantation in the rabbit egg. Nature 258(5537):719–722
10. McGrath J, Solter D (1983) Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220(4603):1300–1302
11. McGrath J, Solter D (1984) Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226(4680):1317–1319
12. Willadsen SM (1986) Nuclear transplantation in sheep embryos. Nature 320(6057):63–65
13. Prather RS et al (1987) Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 37(4):859–866
14. Prather RS, Sims MM, First NL (1989) Nuclear transplantation in early pig embryos. Biol Reprod 41(3):414–418
15. Campbell KH et al (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380(6569):64–66
16. Campbell KH, Ritchie WA, Wilmut I (1993) Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol Reprod 49(5):933–942
17. Campbell KH et al (1994) Improved development to blastocyst of ovine nuclear transfer embryos reconstructed during the presumptive S-phase of enucleated activated oocytes. Biol Reprod 50(6):1385–1393
18. Wilmut I et al (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810–813
19. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
20. Lister R et al (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471(7336):68–73
21. Hussein SM et al (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471(7336):58–62
22. Gore A et al (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471(7336):63–67
23. Ding J et al (2009) Embryonic stem cells derived from somatic cloned and fertilized blastocysts are post-transcriptionally indistinguishable: a MicroRNA and protein profile comparison. Proteomics 9(10):2711–2721
24. Brambrink T et al (2006) ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc Natl Acad Sci USA 103(4):933–938
25. Loi P, Modlinski JA, Ptak G (2011) Interspecies somatic cell nuclear transfer: a salvage tool seeking first aid. Theriogenology 76(2):217–228
26. Vajta G (2007) Handmade cloning: the future way of nuclear transfer? Trends Biotechnol 25(6):250–253
27. Lee JH, Campbell KH (2006) Effects of enucleation and caffeine on maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities in ovine oocytes used as recipient cytoplasts for nuclear transfer. Biol Reprod 74(4):691–698
28. Thornton M, Eward KL, Helmstetter CE (2002) Production of minimally disturbed synchronous cultures of hematopoietic cells. Biotechniques 32(5):1098–1100, 1102, 1105
29. Lee JW et al (2003) Production of cloned pigs by whole-cell intracytoplasmic microinjection. Biol Reprod 69(3):995–1001
30. Song BS et al (2011) Inactivated Sendai-virus-mediated fusion improves early development of cloned bovine embryos by avoiding endoplasmic-reticulum-stress-associated apoptosis. Reprod Fertil Dev 23(6):826–836
31. Heyman Y et al (2002) Frequency and occurrence of late-gestation losses from cattle cloned embryos. Biol Reprod 66(1):6–13
32. Li N et al (2005) Perturbations in the biochemical composition of fetal fluids are apparent in surviving bovine somatic cell nuclear transfer pregnancies in the first half of gestation. Biol Reprod 73(1):139–148
33. Palmieri C et al (2007) Placental abnormalities in ovine somatic cell clones at term: a light and electron microscopic investigation. Placenta 28(5–6):577–584

34. Loi P et al (2006) Placental abnormalities associated with postnatal mortality in sheep somatic cell clones. Theriogenology 65(6):1110–1121
35. Lin J et al (2011) Defects in trophoblast cell lineage account for the impaired in vivo development of cloned embryos generated by somatic nuclear transfer. Cell Stem Cell 8(4):371–375
36. Morgan HD et al (2005) Epigenetic reprogramming in mammals. Hum Mol Genet 14(1):R47–R58
37. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69(6):915–926
38. Beaujean N et al (2004) Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer. Biol Reprod 71(1):185–193
39. Dean W et al (2001) Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci USA 98(24):13734–13738
40. Beaujean N et al (2004) Non-conservation of mammalian preimplantation methylation dynamics. Curr Biol 14(7): R266–R267
41. Shi W, Haaf T (2002) Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. Mol Reprod Dev 63(3):329–334
42. Santos F et al (2003) Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr Biol 13(13):1116–1121
43. Wu X et al (2011) Multiple histone site epigenetic modifications in nuclear transfer and in vitro fertilized bovine embryos. Zygote 19(1):31–45
44. Maalouf WE, Alberio R, Campbell KH (2008) Differential acetylation of histone H4 lysine during development of in vitro fertilized, cloned and parthenogenetically activated bovine embryos. Epigenetics 3(4):199–209
45. Vassena R et al (2007) Tough beginnings: alterations in the transcriptome of cloned embryos during the first two cell cycles. Dev Biol 304(1):75–89
46. Oback B (2008) Climbing mount efficiency – small steps, not giant leaps towards higher cloning success in farm animals. Reprod Domest Anim 43:407–416
47. Latham KE, Solter D, Schultz RM (1992) Acquisition of a transcriptionally permissive state during the 1-cell stage of mouse embryogenesis. Dev Biol 149(2):457–462
48. Nichols J et al (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95(3):379–391
49. Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24(4):372–376
50. Mitsui K et al (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631–642
51. Rappolee DA et al (1994) Expression and function of FGF-4 in peri-implantation development in mouse embryos. Development 120(8):2259–2269
52. Dailey L, Yuan HB, Basilico C (1994) Interaction between a Novel F9-specific factor and octamer-binding proteins is required for cell-type-restricted activity of the fibroblast growth-factor-4 enhancer. Mol Cell Biol 14(12):7758–7769
53. Feldman B et al (1995) Requirement of Fgf-4 for postimplantation mouse development. Science 267(5195): 246–249
54. Tanaka S et al (1998) Promotion of trophoblast stem cell proliferation by FGF4. Science 282(5396):2072–2075
55. Boiani M et al (2002) Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 16(10):1209–1219
56. Bortvin A et al (2003) Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. Development 130(8):1673–1680
57. Fujii T et al (2010) Aberrant expression patterns of genes involved in segregation of inner cell mass and trophectoderm lineages in bovine embryos derived from somatic cell nuclear transfer. Cell Reprogram 12(5):617–625
58. Kirchhof N et al (2000) Expression pattern of Oct-4 in preimplantation embryos of different species. Biol Reprod 63(6):1698–1705
59. He S et al (2006) Developmental expression of pluripotency determining factors in caprine embryos: novel pattern of NANOG protein localization in the nucleolus. Mol Reprod Dev 73(12):1512–1522
60. Cauffman G et al (2005) Oct-4 mRNA and protein expression during human preimplantation development. Mol Hum Reprod 11(3):173–181
61. Kuijk EW et al (2008) Differences in early lineage segregation between mammals. Dev Dyn 237(4):918–927
62. Rodriguez-Alvarez L et al (2010) Changes in the expression of pluripotency-associated genes during preimplantation and peri-implantation stages in bovine cloned and in vitro produced embryos. Zygote 18(3):269–279
63. Long JE, Cai X, He LQ (2007) Gene profiling of cattle blastocysts derived from nuclear transfer, in vitro fertilization and in vivo development based on cDNA library. Anim Reprod Sci 100(3–4):243–256
64. Beyhan Z et al (2007) Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. Mol Reprod Dev 74(1):18–27
65. Niwa H et al (2005) Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell 123(5):917–929
66. Home P et al (2009) GATA3 Is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. J Biol Chem 284(42): 28729–28737
67. Nishioka N et al (2008) Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. Mech Dev 125(3–4):270–283
68. Ralston A et al (2010) Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. Development 137(3):395–403

69. Berg DK et al (2011) Trophectoderm lineage determination in cattle. Dev Cell 20(2):244–255
70. Degrelle SA et al (2005) Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. Dev Biol 288(2):448–460
71. Vejlsted M et al (2005) Ultrastructural and immunohistochemical characterization of the bovine epiblast. Biol Reprod 72(3):678–686
72. Kishigami S et al (2006) Normal specification of the extraembryonic lineage after somatic nuclear transfer. FEBS Lett 580(7):1801–1806
73. Mcgrath J, Solter D (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 37(1):179–183
74. Inoue K et al (2002) Faithful expression of imprinted genes in cloned mice. Science 295(5553):297
75. Humpherys D et al (2002) Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc Natl Acad Sci USA 99(20):12889–12894
76. Rideout WM 3rd, Eggan K, Jaenisch R (2001) Nuclear cloning and epigenetic reprogramming of the genome. Science 293(5532):1093–1098
77. Gao SR et al (2003) Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol Reprod 69(1):48–56
78. Ng RK, Gurdon JB (2005) Epigenetic memory of active gene transcription is inherited through somatic cell nuclear transfer. Proc Natl Acad Sci USA 102(6):1957–1962
79. Smith SL et al (2005) Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. Proc Natl Acad Sci USA 102(49):17582–17587
80. Krisher RL (2004) The effect of oocyte quality on development. J Anim Sci 82(E Suppl):E14–E23
81. Li Y et al (2006) Confocal microscopic analysis of the spindle and chromosome configurations of human oocytes matured in vitro. Fertil Steril 85(4):827–832
82. Rizos D et al (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 61(2):234–248
83. Onishi A et al (2000) Pig cloning by microinjection of fetal fibroblast nuclei. Science 289(5482):1188–1190
84. Gao S et al (2002) Germinal vesicle material is essential for nucleus remodeling after nuclear transfer. Biol Reprod 67(3):928–934
85. Lee JH et al (2010) Generation of mtDNA homoplasmic cloned lambs. Cell Reprogram 12(3):347–355
86. Wakayama S, Cibelli JB, Wakayama T (2003) Effect of timing of the removal of oocyte chromosomes before or after injection of somatic nucleus on development of NT embryos. Cloning Stem Cells 5(3):181–189
87. Polejaeva IA et al (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407(6800):86–90
88. Wakayama T et al (2000) Nuclear transfer into mouse zygotes. Nat Genet 24(2):108–109
89. Baguisi A et al (1999) Production of goats by somatic cell nuclear transfer. Nat Biotechnol 17(5):456–461
90. Egli D et al (2007) Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. Nature 447(7145):679–U8
91. Egli D et al (2009) Reprogramming after chromosome transfer into mouse blastomeres. Curr Biol 19(16):1403–1409
92. Mohammed AA et al (2010) Enucleated GV oocytes as recipients of embryonic nuclei in the G1, S, or G2 stages of the cell cycle. Cell Reprogram 12(4):427–435
93. Memili E, Dominko T, First NL (1998) Onset of transcription in bovine oocytes and preimplantation embryos. Mol Reprod Dev 51(1):36–41
94. Bordignon V, Smith LC (1998) Telophase enucleation: an improved method to prepare recipient cytoplasts for use in bovine nuclear transfer. Mol Reprod Dev 49(1):29–36
95. Wakayama T (2007) Production of cloned mice and ES cells from adult somatic cells by nuclear transfer: how to improve cloning efficiency? J Reprod Dev 53(1):13–26
96. Robl JM et al (1987) Nuclear transplantation in bovine embryos. J Anim Sci 64(2):642–647
97. Tsunoda Y et al (1987) Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. J Exp Zool 242(2):147–151
98. Egli D, Birkhoff G, Eggan K (2008) Mediators of reprogramming: transcription factors and transitions through mitosis. Nat Rev Mol Cell Biol 9(7):505–516
99. Egli D, Eggan K (2010) Recipient cell nuclear factors are required for reprogramming by nuclear transfer. Development 137(12):1953–1963
100. Noggle S et al (2011) Human oocytes reprogram somatic cells to a pluripotent state. Nature 478(7367):70–75
101. Alberts B, Wilson JH, Hunt T (2008) Molecular biology of the cell, 5th edn. Abingdon: Garland Science, New York, xxxiii, 1601, 90 p
102. Campbell KH et al (1996) Cell cycle co-ordination in embryo cloning by nuclear transfer. Rev Reprod 1(1):40–46
103. Leno GH, Downes CS, Laskey RA (1992) The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in Xenopus egg extract. Cell 69(1):151–158
104. Tani T, Kato Y, Tsunoda Y (2001) Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. Biol Reprod 64(1):324–330
105. Wakayama T, Yanagimachi R (2001) Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. Reproduction 122(1):49–60
106. Ono Y et al (2001) Production of cloned mice from embryonic stem cells arrested at metaphase. Reproduction 122(5): 731–736
107. Wells DN et al (2003) Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. Theriogenology 59(1):45–59

108. Eggan K et al (2001) Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci USA 98(11):6209–6214
109. Rideout WM 3rd et al (2000) Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet 24(2):109–110
110. Gao S et al (2003) Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line. Biol Reprod 68(2):595–603
111. Humpherys D et al (2001) Epigenetic instability in ES cells and cloned mice. Science 293(5527):95–97
112. Wakayama T et al (1999) Mice cloned from embryonic stem cells. Proc Natl Acad Sci USA 96(26):14984–14989
113. Yabuuchi A et al (2004) Effects of nuclear transfer procedures on ES cell cloning efficiency in the mouse. J Reprod Dev 50(2):263–268
114. Ono Y, Kono T (2006) Irreversible barrier to the reprogramming of donor cells in cloning with mouse embryos and embryonic stem cells. Biol Reprod 75(2):210–216
115. Zhou Q et al (2001) Developmental potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei (vol 65, pg 412, 2001). Biol Reprod 65(5):1622
116. Shiels PG et al (1999) Analysis of telomere lengths in cloned sheep. Nature 399(6734):316–317
117. Alexander B et al (2007) Telomere length status of somatic cell sheep clones and their offspring. Mol Reprod Dev 74(12):1525–1537
118. Hawkins RD et al (2010) Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell 6(5):479–491
119. Maherali N et al (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1(1):55–70
120. Meshorer E, Misteli T (2006) Chromatin in pluripotent embryonic stem cells and differentiation. Nat Rev Mol Cell Biol 7(7):540–546
121. Ahmed K et al (2010) Global chromatin architecture reflects pluripotency and lineage commitment in the early mouse embryo. PLoS One 5(5):e10531
122. Guenther MG et al (2010) Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. Cell Stem Cell 7(2):249–257
123. Wakayama T, Yanagimachi R (2001) Mouse cloning with nucleus donor cells of different age and type. Mol Reprod Dev 58(4):376–383
124. Eggan K et al (2004) Mice cloned from olfactory sensory neurons. Nature 428(6978):44–49
125. Hochedlinger K, Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. Nature 415(6875):1035–1038
126. Inoue K et al (2005) Generation of cloned mice by direct nuclear transfer from natural killer T cells. Curr Biol 15(12):1114–1118
127. Oback B, Wells DN (2007) Donor cell differentiation, reprogramming, and cloning efficiency: elusive or illusive correlation? Mol Reprod Dev 74(5):646–654
128. Wakayama T et al (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394(6691):369–374
129. Wakayama T, Yanagimachi R (1999) Cloning of male mice from adult tail-tip cells. Nat Genet 22(2):127–128
130. Ogura A et al (2000) Birth of mice after nuclear transfer by electrofusion using tail tip cells. Mol Reprod Dev 57(1):55–59
131. Schatten H, Sun QY (2009) The functional significance of centrosomes in mammalian meiosis, fertilization, development, nuclear transfer, and stem cell differentiation. Environ Mol Mutagen 50(8):620–636
132. Wong C, Stearns T (2003) Centrosome number is controlled by a centrosome-intrinsic block to reduplication. Nat Cell Biol 5(6):539–544
133. Dai Y et al (2006) Fate of centrosomes following somatic cell nuclear transfer (SCNT) in bovine oocytes. Reproduction 131(6):1051–1061
134. Simerly C et al (2003) Molecular correlates of primate nuclear transfer failures. Science 300(5617):297
135. Zhou Q et al (2006) A comparative approach to somatic cell nuclear transfer in the rhesus monkey. Hum Reprod 21(10):2564–2571
136. Anderson S et al (1981) Sequence and organization of the human mitochondrial genome. Nature 290(5806):457–465
137. St John JC, Bowles EJ, Amaral A (2007) Sperm mitochondria and fertilisation. Soc Reprod Fertil Suppl 65:399–416
138. St John JC et al (2004) The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. Reproduction 127(6):631–641
139. Michaels GS, Hauswirth WW, Laipis PJ (1982) Mitochondrial DNA copy number in bovine oocytes and somatic cells. Dev Biol 94(1):246–251
140. Yang XY et al (2006) Improved efficiency of bovine cloning by autologous somatic cell nuclear transfer. Reproduction 132(5):733–739
141. Yan ZH et al (2010) Donor-host mitochondrial compatibility improves efficiency of bovine somatic cell nuclear transfer. BMC Dev Biol 10:31
142. Sansinena MJ et al (2011) Ooplasm transfer and interspecies somatic cell nuclear transfer: heteroplasmy, pattern of mitochondrial migration and effect on embryo development. Zygote 19(2):147–156
143. Kurome M et al (2003) Comparison of electro-fusion and intracytoplasmic nuclear injection methods in pig cloning. Cloning Stem Cells 5(4):367–378
144. Galli C et al (2002) Comparison of microinjection (piezoelectric) and cell fusion for nuclear transfer success with different cell types in cattle. Cloning Stem Cells 4(3):189–196
145. Jouneau A et al (2007) Piezo-assisted nuclear transfer affects cloning efficiency and may cause apoptosis. Reproduction 133(5):947–954

146. Kashir J et al (2010) Oocyte activation, phospholipase C zeta and human infertility. Hum Reprod Update 16(6):690–703
147. Ozil JP et al (2006) Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. Dev Biol 300(2):534–544
148. Alberio R et al (2001) Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. Int J Dev Biol 45(7):797–809
149. Cervera R et al (2010) Effects of different oocyte activation procedures on development and gene expression of porcine preimplantation embryos. Reprod Domest Anim 45(5):e12–e20
150. Schurmann A, Wells DN, Oback B (2006) Early zygotes are suitable recipients for bovine somatic nuclear transfer and result in cloned offspring. Reproduction 132(6):839–848
151. Kishikawa H, Wakayama T, Yanagimachi R (1999) Comparison of oocyte-activating agents for mouse cloning. Cloning 1(3):153–159
152. Ross PJ et al (2008) Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. Bmc Develop Biol 8:16
153. Ross PJ et al (2009) Activation of bovine somatic cell nuclear transfer embryos by PLCZ cRNA injection. Reproduction 137(3):427–437
154. Sparman ML, Tachibana M, Mitalipov SM (2010) Cloning of non-human primates: the road "less traveled by". Int J Dev Biol 54(11–12):1671–1678
155. Vajta G et al (2010) Embryo culture: can we perform better than nature? Reprod Biomed Online 20(4):453–469
156. Zhou Q, Dai XP, Hao J (2009) A modified culture method significantly improves the development of mouse somatic cell nuclear transfer embryos. Reproduction 138(2):301–308
157. Hill JR, Chavatte-Palmer P (2002) Pregnancy and neonatal care of cloned animals. In: Cibelli J, Lanza R, Campbell K, West M (eds) Principles of cloning. Academic, New York, pp 247–266
158. Meirelles FV et al (2010) Delivery of cloned offspring: experience in Zebu cattle (*Bos indicus*). Reprod Fertil Dev 22(1):88–97
159. Enright BP et al (2003) Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2′-deoxycytidine. Biol Reprod 69(3):896–901
160. Maalouf WE et al (2009) Trichostatin A treatment of cloned mouse embryos improves constitutive heterochromatin remodeling as well as developmental potential to term. BMC Dev Biol 9:11
161. Loi P et al (2008) Nuclear reprogramming: what has been done. Bioessays 30(1):66–74
162. Tsuji Y, Kato Y, Tsunoda Y (2009) The developmental potential of mouse somatic cell nuclear-transferred oocytes treated with trichostatin A and 5-aza-2′-deoxycytidine. Zygote 17(2):109–115
163. Kiziltepe T et al (2007) 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Mol Cancer Ther 6(6):1718–1727
164. Ding X et al (2008) Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2′-deoxycytidine and trichostatin A. Theriogenology 70(4):622–630
165. Rathbone AJ et al (2010) Reprogramming of ovine somatic cells with *Xenopus laevis* oocyte extract prior to SCNT improves live birth rate. Cell Reprogram 12(5):609–616
166. Loi P et al (2002) Nuclei of nonviable ovine somatic cells develop into lambs after nuclear transplantation. Biol Reprod 67(1):126–132
167. Gurdon JB (1988) A community effect in animal development. Nature 336(6201):772–774
168. Boiani M et al (2003) Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? EMBO J 22(19):5304–5312
169. Balbach ST et al (2010) Governing cell lineage formation in cloned mouse embryos. Dev Biol 343(1–2):71–83
170. Zhou WL et al (2008) Aggregation of bovine cloned embryos at the four-cell stage stimulated gene expression and in vitro embryo development. Mol Reprod Dev 75(8):1281–1289
171. Matoba S et al (2011) RNAi-mediated knockdown of Xist can rescue the impaired postimplantation development of cloned mouse embryos. Proc Natl Acad Sci
172. Okamoto I et al (2011) Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development. Nature 472(7343):370–U141
173. Inoue K et al (2010) Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. Science 330(6003):496–499

Lodging Resistance in Cereals

Pete M. Berry
Sustainable Crop Management, ADAS UK Ltd, Malton, North Yorkshire, UK

Article Outline

Glossary

Anchorage failure moment Anchorage failure at the point of failure. Also described as anchorage strength.

Base bending moment Wind-induced force acting on the base of the shoot or the anchorage system. Also described as leverage force.

Brackling Lodging resulting from buckling of the upper half of the stems.

Crop management Agronomic methods of growing crops.

Failure wind speed Wind speed at which a plant will lodge.

Hagberg falling number (HFN) Measure of bread making quality.

Lodging Permanent displacement of cereal stems from their vertical position.

Lodging-proof ideotype Plant dimensions required to achieve a lodging-return period of 25 years.

Necking Lodging resulting from buckling of the stem just below the ear.

Plant growth regulators – (PGRs) Chemical growth regulators that reduce the rate of stem extensions.

Root lodging Lodging resulting from failure of the anchorage system.

Stem failure moment Stem strength at the point of failure. Also described as stem strength.

Stem lodging Lodging resulting from buckling of the lower stems.

Definition of the Subject

Lodging is the process by which the shoots of small grained cereals are permanently displaced from their vertical stance. Lodging limits yield potential and reduces grower profits, but it is difficult to control because it is a complex process that is influenced by many factors including wind, rain, topography, soil type, previous crop, crop management, and disease. Significant progress was made during the 1950s, 1960s, and 1970s to reduce lodging risk by the introduction of semi-dwarf varieties. The reduced lodging risk of these shorter varieties enabled them to respond to greater amounts of fertilizers and this was a significant reason for the steady improvement in global cereal grain yields starting in the late 1960s. However lodging is still a major problem in many countries and there is an urgent need to improve lodging resistance to further increase the yield of cereal species.

Introduction

Lodging is the permanent displacement of cereal stems from their vertical position (Fig. 1) and usually only occurs after the ear or panicle has emerged. This can reduce yield by up to 80% and causes several knock-on effects including reduced grain quality, greater drying costs, and slower harvest. It is a problem that limits cereal productivity in both developed and developing countries.

Lodging is a complicated phenomenon that is influenced by many factors including wind, rain, topography, soil type, previous crop, husbandry, and disease. It is frequently associated with conditions that promote plant growth such as an abundant supply of nutrients. Significant progress was made during the 1950s, 1960s, and 1970s to reduce lodging risk by the introduction of semi-dwarf varieties. These shorter varieties had a greater lodging resistance and could respond to greater amounts of fertilizers. For these reasons the introduction of semi-dwarf varieties was one of the most significant reasons for the steady improvement in grain yields starting in the late 1960s, which has resulted in cereal yields increasing by as much as 1 t ha^{-1} per decade in western Europe and 0.5 t ha^{-1} in many American and Asian countries [1]. The continued improvement in yields in some

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Lodging Resistance in Cereals. Figure 1
Lodging in wheat

countries has been significantly aided by the use of plant growth regulators (PGRs) that further reduce crop height making cereals even more resistant to lodging. Four major types of PGRs have been introduced including chlormequat chloride during the mid-1960s, ethephon during the late 1980s, trinexapac-ethyl during the mid-1990s, and prohexadione-calcium in the 2000s. In France, Germany, and the UK, which have among the highest cereal yields in the world, PGRs are now applied to more than 70% of wheat area (W. Rademacher 2004, personal communication).

Dwarfing genes and PGRs have been very effective tools for reducing lodging risk and maintaining steady improvements in yield. However, they have not eradicated lodging and there is evidence that farmers will not be able to rely on these tools for further reductions in lodging risk in order to counter the escalating lodging risk resulting from continued yield increases. Several studies have shown that yield is reduced when crops are shortened too much [2–7]. The reduction in yield appears to be exacerbated by high temperatures or drought stress. Several of these studies indicate that the minimum crop height for optimum yield lies between 0.7 m and 1.0 m. Many modern varieties are already within this height range. While there is scope for further shortening with PGRs through sequential applications, pressure may be brought to bear to reduce their use because some PGRs leave residues in the grain [8]. It is therefore clear that new methods of improving lodging resistance in cereals must be developed.

During the 1990s and 2000s collaborative studies by biologists and engineers have elucidated the mechanisms of lodging in cereals [9]. Crucially it has been demonstrated that lodging may occur by two mechanisms: stem lodging and root lodging. These studies have culminated in models of the lodging process which help to understand how the plant interacts with its environment during the lodging process and identifies the most important plant traits that must be targeted to improve lodging resistance. Several studies have also explained how variety, sowing date, seed rate, nitrogen fertilizer, and PGRs affect lodging. This improved understanding offers the prospect of designing a lodging proof ideotype for cereals which may be achieved through a combination of crop management and plant breeding. This entry on lodging resistance in cereals describes (1) the impact that lodging in different cereal species has on crop yield and grain quality, (2) the mechanisms of the lodging process, (3) the effect of cultivar choice and crop management on lodging resistance, and (4) prospects for improving lodging resistance. This entry is intended to be a concise summary of the most important aspects of lodging resistance in cereals, and for a more comprehensive review readers are directed to Berry et al. [10].

Impact of Lodging on Grain Yield and Quality

Grain Yield

Lodging can reduce cereal yield by reducing the grain size and number or through reducing the amount of crop that can be recovered at harvest. This section deals only with physiological reductions in yield associated with lodging. The greatest lodging-induced reductions in grain yield occur when crops are lodged flat at anthesis or early on in grain filling. Yield reductions from this type of lodging have been reported to reduce yields of wheat by [11] 31–80% [12], barley by 28–65% [13–15], oats by 37% [16], and rice by 38% [17]. All of the above studies, apart from Easson et al. [12], artificially lodged the plants. This was achieved by growing the plants through wire netting and then moving the wires to effect lodging. This method has the advantage of lodging the crops at specific dates and at different angles, but may induce damage not normally incurred with natural lodging. Easson et al. [12] compared the yields of crops grown at high seed rate, which lodged

naturally, with those at low seed rate, which experienced negligible lodging.

Smaller yield losses have been observed when the angle of lodging is less than 90° from the vertical. Lodging at 45° causes between one quarter and one half of the yield losses incurred from 80° lodging in wheat [18], barley [14], and oats [16]. Smaller yield losses also occur when lodging occurs at a later stage of development. Artificial lodging at the ear emergence, milk, soft dough, and hard dough stages reduced yield by 31%, 25%, 20%, and 12%, respectively [11]. Stapper and Fischer [19] supported these observations by showing that about 0.5% of the potential yield was lost for each day of the grain filling period that a crop was lodged flat. Crops that lodge before anthesis often have smaller yield losses than crops that lodge soon after anthesis [18]. This appears to be associated with the upper one of two internodes bending upward to partially re-erect the crop. Crops that lodge after anthesis have completed stem extension and are unable to re-erect themselves. In natural situations, the re-erected crops are very unstable and are usually re-lodged by unexceptional weather conditions [12].

Grain Quality

Artificial lodging has been observed to cause significant reductions in grain quality in terms of the bread making quality (measured as the Hagberg falling number [HFN]), individual grain weight, and the specific weight of the grain [10]. Lodging increases the likelihood of grain sprouting in the ear due to the more moist environment (Fig. 2) and this reduces HFN. Lodging induced during early grain filling reduced grain quality by reducing the HFN from 289 s to 114 s, reducing individual grain weight from 42.2 mg to 37.2 mg, and reducing specific weight from 70.3 kg/hl to 65.8 kg/hl. Lodging after early grain filling caused smaller effects on quality. A HFN of at least 250 s is required to produce good quality bread. Similar effects on grain weight and specific weight have also been observed in wheat [11, 20], barley [13, 21], and oats [22]. In the UK, the harvest year of 1992 was a severe lodging year with 16% of the wheat area lodged [23]. In this year the national average HFN fell from a 5-year average of 287 s to 254 s, thus significantly reducing the amount of bread making grain produced in this year.

Lodging Resistance in Cereals. Figure 2
Sprouting in a lodged wheat crop

Also in this year, the specific weight of wheat grains fell from 77 kg hl^{-1} to 73 kg hl^{-1} and the number of small grains (<2.0 mm) increased from 1.9% to 2.6% [24]. It is likely that at least a proportion of these effects were caused by the greater than usual lodging experienced in this country during this year.

Mechanisms of Lodging

Lodging can either occur through stem buckling (stem lodging) or displacement of the roots within the soil (root lodging) (Fig. 3). During stem lodging the roots are held firmly in a strong soil and the wind force buckles the stem. Stem lodging can occur due to buckling of the lower internodes. Buckling of the middle internodes is commonly known as "brackling" and is often observed in barley (Fig. 4) [25] and oats. Buckling of the peduncle just below the ear is known as "necking" and occurs most frequently in barley [26]. Root lodging becomes more likely when the anchorage strength is reduced by weak soil or poorly developed anchorage roots. Rainfall can reduce soil strength by several fold and has an important influence on the anchorage strength of cereals.

Very few observations have been reported of the lodging process as it occurs and conjecture exists as to whether stem lodging or root lodging predominates in cereals. Wheat and barley have been observed to root lodge [27, 28] and to stem lodge [25]. However, recently a quantitative understanding of root and shoot

Lodging Resistance in Cereals. Figure 3
Wheat plants leaning as a result of root lodging

Lodging Resistance in Cereals. Figure 4
Brackling in barley

lodging has been developed for wheat and barley showing that both types of lodging are possible depending on the circumstances of a particular crop [29, 30]. This has been confirmed in wheat by direct observations of both mechanisms occurring during wind-tunnel experiments on field-grown winter wheat [31].

The lodging models described by Baker et al. [29] and Berry et al. [30] calculate the wind-induced base bending moment of a shoot from plant characteristics and meteorological data. The base bending moment is then compared to the failure moments (strength at the point of failure) of the stem base and of the anchorage system. Stem lodging is assumed to occur when the base bending moment of a single shoot exceeds the failure moment of the stem base. Root lodging is assumed when the base bending moment of all the shoots belonging to a single plant exceeds the failure moment of the anchorage system. The following sections describe current understanding about how the components of lodging (base bending moment, stem failure moment, and anchorage failure moment) may be calculated and what factors influence them.

Base Bending Moment

The wind-induced force acting on the upper sections of a shoot or plant results in a bending moment at the plant's base. This can be described as the shoot leverage. The coherent waving of cereal shoots, apparent even in light winds, provides evidence that cereal shoots are subjected to varying forces and illustrates the importance of including the shoot's motion in any calculation of the applied base bending moment. Baker [32] attempted to account for the dynamic nature of shoot movement by considering the forces that act on an idealized shoot and assuming that the shoot's movement could be modeled as damped harmonic oscillator. The theoretical modeling work described in [32] and [29], which was later validated in wind-tunnel experiments using field crops [31], showed that the wind-induced base bending moment of wheat could be calculated from a range of environmental and plant inputs. These include: the wind speed at crop height, the natural frequency of the shoot (rate at which it oscillates), the damping ratio of the shoot (which describes the rate at which oscillations die out), the height at center of gravity of the shoot, and the projected area and drag coefficient of the ear. These parameters can be used to estimate the bending moment at the base of the shoot for a shoot with a stiff stem such as wheat. Additional parameters are required to estimate the bending moment of more flexible stems such as for barley and include the flexural rigidity of the stem and its fresh weight. A method for calculating the bending moment of flexible stems is described in [30].

Anchorage Failure Moment

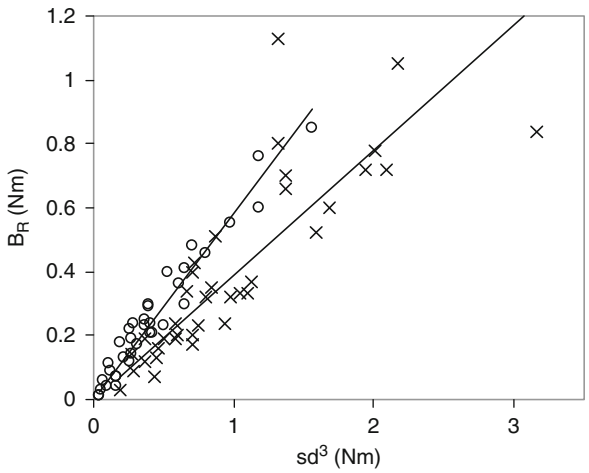
There is uncertainty about the exact mechanism of anchorage failure in cereals due to the obvious difficulty associated with observing the process in field conditions. Ennos [33] showed that anchorage failure of spring wheat involved bending of the crown roots and resistance to axial movement through the soil. Crook and Ennos [27] showed that the upper portions of the crown root system of winter wheat form a cone (Fig. 5) and anchorage failure occurred when the root-soil cone rotated at its windward edge, the soil inside the cone moved as a block and compressed the soil beneath. This idea supported earlier observations by Pinthus [34], who showed that a wider angle of root spread was related to greater resistance to root lodging. Easson et al. [35] suggested that winter wheat roots acted like ropes to withstand root lodging and that anchorage strength would therefore be a function of the tensile strength of the roots on the windward side of the plant.

The model developed by Crook and Ennos [27] has been tested and calibrated with field experiments on wheat [29] and on barley [30] (Fig. 6). These experiments showed that the anchorage strength was linearly related to the product of the diameter of the root cone cubed, the shear strength of the surrounding soil, and a constant specific to wheat or barley. The size of the root plate is identified by the parts of the crown roots that are surrounded by a rhizosheath. The rhizosheath is a dense mat of hairs that cover the upper sections of crown roots. These sections of roots have been shown to have an outer ring of lignified tissue in addition to the lignified central stele [27], which is why the rhizosheath can be used to estimate the length of root that provides anchorage.

The observation that anchorage strength was linearly related to the spread of the diameter of the root cone for both wheat and barley strongly suggests that both species have the same mechanism of anchorage failure first described by Crook and Ennos [27]. However, it was apparent that the constant factor was different between species with a value of 0.39–0.43 for wheat [29, 30] compared with 0.58 for barley demonstrating a greater anchorage strength for a given root plate spread for barley. This may have been caused by the greater number of stems per mm of root plate for barley compared with wheat. Up to 20 mm of the stem base is below ground, so it seems likely that a greater number of stems will increase the rotational resistance of the anchorage system.

A model of soil strength developed by Baker et al. [29] showed that variation in clay content, moisture content, and compaction that is normally found within farmer's fields could each be expected to alter the soil

Lodging Resistance in Cereals. Figure 5
Upper portions of the root system of winter wheat



Lodging Resistance in Cereals. Figure 6
The product of soil shear strength (s) and root plate spread cubed (d^3) plotted against failure moment (B_R) for winter barley (o; y = 0.58x, R^2 = 0.95) and for winter wheat (5; y = 0.39x, R^2 = 0.69) (Adapted from [30])

shear strength by several fold. This indicates that the state of the soil is likely to be of paramount importance for determining lodging given that it has been predicted in the anchorage model described above to be directly proportional to anchorage strength.

Stem Failure Moment Assuming that a typical stem can be considered to be analogous to a cylinder, Baker [32] showed that the stem failure moment (B_S) can be expressed as:

$$B_S = \frac{\sigma \pi a^3}{4}\left(1 - \left(\frac{a-t}{a}\right)^4\right)$$

where σ is the stem failure yield stress (material strength), a is the external radius of the stem, and t is the wall thickness. This formula assumes that the pith in the center of the stem does not contribute to the structural properties of the stem. Experiments by Neenan and Spencer-Smith [25] have shown that the stems of wheat and barley buckle at a certain critical ratio of radius of curvature to the outside diameter of the stem. Buckling was shown to occur suddenly with negligible amounts of plastic deformation. The Young's modulus of wheat and barley was shown to remain reasonably constant for a range of stem curvatures which indicates that negligible plastic deformation occurs and that the limit of proportionality between the applied stress and corresponding strain is seldom exceeded. It may therefore be concluded that stem lodging occurs abruptly and will result in complete structural failure of the stem.

Influence of Crop and Environmental Characteristics on Lodging Risk

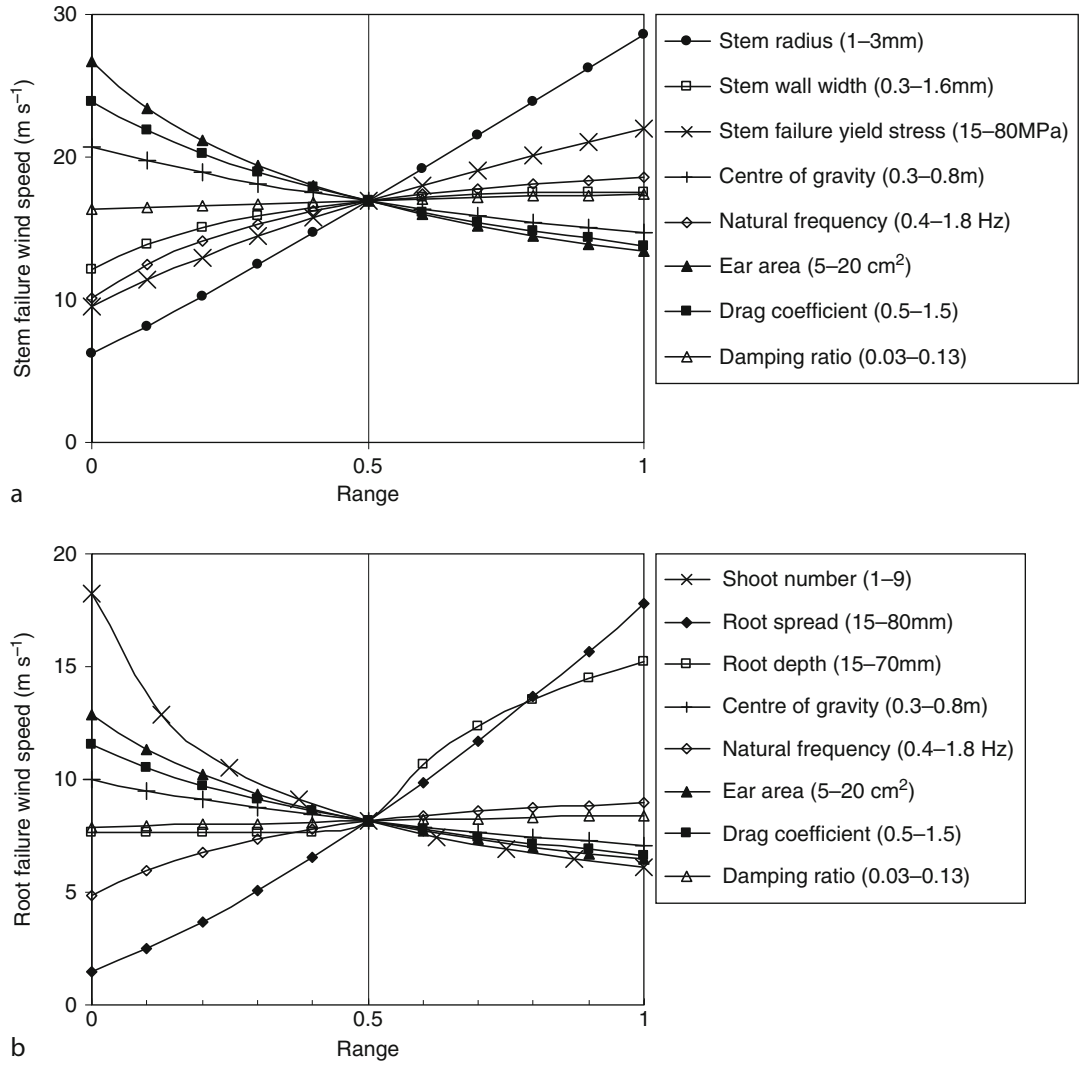
Lodging is a complex process that involves several environmental and plant characteristics. Any assessment of the effect of individual characteristics on lodging risk must account for interrelationships between characteristics. To date the only study to have achieved this involves a sensitivity analysis of the lodging model of Baker et al. [29] and this is described in [9]. This study further developed the model of Baker et al. [29] to account for spatial non-uniformity between plants and temporal changes in plant structure during the growing season. A sensitivity analysis using this model showed that the risk of stem lodging is influenced most by changes in stem diameter and the risk of root lodging is affected most by changes to the diameter of the root cone (also referred to as the spread of the root plate) (Fig. 7). In these analyses, the risk of lodging is measured in terms of the wind speed required to cause lodging, which is termed the "stem" or "root" "failure wind speed." The large effect on the chance of lodging caused by small changes in the failure wind speed is illustrated by the probabilities of experiencing different wind speeds above a UK wheat crop (Fig. 8).

Methods for Controlling Lodging Risk

Crop Management

Cultivations The use of minimal cultivations or direct drilling to prepare seed beds have been shown to reduce lodging compared with more traditional methods which usually involve plowing to about 20 cm depth [36]. It seems likely that observations for direct drilling or minimal cultivations to reduce lodging are mainly caused directly by increased soil strength resulting from greater bulk density [37, 38]. The common observations for high bulk density to impede root extension and increase root thickness [39, 40] appear to be restricted to sections of the cereal root system that play little part in anchorage, namely, the seminal roots or the distal sections of the crown roots.

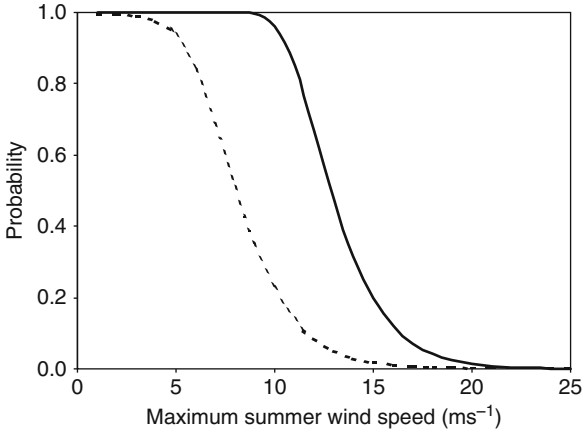
Rolling to consolidate the soil is another management practice that has been shown to reduce lodging [34, 41–43]. This can be done immediately after the primary cultivations or can be done in spring to re-consolidate the top-soil after it has been loosened by cycles of freezing and thawing. Berry et al. [43] showed that rolling a sandy loam in the spring increased shear strength in the top 5 cm by 25% and this effect persisted until harvest. No effects were observed on the biomechanical properties of the wheat roots. This study also showed that rolling before growth stage (GS) 30 [44] reduced lodging, but rolling after GS31 had no effect on lodging. It was hypothesized that this treatment damaged the extending stems, which encouraged extra tillering, and these extra shoots countered the effects of the stronger soil. This theory was supported by rolling experiments to break cereal stems by Peltonen and Peltonen-Sainio [45].



Lodging Resistance in Cereals. Figure 7

Failure wind speeds after 7 mm rain for (**a**) internode 1 and (**b**) anchorage. The ranges (0–1) are judged to represent the combined genetic and environmental range of each parameter within a high yielding wheat crop (Adapted from [9]). Stem radius – radius at the mid-point of the lowest internode; stem wall width – wall width at the mid-point of the lowest internode; stem failure yield stress – material strength of the stem wall of the bottom internode; center of gravity – height at center of gravity of the main shoot; natural frequency – rate at which the shoot oscillates; ear area – projected area of the ear; drag coefficient – resistance offered by the ear to wind; damping ratio – rate at which the shoot's oscillations stop; shoot number – number of fertile shoots per plant; root spread – diameter of the root plate defined by the thickened regions of the crown roots; root depth – depth of the root plate defined by the thickened regions of the crown roots

Sowing Date and Seed Rate The lodging risk of wheat is almost always reduced by delaying sowing [19, 46–49]. Pinthus [50] cites two studies that show reduced lodging in barley when it is sown later, but [47] observed that early sowings could reduce or increase lodging in barley. Reducing the number of plants established also causes a large reduction in the lodging risk of wheat [12, 19, 48, 51–53], and of barley [54].



Lodging Resistance in Cereals. Figure 8
Probabilities of experiencing wind gusts independent of rainfall (—) and wind gusts with $\geq$ 7 mm daily rain (---) between mid-June and mid-August within the main wheat-growing regions of the UK (Adapted from [9])

The sowing date and seed rate effects described above are caused by changes to the structure of the crop. Berry et al. [48] showed that sowing winter wheat 6 weeks earlier increased both root and stem lodging risk by increasing the base bending moment of the shoot by about 30% and by reducing the strength of the stem base and the strength of the anchorage system by about 50%. Stapper and Fischer [19] have shown that early sowing results in a greater number of extended internodes, and this probably caused the longer stems which gave rise to the greater base bending moment. Establishing 200 plants m^{-2} compared with 400 plants m^{-2} reduced lodging risk by increasing the strength of the anchorage system by more than 50% and the strength of the stem base by 15% [48]. The increase in anchorage strength more than compensated for the increase in shoot number on these plants. The greater anchorage strength has been attributed to several morphological changes including more roots per plant [12], stronger and thicker roots [55], and a wider and deeper root cone [48].

Sowing earlier or establishing more plants resulted in weaker stems because the stems were narrower and had thinner walls [48]. The mechanism by which weak stems develop is thought to be due to a greater number of shoots competing for limited photo-assimilate during early stem extension, which reduces the dry matter per unit length of the lower internodes [56]. Sparsely populated plants have many tillers [57] each of which develops up to four crown roots from each of their subterranean nodes. Therefore, it should be of no surprise that establishing fewer plants results in plants with more crown roots. Thicker and stronger roots may be caused by the absence of a strong shade avoidance response by the plant, which stimulates a greater proportion of assimilate to be partitioned to the roots [58]. Similar effects on anchorage strength were observed after later sowing as a result of fewer plants established.

Drilling Depth and Seed Treatment Deeper sowing has also been found to reduce lodging in barley [50], but in general published evidence for sowing depth effects is scarce. This is probably caused by the plants ability to adjust its crown depth to about 40 mm for sowing depths of between 40 and 70 mm [59]. This means that sowing depths over this range are unlikely to affect the depth of the structural roots. However, drilling more shallowly than 40 mm may be expected to raise the crown and its structural roots, thus weakening anchorage.

Evidence that altering crown depth can affect lodging can be found from the effect of seed treatments: fluquinconazole [60] and triadimenol [61]. Studies on fluquinconazole showed that it shortened the sub-crown internode linking the seed to the crown (the part of the plant where the crown roots and tillers emerge). This deepened the crown and the depth of the root plate, which in turn increased anchorage strength and the resistance to root lodging. Observed natural root lodging also showed that the plots treated with the triazole seed treatment were less susceptible to root lodging [60].

Disease Scott and Hollins [62] showed that wheat crops with a greater incidence of sharp eyespot (*Rhizoctonia*), brought about through inoculation, had more lodging. It has been shown that severe levels of either disease can reduce the failure moment of the lower internodes by between 30% and 40%, thus increasing the likelihood of stem lodging [10]. Interestingly, slight or medium levels of disease did not appear to weaken the stems. There is no evidence that take-all root disease increases the risk of lodging.

Nutrition An increased supply of available nitrogen from either mineralization of organic matter or from inorganic fertilizer has frequently been shown to increase lodging in wheat [48, 63, 64], barley [26, 65], and oats [66]. In wheat, the greatest increase in lodging is usually observed in response to early applications of nitrogen fertilizer before the onset of stem elongation [22, 23, 67], with applications after anthesis having no effect [52]. Contrary to this, Chalmers et al. [66] found that lodging in winter oats was reduced by applications of nitrogen before the onset of stem extension compared with later applications at GS30/31.

Both Crook and Ennos [68] and Berry et al. [48] showed that increasing the nitrogen supply to winter wheat, through either greater amounts of soil residual nitrogen at sowing or through larger applications of fertilizer in the spring, reduced the strength of the stem base and, to a lesser extent, reduced the strength of the anchorage system. Increases in crop height were generally small. Reductions in stem strength could be as much as 50% when high levels of residual nitrogen were combined with applications of fertilizer early in the spring [48]. Greater nitrogen supply almost always decreases the dry weight per unit length of the basal internodes of wheat [27, 48], barley [26], oats, and rye [22]. In relation to this, stem diameter and stem wall width are also frequently reduced. Berry et al. [48] showed that high levels of residual nitrogen reduced the strength of the stem wall material. These findings were supported by Crook and Ennos [27], who showed that a component of material strength, Young's modulus (which approximates to the stiffness of the stem), was also reduced by more fertilizer in spring. The cause of these effects may have resulted from a reduced amount of lignified tissue within the sclerenchyma zone and the thickness of the sclerenchyma cell walls [22]. Reductions in anchorage strength in response to more nitrogen can be linked with fewer roots, which are thinner with smaller bending and tensile strengths [55, 68]. Mulder [22] showed that the crown roots of oat plants supplied with large amounts of nitrogen were practically free from lignified cells beneath the epidermis, in contrast with plants supplied with moderate amounts of nitrogen.

In the consideration of nutrition, there should be a differentiation between the effects that result from repairing a deficiency and effects resulting from super-optimal supply. For example, it appears that an increase in nitrogen will increase lodging risk, but the mechanism by which this occurs will depend on the level of nitrogen supply. If a nitrogen deficiency is being repaired then lodging risk increases because the leverage of the shoot and ear increases. It seems likely that the stem strength, and possibly the anchorage strength, will be increased by correcting the deficiency but these effects are outweighed by the greater leverage. Additional nitrogen increases the shoot leverage by progressively smaller amounts, but lodging risk continues to rise because the strength of the stem base and root system begins to decrease as a result of the indirect effects of shading. It is possible that phosphorus behaves in a similar way to nitrogen [67]. However, potassium might be different as the evidence [22, 50, 69] indicates that it can reduce lodging when repairing a deficiency and additional amounts have no effect. This may be due to the important role that this element plays in regulating the turgor of plant tissues.

Plant Growth Regulators Plant growth regulators (PGRs) are synthetic compounds that can be used to reduce lodging in cereal species. They are most commonly used for this purpose in north and western European countries and in North America. In the UK, 89% of the winter wheat is treated with PGRs [70]. PGRs can be classified into two main groups: inhibitors of gibberellic acid biosynthesis and ethylene-releasing compounds. The most commonly used inhibitors of gibberellic acid biosynthesis in cereal crops are chlormequat chloride, mepiquat chloride, trinexapac-ethyl and prohexadione-calcium [71]. Ethephon is the most commonly used ethylene-releasing compound used on cereals [72].

Plant growth regulators have been shown to be a cost-effective method of reducing the incidence of lodging. PGRs applied before the emergence of the ear reduced lodging in almost all of the vast number of published experiments that have studied their effect and in which lodging occurred, with reductions in the percentage area lodged of anything up to 70% [10]. The primary mechanism by which PGRs have been shown to reduce lodging risk is by reducing crop height, with height reductions of up to 40% [10]. The variation on plant height reduction is probably caused by interactions between the type of active ingredient, the cereal

species together with the stage of plant development, and the environmental conditions when the chemical is applied. No evidence has been found for PGRs to increase the strength of the stem or of the anchorage system [48, 68], although it must be recognized that only two studies have investigated the effects of PGRs by directly measuring stem and anchorage strength. Chlormequat has been shown to be effective at reducing lodging in winter and spring wheat, oats, and rye, but less effective on barley [73, 74]. Barley undergoes large height reductions in response to a mixture of ethephon and mepiquat chloride [13, 75].

Summary of Crop Management Effects Many crop management practices result in large changes in lodging risk by either affecting the wind-induced leverage of the shoot, the strength of the stem base, the strength of the anchorage system, or a combination of all three mechanisms. Furthermore, the strengths of the stem base and anchorage system are often changed by different amounts for any change in crop management. This means that certain types of crop management would be expected to reduce one type of lodging (stem or root) more than the other. The effects of several crop management practices on the risk of stem and root lodging have been summarized by Berry et al. [76] in terms of changes to the failure wind speed (Table 1). This shows that stem lodging is best reduced by sowing on soils with less residual nitrogen and by reducing and delaying the amount of fertilizer applied in the spring. Root lodging is best reduced by establishing fewer plants, using a seed treatment with growth regulatory properties and by rolling in the spring to consolidate the soil. Delayed sowing and growth regulators were estimated to reduce stem and root lodging by equal amounts.

Plant Breeding

The *Rht* (Reduced height) alleles began to be introduced into wheat varieties during the 1960s and 1970s and are now part of the germplasm of most high yielding semi-dwarf varieties. In UK and German wheats, *Rht*1 and *Rht*2 alleles can reduce height by 14–17% independently of each other and by 42% when in combination [2]. *Rht*3 can reduce height by 59%, but has not yet been used in commercial varieties. In the UK, the *Rht*1 and *Rht*2 alleles have helped to reduce the height of wheat cultivars from over 1 m to about 0.7–0.9 m between the early 1970s and the mid-1990s. This reduction in height and consequent reduction in leverage has enabled the amount of nitrogen fertilizer applied to wheat to be increased from less than 100 kg ha^{-1} in the early 1970s to nearly 200 kg ha^{-1} in the 1990s [77] without a dramatic increase in the incidence of lodging. Pleiotropic effects have further added to the yield improvements associated with these *Rht* genes in wheat. Reduced stem growth rates allow

Lodging Resistance in Cereals. Table 1 Effect of crop management on the wind speed required to cause stem or root lodging (Adapted from [60, 76]

Factor	Increase in stem failure wind speed ms^{-1}	Increase in root failure wind speed ms^{-1}
Less soil residual N (116–71 kg N ha^{-1})	2.3 (3.9 [a])	1.3
Seed treatment with PGR activity (e.g., Fluquinconazole [60])	0	0.7
Delayed sowing (per week, between 20 September and 1 November)	0.5	0.5
Less plants established (per 100 plants m^{-2}, between 400 and 200 plants m^{-2})	0.8	1.8
PGRs (split chlormequat @ GS30/31)	1.4	1.4
Delayed and less fertilizer N (Target GAI of 5)	1.4	0.8
Spring rolling (pre-GS30)	0	0.8

[a]At 400 plants m^{-2}

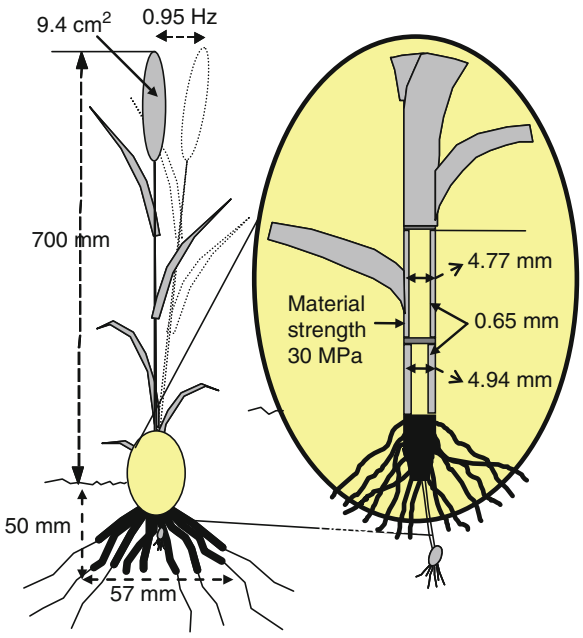
more resources to be allocated to the developing ear which results in a greater number of fertile florets and grains per year.

In oats, the variety S172, released in 1939, has been reported to be Europe's first dwarf cereal variety [78]. However, dwarfness in this and several derived varieties was associated with small grains and a yield penalty. Recently varieties have been released that contain the *DW-6* dwarfing gene which was discovered in a mutation program in Canada [79] and has been shown to shorten the peduncle [80]. This gene has been shown to reduce height by 20–75 cm, have thicker stems, and to reduce lodging by large amounts [81]. Major dwarfing genes are common in spring barley. The *ari-eGP* dwarfing gene was found in cvs Golden Promise and Midas, which comprised over 70% of the Scottish barley crop from the mid-1970s to early 1980s. The *ari-eGP* gene was then superseded by the *sdw1* dwarfing gene, such that by 1989 the percentages of certified seed carrying the sdw1 and ari-eGP genes were 74 and 8, respectively [82].

There is great potential to continue increasing lodging resistance through further height reductions via the introduction of more extreme dwarfing genes such as *Rht3* in wheat. However, several studies have shown that yield is reduced when crops are shortened too much [2–7]. The reduction in yield appears to be exacerbated by high temperatures or drought stress. Several of these studies indicate that the minimum crop height for optimum yield lies between 0.7 m and 1.0 m, a height which many modern varieties have already achieved. There is evidence that the same problem could occur in oats because the *DW-6* dwarfing gene has been associated with small grains, low kernel content and poor extrusion of the panicles from the flag leaves in some genetic backgrounds [81]. It therefore seems unlikely that much further improvement in lodging resistance can be made by continuing to shorten wheat crops and there may be only limited further shortening possible in other cereal species. As a result breeders must target other plant traits, namely, stem strength and anchorage strength, to improve lodging resistance and counter greater yields.

The lodging model of Baker et al. [29] has been used with preliminary datasets describing the dry matter costs of improving traits associated with stem strength and anchorage strength to estimate the dimensions of a wheat plant to make it lodging-proof for the least investment of biomass in the supporting stem and root system [83]. The characteristics required to give a crop yielding 8 t ha^{-1} with 500 shoots m^{-2} and 200 plants m^{-2} a lodging return period of 25 years in a UK environment include a height of 0.7 m, a root plate spread of 57 mm, and for the bottom internode a wall width of 0.65 mm, a stem diameter of 4.94 mm, and a material strength of 30 Mpa (Fig. 9). Observations of a range of varieties grown in the UK showed that the root plate of the best variety was 7 mm less than the ideotype target, the widest stem was 0.5 mm below the ideotype target, other stem character targets were achieved but not all in one variety, and the height target was achievable with the use of plant growth regulators.

It is possible that the lodging-proof ideotype traits could be achieved because large differences among wheat varieties have been observed for the traits that determine stem strength and anchorage strength [42, 76, 84]. The latter study showed that anchorage strength could vary from 206 Nmm to 587 Nmm and stem strength could vary from 122 Nmm to 175 Nmm



Lodging Resistance in Cereals. Figure 9
Description of a lodging-proof ideotype for wheat grown in a UK environment as defined in [83]

between varieties. These differences were caused by a combination of wider, deeper root plates and stiffer roots for anchorage strength, and wider, thicker walled stems with a greater material strength for overall stem strength. Subsequent studies using more varieties and breeding lines showed even greater genetic variation in the traits which determine stem strength and anchorage strength [83, 85]. In barley, differences in culm wall thickness have frequently been positively correlated with varietal differences in lodging resistance [86–88]. If oats and barley have a similar level of genetic variation in stem and anchorage strength as has been observed in wheat then there appears to be significant scope for breeders to improve the strength of the stems and anchorage systems of cereals. However, these traits are time-consuming to measure; therefore, new technologies will be required to help plant breeders to rapidly select them.

Future Directions

The majority of lodging research has concentrated on wheat. This has enabled models of the lodging mechanism to be developed, which have been used to identify the critical plant characters, to quantify the effects of factors on lodging and to elucidate the mechanisms by which these effects are caused. A rudimentary model of lodging has been developed for barley, but further work is required to fully validate this model, particularly the way in which flexural rigidity of the stem is considered. Understanding about lodging in other cereals, such as rice, maize, and oats, lags far behind wheat. In order to replicate the advances made in wheat the next step must be to model the lodging mechanisms in these cereals. This may require fundamentally different types of lodging model due to the different plant structures of these species.

Historically reducing crop height has been the main avenue by which the lodging risk of wheat has been reduced. However, several studies on wheat indicate that the minimum height compatible with high yields is around 0.7 m. This height has been achieved by many wheat varieties. Crop height also has a major impact on the structural dry matter requirements for lodging resistance with each additional centimeter in height increasing the stem dry matter required by 0.23 t/ha [83]. Further work must investigate why there appears to be a minimum height for high yield, whether this barrier can be overcome and whether the minimum height for high yield varies between environments.

Preliminary studies with wheat have indicated that increasing stem strength has a significant dry matter cost which could compete with the formation of grain yield [83]. Further work must quantify the dry matter cost of increasing stem strength, understand the optimum combination of stem diameter, wall width, and material that is required to minimize the dry matter cost of increasing stem strength, and quantify the extent to which this competes with yield formation.

It has been predicted that breeders must increase the stem strength and anchorage strength of wheat in order to achieve a lodging-proof ideotype for wheat. These traits are time-consuming to measure; therefore methods must be developed for rapidly assessing these traits. Berry et al. [89] and Keller et al. [90] have identified more than one quantitative trait loci associated with these traits and indicated that they are controlled by several genes. Further work will be required to better understand the genetic control of the traits associated with lodging and to investigate whether reliable genetic markers can be identified which work across a range of genotypes and environments. Phenotypic screens must also be investigated to assess whether they can offer an alternative method to genetic markers for selecting germplasm.

Recent work has shown that although a wide genetic variation for the key lodging traits is present within UK wheat breeding material [85], few of the target traits required for complete lodging proof have been identified. This indicates that a wider range of germplasm must be assessed to find the target traits.

Currently there is not a reliable method predicting the likelihood of lodging from earlier stages of crop development, when growers can alter lodging risk through their crop management (PGRs, fertilizer, rolling). Therefore, further research must study the development of the lodging-associated plant characters with the objective of predicting lodging from early assessments of the crop. Any prediction scheme must predict lodging risk before, or soon after, the onset of stem elongation to enable growers to alter their crop management accordingly.

PGRs reduce lodging risk by shortening crops, but there is little published evidence that they can

strengthen the stems and anchorage system. Further research should investigate the effect of existing PGRs on stem and anchorage strength as well as focusing on discovering new chemicals that strengthen these traits.

Bibliography

1. Conway G (1997) The doubly green revolution: food for all in the 21st century. Cornell University Press, Ithaca
2. Flintham JE, Borner A, Worland AJ, Gale MD (1997) Optimizing wheat grain yield: effects of *Rht* (gibberellin-insensitive) dwarfing genes. J Agric Sci 128:11–25
3. Kertesz Z, Flintham JE, Gale MD (1991) Effects of Rht dwarfing genes on wheat grain yield and its components under eastern European conditions. Cereal Res Commun 19:297–304
4. Miralles DJ, Slafer GA (1995) Individual grain weight responses to genetic reductions in culm length in wheat as affected by source-sink manipulations. Field Crops Res 43:55–66
5. Allan RE (1986) Agronomic comparisons among wheat lines nearly isogenic for three reduced height genes. Crop Sci 26:707–710
6. Baylan HS, Singh O (1994) Pleiotropic effects of GA-insensitive *Rht* genes on grain yield and its component characters in wheat. Cereal Res Commun 22:195–200
7. Richards RA (1992) The effect of dwarfing genes in spring wheat in dry environments. I. Agronomic characteristics. Aust J Agric Res 43:517–527
8. Spink JH, Berry PM, Wade AP, White EL (2004) Minimising chlormequat residues in harvested grain. Home-Grown Cereals Authority Research Project No. 332. HGCA, London, p 44
9. Berry PM, Sterling M, Baker CJ, Spink JH, Sparkes DL (2003) A calibrated model of wheat lodging compared with field measurements. Agric For Meteorol 119:167–180
10. Berry PM, Sterling M, Spink JH, Baker CJ, Sylvester-Bradley R, Mooney S, Tams A, Ennos AR (2004) Understanding and reducing lodging in cereals. Adv Agron 84:215–269
11. Weibel RO, Pendleton JW (1964) Effect of artificial lodging on winter wheat grain yield and quality. Agron J 56:487–488
12. Easson DL, White EM, Pickles SJ (1993) The effects of weather, seed rate and cultivar on lodging and yield in winter wheat. J Agric Sci 121:145–156
13. Stanca AM, Jenkins G, Hanson PR (1979) Varietal responses in spring barley to natural and artificial lodging and to a growth regulator. J Agric Sci 93:449–456
14. Sisler WW, Olsen PJ (1951) A study of methods of influencing lodging in barley and the effect of lodging upon yield and certain quality characteristics. Sci Agric 31:177–186
15. Jedel PE, Helm JH (1991) Lodging effects on a semidwarf and two standard barley cultivars. Agron J 83:158–161
16. Pendleton JW (1954) The effect of lodging on spring oat yields and test weights. Agron J 46:265–267
17. Setter TL, Laureles EV, Mazaredo AM (1997) Lodging reduces the yield of rice by self-shading and reductions in canopy photosynthesis. Field Crops Res 49:95–106
18. Fischer RA, Stapper M (1987) Lodging effects on high yielding crops of irrigated semi-dwarf wheat. Field Crops Res 17: 245–248
19. Stapper M, Fischer RA (1990) Genotype, sowing date and plant spacing influence on high-yielding irrigated wheat in southern New South Wales. II. Growth, yield and nitrogen use. Aust J Agric Res 41:1021–1041
20. Laude HH, Pauli AW (1956) Influence of yield on lodging and other characteristics in winter wheat. Agron J 48:452–455
21. Day AD, Dickson AD (1958) Effect of artificial lodging on grain and malt quality of fall sown irrigated barley. Agron J 50: 338–340
22. Mulder EG (1954) Effect of mineral nutrition on lodging in cereals. Plant Soil 5:246–306
23. Berry PM, Spink JH, Griffin JM, Sylvester-Bradley R, Baker CJ, Scott RK, Clare RW (1998) Research to understand, predict and control factors affecting lodging in wheat. Home-Grown Cereals Authority Research Project No. 169. HGCA, London, p 131
24. HGCA (1997) Cereal statistics 1997. Home Grown Cereals Authority, London
25. Neenan M, Spencer-Smith JL (1975) An analysis of the problem of lodging with particular reference to wheat and barley. J Agric Sci 85:495–507
26. White EM (1991) Response of winter barley culivars to nitrogen and a plant growth regulator in relation to lodging. J Agric Sci 116:191–200
27. Crook MJ, Ennos AR (1993) The mechanics of root lodging in winter wheat (*Triticum aestivum* L). J Exp Bot 44:1219–1224
28. Graham J (1983) Crop lodging in British wheats and barleys. Ph.D. thesis, University of Reading, Reading, Berkshire, UK
29. Baker CJ, Berry PM, Spink JH, Sylvester-Bradley R, Clare RW, Scott RK, Griffin JM (1998) A method for the assessment of the risk of wheat lodging. J Theor Biol 194:587–603
30. Berry PM, Sterling MS, Mooney SJ (2006) Development of a model of lodging for barley. J Agron Crop Sci 192:151–158
31. Sterling M, Baker CJ, Berry PM, Wade A (2003) An experimental investigation of the lodging of wheat. Agric For Meteorol 119:149–165
32. Baker CJ (1995) The development of a theoretical model for the windthrow of plants. J Theor Biol 175:355–372
33. Ennos AR (1991) The mechanics of anchorage in wheat (*Triticum aestivum* L.) II. Anchorage of mature wheat against lodging. J Exp Bot 42:1607–1613
34. Pinthus MJ (1967) Spread of the root system as indicator for evaluating lodging resistance of wheat. Crop Sci 7:107–110
35. Easson DL, White EM, Pickles SJ (1992) A study of lodging in cereals. HGCA Research Project No. 52. Home-Grown Cereals Authority, London
36. Ellis FB, Christian DG, Graham JP, Jackson R, Wakel YT (1978) Long-term tillage experiments – agronomic results. Annual Report for 1977, ARC Letcombe Laboratory, Wantage
37. Schjonning P, Rasmussen KJ (2000) Soil strength and soil pore characteristics for direct drilled and ploughed soils. Soil Tillage Res 57:69–82

38. Finney JR, Knight BAG (1973) The effect of soil physical conditions produced by various cultivation systems on the root development of winter wheat. J Agric Sci 80:435–442
39. Wilson AJ, Robards AW (1977) Effects of mechanical impedance on root growth in barley, *Hordeum vulgare* L. J Exp Bot 28:1216–1227
40. Materechera SA, Dexter AR, Alston AM (1991) Penetration of very strong soils by seedling roots from different plant species. Plant Soil 135:31–41
41. Kopecky H (1970) The effect of compacting the soil on the formation of yield and on the quality of malting barley. Rostl Vyroba 16:309–319
42. Crook MJ, Ennos AR (1994) Stem and root characteristics associated with lodging resistance in four winter wheat cultivars. J Agric Sci 123:167–174
43. Berry PM, Spink JH, Sylvester-Bradley R, Pickett A, Sterling M, Baker C, Cameron N (2002) Lodging control through variety choice and management. In: Proceedings of the 8th Home-Grown Cereals Authority R&D Conference on Cereals and Oilseeds. HGCA, London, pp 7.1–7.12
44. Tottman DR (1987) Decimal code for the growth stages of cereals. Ann Appl Biol 110:683–687
45. Peltonen J, Peltonen-Sainio P (1997) Breaking uniculm growth habit of spring cereals at high latitudes by crop management II: tillering, grain yield and yield components. J Agron Crop Sci 2:87–95
46. Green CF, Ivins JD (1985) Time of sowing and the yield of winter wheat. J Agric Sci 104:235–238
47. Fielder AF (1988) Interactions between variety and sowing date for winter wheat and winter barley. HGCA Research Project No. 6. Home-Grown Cereals Authority, London
48. Berry PM, Griffin JM, Sylvester-Bradley R, Scott RK, Spink JH, Baker CJ, Clare RW (2000) Controlling plant form through husbandry to minimise lodging in wheat. Field Crops Res 67:59–81
49. Spink JH, Foulkes MJ, Gay A, Bryson R, Berry PM, Sylvester-Bradley R, Semere T, Clare RW, Scott RK, Kettlewell PS, Russell G (2000a) Reducing winter wheat production costs through crop intelligence information on variety and sowing date, rotational position, and canopy management in relation to drought and disease control. HGCA Research Project No. 235. Home-Grown Cereals Authority, London
50. Pinthus MJ (1973) Lodging in wheat, barley and oats: the phenomenon, its causes and preventative measures. Adv Agron 25:209–263
51. Lowe LB, Carter OG (1972) Response of four wheat cultivars to applications of (2-chloroethyl) trimethyl ammonium chloride (CCC). Aust J Exp Agric Anim Husb 12:75–80
52. Webster JR, Jackson LF (1993) Management practices to reduce lodging and maximise grain yield and protein content of fall-sown irrigated hard red spring wheat. Field Crops Res 33:240–259
53. Spink JH, Whaley JM, Semere T, Wade AP, Sparkes DL, Foulkes MJ (2000b) Prediction of optimum plant population in winter wheat. HGCA Research Project No. 234. Home Grown Cereals Authority, London
54. Kirby EJM (1967) The effect of plant density upon the growth and yield of barley. J Agric Sci 68:317–324
55. Easson DL, Pickles SJ, White EM (1995) A study of the tensile strength required to pull wheat roots from soil. Ann Appl Biol 127:363–373
56. Berry PM (1998) Predicting lodging in winter wheat. Ph.D. thesis, University of Nottingham, Nottingham, UK
57. Whaley JH, Sparkes DL, Foulkes MJ, Spink JH, Semere T, Scott RK (2000) The physiological response of winter wheat to reductions in plant density. Ann Appl Biol 137:165–177
58. Kasperbauer MJ, Karlen DL (1986) Light-mediated bioregulation of tillering and biosynthate partitioning in wheat. Physiol Plant 66:159–163
59. Kirby EJM (1993) Effect of sowing depth on seedling emergence, growth and development in barley and wheat. Field Crops Res 35:101–111
60. Berry PM (2008) Effects of seed treatments on wheat lodging. In: Proceedings of the Crop Protection in Northern Britain Conference, Dundee
61. Montfort F, Klepper BL, Smiley RW (1996) Effects of two triazole seed treatments, triticonazole and triadimenol, on growth and development of wheat. Pestic Sci 46:315–322
62. Scott PR, Hollins TW (1974) Effects of eyespot on the yield of winter wheat. Ann Appl Biol 78:269–279
63. Bremner PM (1969) Effects of time and rate of nitrogen application on tillering, "sharp eyespot" (*Rhizoctonia solani*) and yield in winter wheat. J Agric Sci 72:273–280
64. Pandey DS, Kumar D, Misra RD, Prakash A, Gupta VK (1997) An integrated approach of irrigation and fertilizer management to reduce lodging in wheat (*Triticum aestivum*). Indian J Agron 42:86–89
65. Widdowson FV (1962) Applying fertiliser for spring barley. Ann Appl Biol 50:356–360
66. Chalmers AG, Dyer CJ, Sylvester-Bradley R (1998) Effects of nitrogen fertilizer on the grain yield and quality of winter oats. J Agric Sci 131:395–407
67. Miller FL, Anderson L (1963) Relationship in winter wheat between lodging physical properties of stems and fertilizer treatments. Crop Sci 3:468–471
68. Crook MJ, Ennos AR (1995) The effect of nitrogen and growth regulators on stem and root characteristics associated with lodging in two cultivars of winter wheat. J Exp Bot 46:931–938
69. Gaspar PE, Reeves DL, Schumacher TE, Fixen PE (1994) Oat cultivar response to potassium chloride on soils testing high in potassium. Agron J 86:255–258
70. Garthwaite DG, Thomas MR, Heyward E, Battersby A (2006) Arable crops in Great Britain 2006, Pesticide Usage Survey Report 213. Department for the Environment Food and Rural Affairs, London
71. Rademacher W (2000) Growth retardents: effects on gibberellin biosynthesis and other metabolic pathways. Annu Rev Plant Physiol Plant Mol Biol 51:501–531

72. Rademacher W (1993) PGRs- present situation and outlook. Acta Hortic 329:296–308
73. Humphries EC (1968) CCC and cereals. Field Crop Abstr 21: 91–99
74. Matthews S, Thompson WJ (1983) New roles for growth regulators in cereal production. In: Wright DW (ed) The yield of cereals. Royal Agricultural Society of England, London, pp 25–31
75. Herbert CD (1982) Growth regulation in cereals- chance or design? In: McLaren JS (ed) Chemical manipulation of crop growth and development. Butterworth Scientific, London, pp 315–327
76. Berry PM, Spink JH, Gay AP, Craigon J (2003) A comparison of root and stem lodging risks among winter wheat cultivars. J Agric Sci 141:191–202
77. Gooding MJ, Davies WP (1997) Wheat production and utilization: systems, quality and environment. CAB International, Wallingford, p 355
78. Valentine J, Cowan S, Howarth C, Langdon T (2003) One hundred years of progress in cereal breeding from Mendel to molecular selection – oats. In: Faccioli P, Marè C, Stanca AM (eds) Proceedings of the EUCARPIA Cereal Section Meeting. From biodiversity to genomics: breeding strategies for small grain cereals in the Third Millennium, Salsomaggiore, pp 21–25
79. Brown PD, McKenzie RIH, Mikaelson K (1980) Agronomic, genetic and cytologic evaluation of a rigorous new semi-dwarf oat. Crop Sci 20:303–306
80. Milach SCK, Rines HW, Philipps RL (2002) Plant height components and gibberelic acid response for oat dwarf lines. Crop Sci 42:1147–1154
81. Valentine J, Jones DM, Jones JE, Griffiths TER, Middleton B (1997) Genetic improvement in oats with specific reference to winter-hardiness and lodging resistance of winter oats and improvement of naked oats. HGCA Research Project No 145. Home-Grown Cereals Authority, London
82. Thomas WTB, Powell W, Swanston JS (1991) The effects of major genes on quantitatively varying characters in barley. 4. The gpert and denso loci and quality characters. Heredity 66:381–389
83. Berry PM, Sylvester-Bradley R, Berry S (2007) Ideotype design for lodging-proof wheat. Euphytica 154:165–179
84. Spink JH, Berry PM, Fenwick R, Gay AP (2003) To establish separate standing power ratings for stem and root lodging in the UK recommended lists for wheat. HGCA Research Project No. 305. Home-Grown Cereals Authority, London
85. Berry PM, Berry S (2008) Breeding for improved lodging resistance in wheat. In: Plant Genetic Resources for Food & Agriculture. AAB, 22 May 2008, Warwick HRI, UK
86. Dunn GJ, Briggs KG (1989) Variation in culm anatomy among barley cultivars differing in lodging resistance. Can J Bot 67:1838–1843
87. Jezowski S (1981) Variation, correlation and heritability of characters determining lodging of spring barley (*Hordeum vulgare* L.). II. Analysis of relationship between lodging grade and some morphological characters of spring barley varieties. Genet Pol 22:45–61
88. Tandon JP, Jain KB, Singh SB (1973) Relationships between lodging resistance and some morphological characteristics in barley. Indian J Genet Plant Breed 33:347–354
89. Berry PM, Berry ST, Spink JH (2008) Identification of genetic markers for lodging resistance in wheat. Home-Grown Cereals Authority Project Report No 441. HGCA, London, p 16
90. Keller M, Karutz C, Schmid JE, Stamp P, Winzler M, Keller B, Messner MM (1999) Quantitative trait loci for lodging resistance in a segregating wheat x spelt population. Theor Appl Genet 98:1171–1182

Mariculture Systems, Integrated Land-Based

Muki Shpigel
Israel Oceanographic and Limnological Research, National Center for Mariculture, Eilat, Israel

Article Outline

Glossary

Detritivores (also known as *saprophages*) They are heterotrophs that obtain nutrients by consuming detritus (decomposing organic matter).

Halophyte Salt-loving plants that can be grown at higher salinities than most traditional crop plants.

IMTA The Integrated Multi-Trophic Aquaculture System (IMTA) is an aquaculture practice in which excretions of one or more organisms are utilized by other cultured organisms from different trophic (nutritional) levels within the system.

Land-based and offshore mariculture systems Two methods of seawater aquaculture (mariculture); the former on land and the latter in the ocean.

Polyculture An aquaculture practice which involves culture of two or more species from the same or different trophic levels in the same water reservoir.

RAS Recirculated Aquaculture System (RAS) is an aquaculture practice for the rearing of aquatic organisms wherein 90% or more of the water is recycled within the system.

Sludge Solid/particulate waste that includes, among other components, feces, uneaten feed, algae and bacteria, which sinks to the bottom of aquaculture water reservoirs.

Definition of Subject

The Integrated Multi-Trophic Aquaculture System (IMTA) is an aquaculture practice in which excretions of one or more organisms are utilized by other cultured organisms from different trophic (nutritional) levels. IMTA systems are distinct from polyculture systems, which involve two or more species from the same or different trophic levels in the same water reservoir. In a typical IMTA, the various species are cultured in separate spatial entities, permitting intensification and optimization of production. The IMTA concept has been increasingly adopted in modern day aquaculture, including land-based (Fig. 1) [1–5] and offshore mariculture [6, 7].

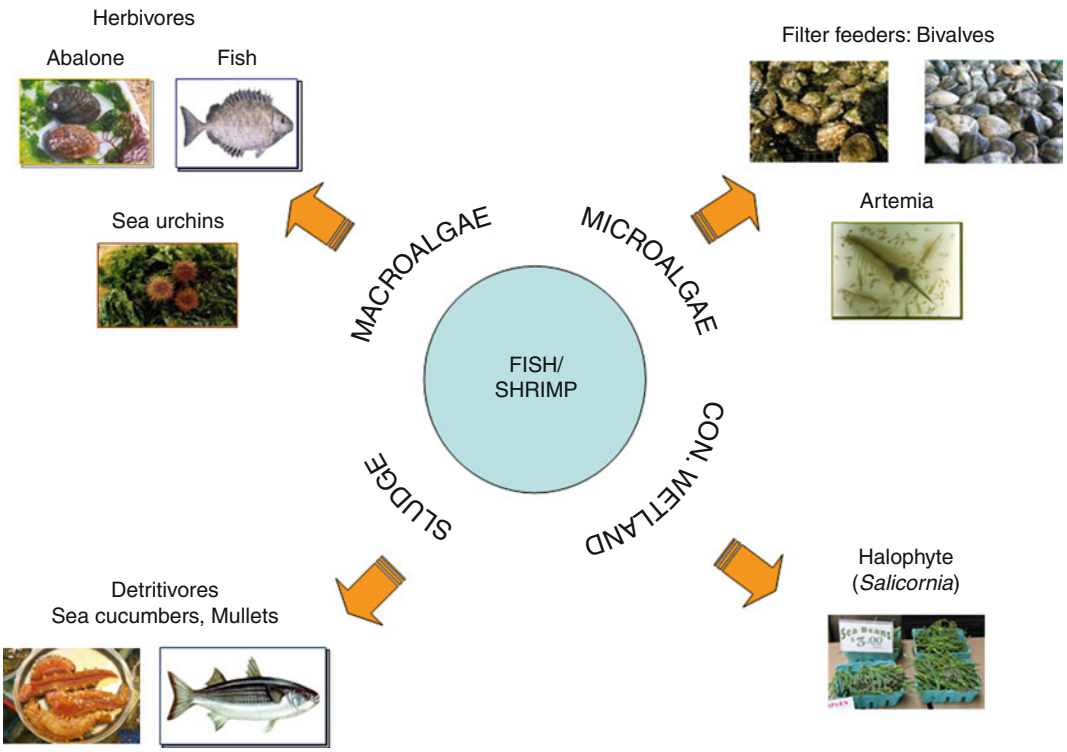
In land-based IMTA systems, seawater is pumped from the sea to fish or shrimp ponds. A pelleted diet is the only source of nutrients for the animals in the system. Nutrient-rich effluent water from these ponds can take three directions: microalgae ponds, macroalgae ponds, and constructed wetlands with halophyte plants. The microalgae can be utilized by filter feeders such as *Artemia* or/and bivalves. The macroalgae can be utilized by macroalgivores such as abalone or sea urchins, and detritus can be utilized by detritivores such as mullets, sea cucumbers, or polychaete worms (Fig. 1).

Introduction

The concept of polyculture and IMTA systems is not new. Such systems of different species of fish, or combinations of invertebrates and fish, have been existing in ancient Egypt and China for thousands of years. Artificial enclosures or natural ponds in tidal zones were generally used. Extensive traditional IMTA and polyculture systems are still practiced today in various parts of Asia in fresh and salt water. Rice and fish are cultured together in China. Earthen ponds, in association with wild or agricultural plants, are used on a wide scale in fish and shrimp farming in China, Indonesia, Taiwan, Thailand, Japan, Vietnam, India, the Philippines, and Ecuador. In Europe, ducks, fish, and crayfish have been raised together in freshwater ponds. This type of extensive production has proven sustainable, because it utilizes organisms that feed on different levels of the food web, and maintains a clean environment.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Mariculture Systems, Integrated Land-Based. Figure 1
Schematic design of land-based IMTA systems (con. = constructed)

The traditional IMTA and polyculture systems are more environmentally friendly than modern intensive mono-aquaculture systems. These systems utilize fewer resources and do not pollute surrounding waters with waste products, because they generally sustain relatively low stocking densities and do not employ fertilizers. Most of them rely on natural production of food. This concept has increasingly been adopted for modern aquaculture, including land-based and sea-cage mariculture. With dramatic increases in global human population, food demand, and overfishing problems, traditional extensive aquaculture cannot satisfy present demand, and much less so the projected future demand, for sea products.

Modern intensive monoculture systems require high levels of resources and produce undesirable wastes. They are dedicated to a few expensive species and do not generate a large amount of food. Intensive aquaculture uses extensive amounts of resources such as water, feeds, fertilizers, chemicals, and energy, while discharging fecal material, uneaten feed, excretions, and drugs into the environment. In turn, this creates eutrophication of the water, has deleterious effects on marine life, increases the risks of antibiotic resistance in organisms, has an adverse effect on biodiversity, and contributes to habitat destruction. The economic success of intensive monoculture in sea cages or land-based facilities has much to do with the fact that, even today, pollution of the environment involves little or no monetary outlay or penalty for the growers. In most countries, aquaculture does not yet include the cost of effluent treatment. However, in the industrialized nations, this age is coming to a timely end and in Europe, there are already laws and regulations requiring effluent treatment and imposing fines for noncompliance. In some countries, this cost can be as high as €0.5–1 kg^{-1} feed, resulting in an expense of €250,000–350,000 per annum for medium-scale RAS (Recirculating Aquaculture System) farms (250 t/year). Awareness is growing among scientists, industry, the public, and politicians that technologies disregarding environmental impact are neither sustainable nor acceptable.

History

The development of modern land-based IMTA using extractive organisms such as shellfish, microalgae, and seaweeds began in the 1970s with the pioneer work of Goldman et al. [1] and Ryther et al. [2] in the treatment of household effluents. Phytoplankton was cultured in a mixture of domestic wastewater effluent and seawater, fed to suspension-feeder molluscs, and the dissolved remnants of nutrients in the final effluent were assimilated by seaweeds. As the food value of organisms grown on human waste effluents was questionable, adaptations of this principle to the treatment of intensive aquaculture effluents in both inland and coastal areas were proposed [8] and were followed by the integration into a system of carnivorous fish and abalone (e.g., [9]). The first practical and quantitative integrated land-based cultures of marine fish and shellfish, with phytoplankton as biofilter and food for shellfish, were constructed in Israel by Hughes-Games [10] and Gordin et al. [11]. A semi-intensive seabream and gray mullet pond system with silicate-rich green water, located on the coast of the Gulf of Eilat (Red Sea), supported dense populations of diatoms, excellent for feeding oysters [12, 13]. Later, the development of a practical intensive culture of bivalves in phytoplankton-rich effluents was described in a series of articles [3, 14–18]. Lefebvre et al. [19] showed that detritical waste from intensive fish farming can contribute to the growth of bivalves and reduce particulate matter in the water. Jones et al. [20], using the Sydney rock oyster *Saccostrea commercialis*, significantly reduced the concentration of suspended particulates including algae, bacteria, and inorganic particles in integrated systems.

Studies showing the performance of seaweed in land-based IMTA, initially at laboratory scale and later expanded to outdoor pilot scale, began to appear in the 1970s [21, 22]. The theoretical and practical principles of intensive large-scale land-based seaweed culture were studied and developed first at Woods Hole and later at Harbor Branch Oceanographic Institution in Florida – U.S.A. [8, 23–25]. The quantitative aspects of their functioning have been described [14, 16, 26–29]. Fish, abalone, and seaweed IMTA systems were studied by Shpigel et al. [30], Butterworth [31], and Nobre et al. [32]. The aspects of bioeconomics of land-based IMTA are described by Nobre et al. [32], Neori et al. [33], and Bunting and Shpigel [34].

Offshore IMTA system is a relatively new concept that started in the late nineties and is a modification of the land-based IMTA. In coastal integrated mariculture, shellfish and seaweed are cultured in proximity to cage fish culture [6, 7]. Kelp (brown algae) [35, 36] and red algae [37, 38] efficiently take up dissolved inorganic nitrogen excreted by the fish [39], so that seaweed production and quality are often higher in areas surrounding fish cages than elsewhere [6, 40–42]. However, nutrient removal efficiency in offshore IMTA is still relatively low, ranging between 15% and 25% [43].

The concept of IMTA systems is generic and can be applied to cold, warm, and temperate waters, in intensive, semi-intensive, and extensive systems, in sea cages or land-based facilities, in fresh water in land-based facilities or lakes, and all of the above in closed, semi-closed, or flow-through systems.

In recent years, several enterprises and research facilities have begun setting up land-based IMTA; most of the systems are pilot scale or R&D facilities. The IMTA typically include two or three species. In most of the studies, seaweed and microalgae are used as biofilters for the dissolved nutrients (review by Neori et al. [33] and Soto [44]). A broad spectrum list of selected organisms being used in farms and in R&D is presented in Fig. 2. Key species in cold water are salmon, mussels, and the seaweeds *Gracilaria, Laminaria*, and *Porphyra*. For temperate and warm seawater, sea bream, sea bass, oysters, clams, and *Ulva lactuca* are the predominant cultured species (Fig. 2).

Over 200 species are currently the object of R&D projects and in commercial farms and research institutes around the world, in various climate conditions. A significant number of fish and shellfish are cultured in temperate water, and a relatively low number of fish and large number of seaweeds in cold-water climates (Fig. 3).

Nutrient Budget in Land-Based IMTA

Protein in fish or shrimp feed is the most expensive component of nitrogen input into the IMTA systems. In conventional cages or ponds, fish or shrimps assimilate only 20–30% of the nitrogen, while the rest

KEY SPECIES CULTURED IN POLYCULTURE AND MULTI-TROPHIC SYSTEMS

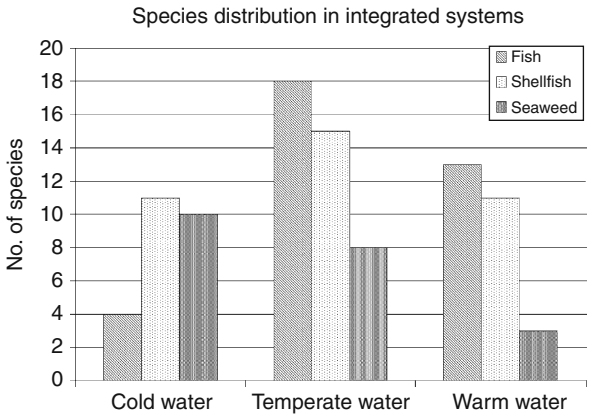
Cold Seawater	Temperate Seawater	Warm Seawater
Oncorhynchus sp.	*Pagrus major*	*Sparus aurata*
Crassostrea sp.	*Sparus aurata*	*Lates calcarifer*
Mytilus edulis	*Dicentrarchus labrax*	*Mugil cephalus*
Haliotis rufescens	*Mercenaria mercenaria*	*Penaeus* sp.
Gracilaria sp.	*Ostrea edulis*	*Crassostrea gigas*
Laminaria sp.	*Ruditapes decussates*	*Tapes japonica*
Macrocystis sp.	*Gracilaria* sp.	*Haliotis diversicolor*
Porphyra sp.	*Ulva* sp.	*Gracilaria changii*
	Penaeus sp.	*Ulva lactuca*
	Crassostrea sp.	

Temperate Brackish Water	Temperate Freshwater	Warm Freshwater
Oreochromis sp.	*Hypophthalmichthys* sp.	*Clarias* sp.
Mugil cephalus	*Macrobrachium rosenbergii*	*Cyprinus carpio*
Sparus aurata		*Oreochromis* sp.
Dicentrarchus labrax		*Mugil cephalus*
Penaeus monodon		*Penaeus* sp.

Mariculture Systems, Integrated Land-Based. Figure 2
Key species cultured in IMTA and polyculture systems for marine and freshwater environment

is excreted into the water, mainly as dissolved ammonia, feces, and uneaten feed.

Two main practical approaches are emerging for handling the organic and nitrogenous wastes: bacterial dissimilation into gasses in "Recirculating Aquaculture Systems" (RAS), or plant assimilation into biomass (IMTA). Bacterial biofilters are dissimilative. Through a process of nitrification followed by denitrification, bacteria break down the organic pollutants into N_2 and CO_2 gasses. Bacterial biofilters are technically rather effective for aquaculture and allow significant water recirculation. However, the technology is relatively expensive, and not simple. Bacterial biofilter technologies are suitable for relatively small intensive land-based culture of lucrative organisms. There are no suggestions as to how such technologies can be integrated with large-scale, low cost fish or shrimp production. In addition, this system wastes expensive nitrogen by converting this valuable resource into gas, which is lost into the atmosphere.



Mariculture Systems, Integrated Land-Based. Figure 3
Fish, shellfish, and seaweed species combination in IMTA systems in different bio-geographical regions around the world

Nutrient assimilation by other organisms is a more promising method of water treatment. In land-based IMTA ponds, seawater is pumped from the "nuclear species" (fish or shrimp) into the ponds/tanks of secondary organisms or macro-/microalgae. A pellet diet is the only source of nutrients for the primary animals in the system. Nutrient-rich effluent water from these ponds can take three directions: microalgae ponds, macroalgae ponds, or to irrigate halophyte crops (e.g., *Salicornia* sp.). The microalgae can be utilized by filter

feeders such as artemia or bivalves. The macroalgae can be utilized by macroalgivores such as abalone, sea urchins, or herbivorous fish. Halophytes such as *Salicornia* can be used as a food product. The remaining detritus can be fed to detritivores such as mullets, sea cucumbers, or polychaete worms, singly or in combination.

Optimization of the IMTA is typically based on the highest value "nuclear" product at any given time. This "nuclear" product may be shifted according to climatic conditions and economic considerations. For example, in a fish-abalone-seaweed integrated system, abalone is the most valuable species, and the entire system is centered around this species. Abalone will be the first organism to receive the incoming water. From the abalone, the water will drain to the ammonia producers and from there to the biofilters.

The biological and chemical processes in the IMTA system should be balanced between nutrient production by the main organism and nutrient uptake capacity of the micro- and/or macroalgae and downstream by the micro- and macroalgivores. In such systems evaluated in Eilat, Israel, macro-and microalgae were able to assimilate 1–5 g N m^{-2} day^{-1}, while algivores and filter feeders assimilated 0.5–1 g N kg $(WW)^{-1}$ day^{-1} (Table 1 and references therein). However, there will be variation in nutrient uptake depending on season and climate, as algal biomass is influenced by day length (i.e., light hours), water temperature, and the nutrient levels in the water.

For example, in a fish-bivalve-seaweed IMTA system in Eilat, 63% of the nitrogen from the feed was assimilated by edible organisms, 32% sank to the bottom as biodeposit (sludge), and only 4.1% was discharged back to the sea (Fig. 4) [3].

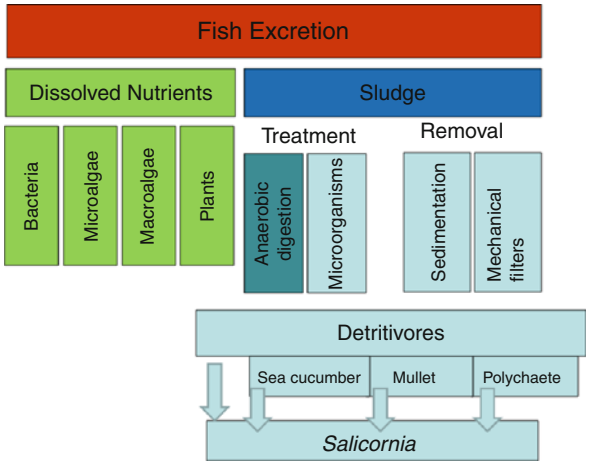
Nitrogen, phosphate, and silicate ratios can vary according to local farm conditions.

Nutrient composition is also affected by additional biochemical processes in the effluent water such as nitrification, denitrification, and ammonification which occurs in the sedimentation pond as well in the pond walls and in the water pipes. These processes can be accelerated or affected by water temperature, nutrient loads, flow rates, and fish feed biochemical composition. Local natural microfauna in the ponds (e.g., zooplankton) and microflora, as well as bloom and crash phenomena, can affect the water quality as well. In most cases, effluent water from fishponds is characterized by a mixture of ammonia, nitrate, and nitrite.

Mariculture Systems, Integrated Land-Based. Table 1 Assimilation rates of the uptake organisms in land-based IMTA in Eilat, Israel

	Assimilation rates	References
Microalgae	1–3 g N m^{-2} day^{-1}	Shpigel and Blaylock (1991)
		Shpigel et al. (1993a)
		Shpigel et al. (2007)
Macroalgae/ *Salicornia*	3–5 g N m^{-2} day^{-1}	Neori et al. (1991)
		Boarder and Shpigel (2001)
		Schuenhoff et al. (2003)
		Neori et al. (2004)
Bivalves/ Artemia	0.3 g N kg^{-1} day^{-1} 6 g N kg^{-1} m^{-3} day^{-1} (20 kg m^{-3})	Shpigel and Blaylock (1992)
		Shpigel et al. (1993a,b, 1994, 1996)
		Zmora and Shpigel (2006)
		Neori et al. (2004, 2006)
Abalone/sea urchins	0.5 g N kg WW day^{-1}	Shpigel et al. (1996, 1999, 2005, 2006)
		Neori et al. (2001)
		Stuart and Shpigel (2009)
Salicornia wetland	2–5 g N m^{-2} day^{-1}	Envirophyte (2010)
		Stuart and Shpigel (2009)

While macro- and microalgae have proven effective components in land-based systems, neither removes 100% of the dissolved matter and they do not remove particulate matter at all. The remaining waste that includes, among other components, feces, uneaten feed, algae and bacteria, sinks to the bottom and becomes what is known as sludge. This sludge contains valuable ingredients, but can also be toxic to the cultured organisms. It can increase stress and



Mariculture Systems, Integrated Land-Based. Figure 4 Different pathways to treat sludge from fishponds

disease risk, and reduce the quality of the water both in situ and for reuse. Ignoring the negative effects of the sludge can thus create serious problems and cause financial losses to the farmers. Removing and dumping sludge into the environment would similarly cause damage, even if moderated by dilution, and "foul the fish farmer's own nest" should he use seawater pumped in from the same area. Using detritivores is a novel option for land-based IMTA. Detritivore organisms such as mullets, cockles, and sea cucumbers will assimilate the waste into their bodies, thereby generating a significant saving in treatment costs, while additionally serving as valuable products in their own right, without requiring the purchase of feed for their culture.

The halophyte *Salicornia* sp. as a biofilter in constructed wetlands was evaluated in the "Genesis" and "Envirophyte" EU projects [34, 45, 46]. Using constructed wetlands (CW) planted with halophytes, which would take up the nutrient-rich wastewater and convert it into valuable plant biomass, is a new option for land-based IMTA. This system was developed to a practical stage for cold (UK) and warm (Israel) water. It was found that CW is efficient in clearing water of nutrients and suspended solids, some materials being purified through incorporation into the plants' biomass and others attaching to the substrate or being broken down by bacteria living therein. CW has the benefit of being low cost, is simple to operate, and can be given an aesthetically pleasing appearance. These plants have commercial value as a health food and are potential candidates for the health, beauty, and nutraceutical industries.

Pilot Scale Systems

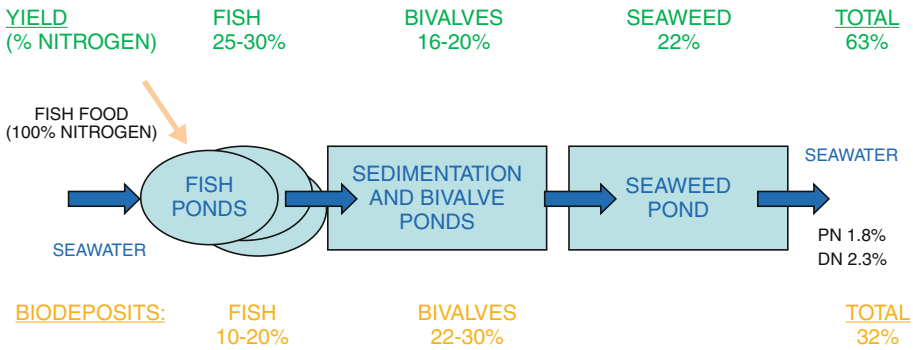
In R&D projects in Eilat, Israel, three different types of IMTA systems were developed:

1. Fish (seabream *Sparus aurata*) – seaweed (*Ulva lactuca*)
2. Fish (seabream *Sparus aurata*) – abalone (*Haliotis discus hannai*)/sea urchin (*Paracentrotus lividus*) (macroalgivores) – seaweed (*Ulva lactuca*)
3. Fish (seabream *Sparus aurata*) – bivalve (*Crassostrea gigas* and *Tapes philippinarum*) – seaweed (*Ulva lactuca*)

In the seabream-*Ulva* system, a daily ration of 1.3 t of feed supported 250 t of fish. This amount of food is equivalent to 64 kg of nitrogen. The fish assimilate around 16 kg of nitrogen. About 9.6 kg of the nitrogen is drained as particulate nitrogen, and 38.4 kg is drained as dissolved nitrogen. One hectare (ha) of macroalgae (*Ulva lactuca*) is required to remove most of the dissolved nitrogen from the water. This system using 500 t of food per year would require an area of 3.4 ha, at a ratio of 1 ha fish to 2.5 ha *Ulva*. Expected yield is approximately 220 t of fish and 1,600 t of *Ulva* (modified from [5] and [47]) (Table 2).

In the seabream-*Ulva*-macroalgivores (sea urchins/abalone) IMTA system, 1 ha of macroalgae produces 1,600 t of *Ulva* annually. This *Ulva* supports 133 t (WW) of abalone (*Haliotis discus hannai*) or 200 t of sea urchins (*Paracentrotus lividus*). A seabream-*Ulva*-sea urchins/abalone IMTA system in Eilat, Israel, using 500 t of food per year will need an area of 5.3 ha, at a ratio of 1 ha for fish, 2.5 ha for *Ulva*, and 1.8 ha for the macroalgivores (modified from [5] and [47]) (Table 2).

In the seabream, microalgae, and bivalves (*Crassostrea gigas* and *Tapes philippinarum*) IMTA system, a daily ration of 1.3 t of feed supports 250 t of fish. The fish assimilate around 16 kg of nitrogen; 38.4 kg of nitrogen is drained as dissolved nitrogen. This system using 500 t of food per year would need an area of 2 ha of phytoplankton pond (with assimilation efficiency of 1–2 g N m^{-2} day^{-1}) to support



Mariculture Systems, Integrated Land-Based. Figure 5
Nitrogen budget of fish-bivalve-seaweed IMTA system in Eilat, Israel

Mariculture Systems, Integrated Land-Based. Table 2 Expected performance of land-based IMTA (WW = wet weight)

IMTA system	Organism	Pond size Ratio/ha	Yield (WW t year^{-1})	Yield (kg WW m^{-2} year^{-1})
Fish-*Ulva* (500 t feed y^{-1})	Seabream	1	220	22
	Ulva	2.5	1,600	64
	Total		1,820	86
Fish-*Ulva* abalone/sea urchin (500 t feed y^{-1})	Seabream	1	220	22
	Ulva	2.5	1,600	64
	Abalone	1.8	185	10
	Sea urchins	1.8	140	8
	Total		1960–2005	94
Fish-*Ulva*-clam/oyster (500 t feed y^{-1})	Seabream	1	220	20
	Clams/*oysters*	4	140	8
	Ulva	0.5	70	64
	Total		430	92

production of 140 t bivalves and 70 t of seaweed (modified from [5] and [47]) (Table 2).

The economics of these types of land-based IMTA systems were summarized in [5]. However, the economics of a land-based IMTA are site specific since they depend on variables including local construction and operating costs and market prices for the farm's products at any given time [34].

Additional anticipated parameters based on the same model of using 500 t feed per year in each of the three IMTA systems tested in Eilat, Israel, with the projected yields as depicted in Table 2, can be seen in Table 3.

Future Directions: Challenges and Constraints

Although considerable information is already available for putting land-based IMTA systems into practice, much of it is designed around commercial exploitation of a few high value species that are not affordable for the masses. The challenge for the future is to produce a large quantity of aquaculture products that will be cost-effective for producers, at a reasonable price for consumers, and ecologically sustainable.

Additional studies are required to overcome further constraints, including biological, engineering, and economical aspects:

Mariculture Systems, Integrated Land-Based. Table 3 Anticipated parameters for organisms in the three IMTA systems tested in Eilat, Israel, based on 500 t feed per year

Seabream
FCR = 1.9; Feed protein content = 49%
Fish stocking density = 200 t ha^{-1}; Annual fish yield −300 t ha^{-1}
Seabream farm gate price = €4 kg^{-1}
Seaweed
Ammonia uptake rate –4 g m^{-2} day^{-1}; ammonia uptake efficiency = 85%
Annual Ulva yield = 900 t ha^{-1}
Seaweed (WW) price = €0.5 kg^{-1}
Abalone
FCR = 12; stocking density = 25 kg m^{-2}
Annual yield = 10 kg m^{-2};
Farm gate price = €35 kg^{-1}
Sea urchins
FCR = 8 t *Ulva* 1 t of production; stocking density = 10 kg m^{-2}
Annual yield = 8 kg m^{-2}
Farm gate price = €10 kg^{-1}
Clams/Oysters
Clam annual yield = 6–8 kg m^{-2}
Clams farm gate price = €4.5 kg^{-1}
Oyster annual yield = 25 kg m^{-3}
Oyster farm gate price = €3.5 kg^{-1}

Biological Aspects

- To acquire the knowledge necessary to maintain the correct balance between nutrient production by the system's core organism, nutrient uptake capacity of microalgae and macroalgae, shellfish filtering efficiency, and macroalgivores' activity in the system
- To acquire the knowledge necessary to maintain steady populations of microalgae (mainly diatoms) for the filter feeders and of macroalgae for the macroalgivores within the system in order to avoid blooms and crashes
- To acquire the knowledge necessary for the efficient regeneration of the biodeposit (sludge) from the bottom back to dissolved nutrients for the macro- and microalgae
- To effectively control diseases of the cultured organisms in IMTA systems and transmission of pathogens between components of the system

Engineering Aspects

- To reduce construction and operating costs by engineering improvements
- To minimize heat loss or gain in downstream components of the system
- To increase the use of greenhouse-covered modular systems, gravitation, low head upwelling, water semi-recirculation and other promising energy-saving methods

Economical Aspects

- To render cost effective the use of the extensive areas required for cultivating micro- and macroalgae which cannot be done in a fully recirculating system and for which the facilities must thus be located not too far from the sea
- To develop and diversify the market of seaweed for human consumption from IMTA in Europe and North America
- To develop new markets and consumer acceptance of IMTA products

With the dramatic increase in population and food requirements, traditional extensive production systems cannot satisfy present and future market needs. Modern intensive monoculture systems are not ideal for mass production because they focus on few and expensive species, require high levels of resources, and produce undesirable wastes. To achieve high production rates and environmental conservation, food production using land-based IMTA systems is one of the most promising routes. The IMTA method assimilates expensive nitrogen waste into a valuable product that will increase profit for the farmer, improve FCR, diversify the mariculture products, create additional jobs, and, most importantly, reduce environmental pollution.

Bibliography

1. Goldman JC, Tenore RK, Ryther HJ, Corwin N (1974) Inorganic nitrogen removal in a combined tertiary treatment-marine aquaculture system. I. Removal efficiencies. Water Res 8:45–54
2. Ryther JH, Goldman JC, Gifford JE, Huguenin JE, Wing AS, Clarner JP, Williams LD, Lapointe BE (1975) Physical models of integrated waste recycling-marine polyculture systems. Aquaculture 5:163–177
3. Shpigel M, Neori A, Popper DM, Gordin H (1993) A proposed model for 'environmentally clean' land-based culture of fish, bivalves and seaweeds. Aquaculture 117:115–128
4. Shpigel M (2005) The use bivalves as biofilters and valuable product in land based aquaculture systems-review. In: Dame R, Olenin S (eds) The comparative roles of suspension-feeders in ecosystems. Kluwer, Dordrecht, The Netherlands, p 400
5. Shpigel M, Neori A (1996) The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: I. Proportion of size and projected revenues. Aquacult Eng 155:313–326
6. Troell M, Halling C, Nilsson A, Buschmann AH, Kautsky N, Kautsky L (1997) Integrated marine cultivation of *Gracilaria chilensis* (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. Aquaculture 156:45–61
7. Troell M, Norberg J (1998) Modelling output and retention of suspended solids in an integrated salmon-mussel culture. Ecol Model 110:65–77
8. Huguenin JH (1976) An examination of problems and potentials for future large-scale intensive seaweed culture systems. Aquaculture 9:313–342
9. Tenore KR (1976) Food chain dynamics of abalone in a polyculture system. Aquaculture 8:23–27
10. Hughes-Games WL (1977) Growing the Japanese oyster (*Crassostrea gigas*) in sub-tropical seawater fishponds. I. Growth rate, survival and quality index. Aquaculture 11:217–229
11. Gordin H, Motzkin F, Hughes-Games A, Porter C (1981) Seawater mariculture pond – an integrated system. Eur Aquacult Spec Publ 6:1–13
12. Krom MD, Erez J, Porter CB, Ellner S (1989) Phytoplankton nutrient uptake dynamics in earthen marine fishponds under winter and summer conditions. Aquaculture 76:237–253
13. Erez J, Krom MD, Neuwirth T (1990) Daily oxygen variations in marine fish ponds, Elat, Israel. Aquaculture 84:289–305
14. Shpigel M, Fridman R (1990) Propagation of the manila clam *Tapes semidecussatus* in the effluent of marine aquaculture ponds in Elat, Israel. Aquaculture 90:113–122
15. Shpigel M, Blaylock RA (1991) The Pacific oyster, *Crassostrea gigas*, as a biological filter for a marine fish aquaculture pond. Aquaculture 92:187–197
16. Shpigel M, Lee J, Soohoo B, Fridman R, Gordin H (1993) The use of effluent water from fish ponds as a food source for the pacific oyster *Crassostrea gigas* Tunberg. Aquac Fish Manage 244:529–543
17. Neori A, Shpigel M (1999) Using algae to treat effluents and feed invertebrates in sustainable integrated mariculture. World Aquac 302:46–51
18. Neori A, Shpigel M, Scharfstein B (2001) Land-based low-pollution integrated mariculture of fish, seaweed and herbivores: principles of development, design, operation and economics. Aquaculture Europe 2001 book of abstracts, European Aquaculture Soc. Special Publ. No. 29, pp 190–191
19. Lefebvre S, Barille L, Clerc M (2000) Pacific oyster (*Crassostrea gigas*) feeding responses to a fish-farm effluent. Aquaculture 187:185–198
20. Jones AB, Dennison WC, Preston NP (2001) Integrated mariculture of shrimp effluent by sedimentation, oyster filtration and macroalgal absorption: a laboratory scale study. Aquaculture 193:155–178
21. Haines KC (1975) Growth of the Carrageenan-producing tropical red seaweed *Hypnea musciformis* in surface water, 870 m deep water, effluent from a clam mariculture system, and in deep water enriched with artificial fertilizers or domestic sewage. In: 10th European Symposium on Marine Biology, 1, pp 207–220
22. Langton RW, Haines KC, Lyon RE (1977) Ammonia nitrogen produced by the bivalve mollusc *Tapes japonica* and its recovery by the red seaweed *Hypnea musciformis* in a tropical mariculture system. Helgoländer wiss Meeresunters 30:217–229
23. Lapointe BE, Ryther HJ (1978) Some aspects of the growth and yield of *Gracilaria tikvahiae* in culture. Aquaculture 15: 185–193
24. DeBusk TA, Blakeslee M, Ryther JH (1986) Studies on the outdoor cultivation of *Ulva lactuca*. L Bot Mar 29:381–386
25. Bird K (1989) Intensive seaweed cultivation. Aquaculure Mag November/December 1989:29–34
26. Vandermeulen H, Gordin H (1990) Ammonium uptake using *Ulva* (Chlorophyta) in intensive fishpond systems: mass culture and treatment of effluent. J Appl Phycol 2:363–374
27. Cohen I, Neori A (1991) *Ulva lactuca* biofilters for marine fishponds effluents. Bot Mar 34:475–482
28. Neori A, Ellne SP, Boyd CE, Krom MD (1993) The integration of seaweed biofilters with intensive fish ponds to improve water quality and recapture nutrients. In: Moshiri GA (ed) Constructed wetlands for water quality improvement. Lewis, Boca Raton, pp 603–607
29. Schuenhoff A, Shpigel M, Lupatsch I, Ashkenazi A, Msuya FE, Neori A (2003) A semi-commercial, integrated system for the culture of fish and seaweed. Aquaculture 2211–4:167–181
30. Shpigel M, Neori A, Marshall A (1996) The suitability of several introduced species of abalone Gastropoda: Haliotidae for land-based culture with pond grown seaweed in Israel. Israeli J Aquaculture/Bamidgeh 484:192–200
31. Butterworth A (2010) Integrated Multi-Trophic Aquaculture systems incorporating abalone and seaweeds. *A report for Nuffield Australia Farming Scholars, Nuffield Australia Project no. 914*
32. Nobre AM, Robertson-Andersson D, Neori A, Sankar K (2010) Ecological-economic assessment of aquaculture options:

comparison between abalone monoculture and integrated mult-trophic aquaculture of abalone and seaweeds. Aquaculture 306:116–126
33. Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Shpigel M, Yarish C (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361–391
34. Bunting SW, Shpigel M (2009) Evaluating the economic potential of horizontally integrated land-based marine aquaculture. Aquaculture 294:43–51
35. Subandar A, Petrell RJ, Harrison PJ (1993) *Laminaria* culture for reduction of dissolved inorganic nitrogen in salmon farm effluent. J Appl Phycol 5:455–463
36. Ahn O, Petrell R, Harrison PJ (1998) Ammonium and nitrate uptake by *Laminaria saccharina* and *Nereocystis luetkeana* originating from a salmon sea cage farm. J Appl Phycol 10:333–340
37. Buschmann AH, Troell M, Kautsky N, Kautsky L (1996) Integrated tank cultivation of salmonids and *Gracilaria chilensis* (Gracilariales, Rhodophyta). Hydrobiologia 326(327):75–82
38. Chopin T, Yarish C (1998) Nutrients or not nutrients? That is the question in seaweed aquaculture... and the answer depends on the type and purpose of the aquaculture system. World Aquaculture Mag 29(31–33):60–61
39. Troell M, Rönnbäck P, Halling C, Kautsky N, Buschmann A (1999) Ecological engineering in aquaculture: use of seaweeds for removing nutrients from intense mariculture. J Appl Phycol 11:89–97
40. Ruokolahti C (1988) Effects of fish farming on growth and chlorophyll content of *Cladophora*. Mar Pollut Bull 19:166–169
41. Rönnberg O, Ådjers K, Roukolathi C, Bondestam M (1992) Effects of fish farming on growth epiphytes and nutrient content of *Fucus vesiculosus* L. in the Åland archipelago, northern Baltic Sea. Aquat Bot 42:109–120
42. Chopin T, Yarish C, Wilkes R, Belyea E, Lu S, Mathieson A (1999) Developing *Porphyra*/salmon integrated aquaculture for bioremediation and diversification of the aquaculture industry. J Appl Phycol 11:463–472
43. Folke C, Kautsky N (1989) The role of ecosystems for a sustainable development of aquaculture. Ambio 18:234–243
44. Soto D (2009) integrated mariculture, a global review. *FAO Fisheries and aquaculture technical paper 529*
45. GENESIS (2004) Development of a generic approach to sustainable integrated marine aquaculture for European environments and markets. (European Economic Community; IPS-2000-102)
46. ENVIROPHYTE (2009) Improvement of the cost effectiveness of marine land-based aquaculture facilities through use of Constructed Wetlands with *Salicornia* as an environmentally friendly biofilter and a valuable by-product. (European Economic Community; SME – 37162)
47. Neori A, Ragg NLC, Shpigel M (1998) The integrated culture of seaweed, abalone, fish and clams in intensive land-based systems: II. Performance and nitrogen partitioning within integrated abalone *Haliotis tuberculata* and macroalgae *Ulva lactuca* and *Gracilaria conferta* culture system. *Aquacultural Engineering* 17(4):215–233

Marine Aquaculture in the Mediterranean

Dror L. Angel
Leon Recanati Institute for Maritime Studies, University of Haifa, Mt Carmel, Haifa, Israel

Article Outline

Glossary

Bioassay (BIOlogical ASSAY) A procedure to test the effect of a substance on living organisms, e.g., the effect of plant nutrients on plant growth rate.

Chemotherapeutants The use of chemicals to treat disease.

Dead zones Coastal areas that undergo seasonal hypoxia (low-oxygen), generally related to eutrophication events, whereafter many of the local (mainly benthic) animals die.

Exotic species An introduced or alien species living outside its natural range, which has been introduced by deliberate or accidental human activity.

FCR (feed conversion ratio) The efficiency at which an animal converts its food into biomass (body mass); FCR = mass of food eaten/increase in biomass.

Immunostimulants Chemicals used to stimulate the immune system by inducing activation or increasing activity of any of its components.

Marine protected areas Areas that restrict human activity (e.g., fishing, boating, coastal development) to protect living, nonliving, cultural, and/or historic resources.

NIMBYism "Not In My Back Yard"-ism; the practice of objecting to a human activity (generally commercial or industrial) that will take place near one's home.

Oligotrophic Waters that have low levels of nutrients and algae, high level of dissolved oxygen, and deep light penetration (i.e., clarity).

Prebiotics Food ingredients (e.g., soluble fiber) that stimulate the growth and/or activity of bacteria in the digestive system which are beneficial to the health of the body.

Probiont Living bacteria added to the environment and feed of reared animals and thought to benefit them by improving intestinal microbial balance, thereby inhibiting pathogenic bacteria.

Protista Unicellular (single-cell) eukaryotic organisms, e.g., foraminifera.

Definition of the Subject

Fisheries and aquaculture play an important role in the economies of many countries; yet this fact is often overlooked as the focus, in many nations, is on provision of food primarily, if not exclusively, from terrestrial agriculture. The value of seafood products as a source of foreign currency is especially important in developing countries and in many cases may exceed the profits from certain agricultural products [1], though this fact also tends to evade common knowledge. The Mediterranean aquaculture sector continues to grow at a rate of close to 9% per year (since 1970) as compared to 3% per year for farmed meat production systems. If the growth of the aquaculture sector can be sustained, it is likely to fulfill the demand for aquatic food supplies by supplying >50% of the total aquatic food consumption within the next 5 years! Therefore, the emphasis here is on the review of the sustainable growth of a commercial activity within an enclosed sea with many conflicting multinational interests. Aquaculture includes the cultivation of finfish, shellfish, crustaceans, and algae; however, this review will focus primarily on Mediterranean finfish farming since many of the sustainability issues revolve around fish farms. There are many different facets (e.g., ecological, social, political, economic) to sustainable commercial activities and this review will touch on several, though not all, of the issues related to aquaculture and its sustainable development in the Mediterranean Sea region.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Introduction

The Mediterranean Sea Environment

Although the term "environment" is often used to mean "ecology," the following description embraces the more holistic meaning, which includes the socio-economic aspects as well. The Mediterranean is a large, semi-enclosed sea bordered by 22 countries, with two distinct basins divided by a narrow, relatively shallow channel between Sicily in the north and Tunisia in the south. The areal division of the sea between the western and eastern basin is roughly 1/3:2/3. The eastern basin is somewhat more saline than the western basin, especially in the vicinity of the Suez Canal. The Mediterranean Sea has a wide range of seawater temperatures, from as low as 5°C in the Gulf of Trieste in the winter to 31°C off the coast of Libya in the summer [2]. The sea is oligotrophic and phosphorus limited [3] though some limited areas (such as parts of the northern Adriatic) may be eutrophic and it is warmer and more oligotrophic in its southern and eastern areas. Whereas the Mediterranean Sea accounts for only 1% of the world's ocean, it contains 6% of the world's marine species, including >400 endemic species of plants and animals [4]. Despite this impressive biodiversity, biomass is relatively low, mainly due to low primary production.

There are approximately 82 million people in the Mediterranean coastal zone: most in coastal cities and 32% of the population is in North Africa. Levels of development vary widely over the region. Tourism brings >100 million visitors to coastal areas annually, serving as a major source of seasonal population pressure and income and is thus a major competing sector with aquaculture. The Mediterranean Sea is a major shipping route, bridging between Europe and the Middle East and is a base for capture fisheries and mariculture. There are 75 marine protected areas (MPA) in the region, designed to protect unique and threatened resources and habitats such as the seagrass *Posidonia oceanica*, and breeding and nesting sites for endangered species, such as the loggerhead sea turtle (*Caretta caretta*). MPAs were also designated to encourage specific uses, such as sustainable tourism and regenerating fish stocks [5].

A Brief History of Mediterranean Aquaculture

The earliest evidence of aquaculture activity in the Middle East is from the ancient Egyptians. An Egyptian frieze, dated from 2500 B.C., depicted men gathering fish from a pond in what may be the earliest record of such activities in this region [6, 7]. In the sixth and fifth centuries B.C., the Etruscans reared fish in marine farms and the Greeks grew mollusks [8]. Throughout the Roman empire, marine fish (mainly sea bass, sea bream, and mullets) and oysters were reared in special enclosures (e.g., piscines) along the coast [9–11], but this practice seems to have died out with the collapse of the empire and did not appear in the Mediterranean until the middle ages. It is not clear precisely when it began, but there are records of extensive aquaculture in lagoons in Italy, also known as valliculture, starting from around the fifteenth century. Europeans traditionally collected shellfish along the shores, but since the eighteenth century the French oyster industry added a more reliable source – shellfish reared in specialized gear in the intertidal zone. Shellfish aquaculture expanded in the nineteenth century and coastal cultivation spread throughout the Western Mediterranean and the northern Adriatic Sea.

In the second half of the twentieth century, aquaculture developed rapidly, mainly as a result of successful research into the life cycle of the farmed animals (reproduction and larval rearing), as well as physiology, nutrition, and engineering of farming systems [8].

Main Forms of Mariculture (Culture Types and Species) in the Mediterranean

On a global scale, aquaculture production in the Mediterranean Sea is small, but not insignificant – especially with regard to the European demand for fresh seafood. Total aquaculture production in the Mediterranean Sea in 2006 was about 370,000 t [1] with 14% growth from 2000 to 2006, outpacing the growth of capture fisheries. It is noteworthy that the interannual variability in aquaculture production is lower than in capture fisheries (these have reached a plateau in terms of annual harvest), which may be a consideration of prime significance for business and decision-makers concerned with food security, coastal communities, and development.

Within the Mediterranean aquaculture sector, the most striking feature of production is the rate at which finfish have overtaken mussels as the dominant product. In 1990, finfish production accounted for less than 10,000 t as compared to approximately 90,000 t of mussels. In 2003, 180,000 t finfish and 150,000 t mussels were produced (49% and 40% of total production, respectively). Clam and oyster production were only 7% and 2%, respectively, and the remainder of production (∼2%) was crustaceans and seaweed. The main cultivated finfish species in the region are gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), and flathead gray mullet (*Mugil cephalus*). Greece, Turkey, Spain, and Italy were the four largest producers of sea bream and bass in 2006, comprising >90% of total Mediterranean production. Sea bream and bass are predominantly reared in net cages in coastal waters, whereas mullets are generally reared in ponds. The major producers of mullets are Egypt and Italy with Egypt generating more than 90% of global mullet production.

A fairly recent development is the farming of bluefin tuna in the Mediterranean, which mainly serves the Japanese sushi market. Tuna farming falls in between the definitions of a standard fishery, which is defined as "capture of wild stock" and aquaculture where fish are both bred and reared in captivity. Because tuna farming is a "postharvest" practice, it is not governed by the regulations of GFCM or ICCAT [12] and as a result there was unregulated growth in this sector, putting heavy pressure on the endangered Mediterranean wild stocks. Concerted efforts are being made to create brood stocks and hatcheries to enable the cultivation of bluefin tuna by the traditional aquaculture methods to release pressure on the endangered Mediterranean wild stocks.

Sustainable Marine Aquaculture in the Mediterranean

One of the features of marine aquaculture in the Mediterranean is that it is developing rapidly in response to a large and ever-growing demand for seafood. This demand was traditionally supplied by fisheries, but the drop in landings in recent decades as a result of overfishing has opened the path for sustainable alternatives to provision of seafood, namely aquaculture. That said, mariculture needs to operate in a manner that will minimize negative impacts on the marine environment, on wild stocks, and on other uses of the seas. Thus, sustainable aquaculture must ensure "*economic viability, social equity and acceptable environmental impacts*" [13].

It is obvious that aquaculture activity must be profitable to succeed, but there are many criteria to profitability and *economic viability* and these may vary considerably in countries that are at different stages of economic development (the process whereby an economic activity develops the technology and experience needed to operate successfully) or that have different interests in mind. In some developing countries, aquaculture may serve as a much needed food and protein source for local consumption, whereas other developing countries may prefer to export their aquaculture production for economic benefit.

Another component of sustainability is *social equity*. Societal equity depends on cultural norms and tendencies of society and varies considerably among the Mediterranean countries. It is probably the most difficult aspect of sustainability to consider because of its intrinsic variability.

Environmental "*acceptability*" is also a difficult issue because of the obvious question: "acceptable by whom?" In order to address this, one needs to consider where the aquaculture activity takes place, who are the stakeholders and how this activity may be conducted in such a manner that it will be acceptable by as many stakeholders as possible. The first aspect of sustainability, discussed below, is the public perception of aquaculture since public opinion may play an important role in the success or failure of the industry. In addition to the various social ramifications, "environmental acceptability" includes the effects of aquaculture on its surroundings and on the ecosystem. The following sections list several of the environmental issues that affect or are affected by Mediterranean aquaculture and a discussion of what is being done about them to enhance the sustainability of this sector.

Public Perception of Aquaculture

The *image* of fish farming varies considerably among different countries and can have a strong effect on the

sustainability of the industry. In some northern European countries, the public considers aquaculture in a positive light as a means to enhance food safety and security. In comparison, many southern European countries have a generally negative attitude toward farmed fish as these are considered inferior in taste and health value in comparison to wild-caught ("natural") fish [14, 15]. Numerous negative connotations are associated with marine aquaculture, including: "pollution causing eutrophication," "discharge of antibiotics and harmful chemicals into the environment," "genetic dilution/pollution of wild fish stocks," and "negative visual impact on the coasts."

The public perception is very important for both producers and coastal zone managers since there are many factors that are stacked against the aquaculture sector [16, 17]. These include lack of knowledge on many aspects of the coastal environment, the weakness of a small industry, competition with tourism and other coastal stakeholders, and increasing political power of local environmental lobbies and associations. These lead to non-sustainable situations, including loss of licenses, leases and markets, and reduced diversity in the coastal economy.

The social acceptability of aquaculture was examined at two Greek islands [18] and revealed that residents were more likely to be opposed to aquaculture if they thought that the fish farms would pollute the environment. A study conducted in Israel [19] evaluated public attitudes toward aquaculture and concluded that although most citizens were not terribly well informed in the implications of aquaculture on tourism and environmental issues, the majority are in favor of marine aquaculture. It is noteworthy that this lack of familiarity with aquaculture and aquaculture implications was also observed among the public surveyed in such countries as Scotland [20], Australia [16], and Germany (Schultz, unpublished).

Although the above focuses on the attitudes of the lay public toward aquaculture, it is possible that the opinion of stakeholders is equally (or more) important, despite the fact that the number of stakeholders is usually smaller. Competition over the coastal zone is one of the major sustainability issues that Mediterranean aquaculture faces on a regular and large-scale basis. The competition is especially severe between aquaculture and tourism since the Mediterranean attracts about 30% of the volume of global tourism annually and this is expected to increase over time. There are many examples of such competition, and one of the more recent clashes between the tourism and aquaculture sectors occurred in Turkey in 2008–2009, resulting in a major shift in legislation and in aquaculture lease requirements.

Measures to Improve the Public Attitudes Toward Aquaculture The negative attitudes toward aquaculture are largely a result of ignorance. The media often presents NGO views and opinions in their description of the fish-farming industry, and many of the facts presented are incorrect. The way to correct some of the misconceptions surrounding aquaculture is by preparing a well-planned outreach and educational program geared to reach as many households as possible. There are myths and misconceptions regarding such things as how fish are reared and the densities at which they are stocked, the safety of the feed used, the quality and healthiness of farmed versus wild fish, etc. Preparation of an aquaculture "module" to be taught at schools is an effective way to reach and educate future stakeholders and decision-makers. Another measure that could reduce conflict between aquaculture and other coastal stakeholders is a search for synergies among the stakeholders that would enable multiple use of the coastal zone [21]. Promotion of organic and other types of certification programs to increase public confidence in aquaculture practices and products would also improve public attitude toward this sector.

Benthic Impacts

In the 1990s, the study of the interactions of Mediterranean marine aquaculture with the environment focused on the negative impacts of the industry since most of the early research on salmon farms documented heavy benthic loading, which caused serious damage to underlying seafloor communities and in some cases to the water column as well [22–26]. Benthic organic enrichment that often occurs under intensive finfish farms rapidly leads to hypoxia and anoxia in the sediments. Anoxic sediments support bacterial sulfate reduction, generally leading to an increase in sediment hydrogen sulfide [27]; conditions that are

noxious, at best and often lethal to macro- and meiofauna [28]. Although abundances of macrofauna in Mediterranean sediments are considerably lower than the abundances found in temperate regions [29–31], defaunation under fish farms strongly reduces benthic bioturbation (i.e., aeration of the sediments) and leads to accumulation of reduced compounds and organic matter therein. If the farm is situated at a site with limited flushing and circulation, the depth and aerial extent of the impacted sediments may grow with time, creating localized "dead zones." Moreover, when methane accumulates in and bubbles out of anoxic sediments, noxious chemicals such as ammonia and hydrogen sulfide may affect the cultivated fish in the overlying cages.

Because the Mediterranean Sea is largely oligotrophic, and fish farming is generally not practiced at sites with poor flushing, the phenomena described above are not common. At a few sites with limited water circulation, for example, some farms in Croatia and Greece, organic enrichment of the seafloor and local impacts were observed, but these were exceptional and sediment conditions under Mediterranean fish farms are generally less impacted.

At those sites that showed evidence of impacted sediments, the visible effects generally did not extend beyond tens of meters from the edge of the perimeter of the farm [32], though the situation at each farm is different as a result of site-specific currents, depth, bathymetry, etc. The determination of the extent of impacted sediments and benthos (distance from the farm) is subjective and may be strongly affected by the method used. Organic matter determinations, visual inspection, and macrofauna indices are often the methods used to assess the state of the sediments and these clearly show a local effect that diminishes with increasing distance from the point source. However, more sophisticated analyses involving stable isotope signatures of farm effluents indicate that the aquaculture effluents may be detected as far away as 1–2 km from the farms [33–35]. It is very important to qualify the meaning of these measurements because they may be used to make a point about the extent of fish farm effects, but the real issue at hand is the extent of "significant impact." The distribution of small suspended particles over great distances will only constitute a significant impact if the flux of these particles is large and in the case of Mediterranean fish farms, the flux of very small suspended particles is small [36]. Therefore – it is essential to emphasize the difference between qualitative and quantitative effects.

Measures to Reduce Benthic Impacts Despite the fact that benthic loading is generally not a major issue in the Mediterranean, a number of different approaches are employed to increase feeding efficiency and reduce benthic loading. Feeding efficiency is not only an environmental issue, but also a major economic consideration since one of the greatest cost factors in intensive fish farming is the formulated feed. Feeding efficiency includes optimizing the composition of the feed (optimal digestibility) to maximize growth and minimize waste at the lowest possible cost, as well as feed delivery. Considerable efforts are invested by feed companies and fish nutritionists to optimize feed for the various strains of cultivated Mediterranean finfish [37, 38] and during recent years, sea bream and sea bass feed conversion ratios (FCR) have been substantially improved, largely (though not exclusively) due to improved diets and feed delivery. Feed delivery includes the optimal feeding regime whereby feed is provided to the caged fish in suitable portions and at the correct intervals to both maximize growth and health and minimize loss to the surrounding waters. Low-tech feeding involves delivery of pelleted feed to fish either manually by hand, or with the aid of a compressor and regulating the amount according to the response of the fish. High-tech systems include feeding programs that are computerized and customized to each individual cage to optimize delivery of feed to the stock. Another sophistication is the use of submerged Doppler systems (e.g., Doppler Pellet Sensor) that detect when fish stop feeding (increase in the flux of pellets to the bottom of the cages), and send signals to cause the automated feeders to cease feeding (http://www.akvagroup.com/). Many of the above are technologies that were developed outside of the Mediterranean, but as they are also applicable to sea bream and bass production, they are widely used by this sector. One of the more recent developments in Mediterranean aquaculture was the tuna-fattening process, which offered large profits to the farmers. Although it is arguable whether this process should actually be

qualified as aquaculture, the environmental ramifications were clear. The penned fish are fed freshly caught or frozen fish rather than pelleted feed and release large amounts of waste (greater than would be released from pelleted food) to the seafloor and have rather high feed conversion ratios (FCRs) 10:1–20:1 as compared to the FCR of sea bream (2:1) or salmon (1:1). Research is currently ongoing to develop artificial diets to create a better FCR for the tuna and to reduce the reliance and fishing pressure on small pelagic fish (e.g., [39]).

Water Quality

The sustainability of any human activity is a function of the nature of the receiving or host environment and in the case of aquaculture this is the basis for estimating the assimilative, holding, or carrying capacity [40]. At a few sites with restricted water exchange, for example, lagoons, there were reports of eutrophication problems [41–43] as the loading of organic and inorganic nutrients clearly exceeded the capacity of the environment to assimilate these [44]. Sites where such self-pollution problems emerge suggest that the preliminary environmental impact assessment and site selection procedures were not carried out properly. In the oligotrophic waters of the eastern Mediterranean, there are generally no reports of eutrophication or degraded water quality related to finfish or shellfish farms [45–47] and this was interpreted as the ability of the oligotrophic system to successfully assimilate the nutrients released by the farms. In an effort to understand whether nutrient release from aquaculture might have large-scale effects on the Mediterranean ecosystem, Karakassis et al. [48] employed a model to examine various production scenarios. They concluded that if aquaculture continues to grow and expand at present rates, farm wastes may increase overall nutrient (mainly N and P) levels by 1%, however, this is a general assessment and does not take into account localized effects. As suggested by Pitta et al. [49] in a study of three different Greek farms, it is likely that dispersion and dilution of the nutrients, combined with efficient herbivore grazing of algae (that develop from the released nutrients) were the reason for the absence of eutrophication around fish farms.

Although water quality is generally not affected, fish farms that operated over or near seagrass beds (especially *Posidonia oceanica*) exerted a clear effect on these [50, 51] and it was proposed that this may be related to the enhanced flux of dissolved and particulate nutrients from aquaculture. In an attempt to identify the effect of the plume of nutrients released from fish farms on water quality, Dalsgaard et al. [52] devised an innovative "bioassay" to measure the effect of dissolved nutrients released from fish farms on micro- and macro-algal production. They determined that primary productivity decreased with distance from the fish farms, yet by comparing bioassays with and without grazer exclusion, Pitta et al. [53] found that planktonic grazers (probably protista) play a key role in transferring nutrients up the food web.

Measures to Reduce Effects on the Water Column

One of the primary considerations when evaluating the suitability of sites for aquaculture is how they will interact with the surrounding marine system [54]. It does not pay, for example, to place net cage farms in shallow, poorly flushed waters (e.g., lagoons) because the organic and inorganic enrichment may affect both the marine ecosystem and the farmed organisms. Nevertheless, some farms have been deployed in unsuitable locations and these need to be relocated to allow the environment to recover and to enable the healthy growth of farmed finfish.

One of the early water quality problems associated with Mediterranean fish farms was the presence of an oily film around the cages. This was generally related to the large percentage of dust (pulverized feed pellets) in the pelleted food, which is not available to the farmed fish. Because this causes considerable loss to the farmers, and reduced water quality (stimulated bacterial growth also depletes the water of essential dissolved oxygen), the problem was rapidly addressed and most of the pelleted feeds are now extruded to improve pellet integrity and reduce feed loss and feed dust is collected and recycled.

A similar problem was identified in the tuna-penning industry. Unlike sea bream and bass that feed on formulated pellets, tuna are fed whole (preferably oily) fish such as sardine, anchovy, and mackerel. When these fish are offered to the tuna, the water around the pens often has an oily film and emits a strong smell. Moreover, in some cases, divers have complained of poor visibility near the pens. As described above,

research is ongoing to develop artificial diets for tuna [55] that will address not only the problems related to feeding with fresh fish but also the water quality problems.

Disease

Intensive aquaculture systems are very susceptible to disasters such as loss of the farmed stock. Among the various causes of such disasters, disease outbreaks rank highest [56] and may lead to great losses within a very short period of time.

Most finfish cage farms in the Mediterranean are intensive, that is, they have high stocking density in order to be economically profitable and to compensate for the low profit margin of sea bream and sea bass, the main species reared in this region. Although cage stocking densities are usually <25 kg m^{-3}, in some farms stocking densities are higher and such conditions may cause a reduction in fish growth rates, suppression of immune mechanisms [57–59], and ultimately greater susceptibly to disease agents, including opportunistic bacterial and viral pathogens and eukaryotic parasites [60]. Current estimates of average mortalities for farmed sea bream and sea bass as a result of disease are 10% and 20%, respectively, for growth from juvenile to market size (350 g) fish. In many cases, the profit margin for these fish is not much higher than 10–20%, which has therefore obliged the aquaculture sector to consider various options to address this problem. Moreover, there is concern regarding the potential transmission of disease from the farmed stock to wild fish, based on studies of disease transfer among Atlantic salmon (e.g., [61]). It is noteworthy that although there are numerous examples of disease exchange between caged and wild fish (e.g., [62–64]) in the Mediterranean, and other seas, most of these are not clearly understood [65, 66] and their effect on native stocks is unclear.

Measures to Reduce Disease Outbreaks Numerous antibiotics have been tested against the common farmed fish diseases and there are currently treatments available for most bacterial fish pathogens [67]. However, the routine use of antibiotics in marine aquaculture is problematic and has declined for a number of reasons. First, as specified above, there are concerns related to human and environmental health and safety. Second, although many of these drugs work well in freshwater, some of the major antibiotics, such as quinolones and tetracyclines interact with the divalent cations that are abundant in seawater (mostly Mg^{2+} and Ca^{2+}) which massively reduces their function and efficacy [68, 69]. Moreover, there is no "harmonization" regarding antibiotics use among Mediterranean countries and the list of pharmaceuticals licensed for fish varies from country to country, complicating international trade and marketing.

In addition to bacterial pathogens, there are several parasitic diseases that may stunt growth rates, cause loss of fecundity, and even mortality in Mediterranean fish. These include various protozoa and metazoa, which are classified as ecto- and endoparasites according to their distribution on/in the fish. Pathologists consider the myxosporeans *Myxidium leei*, *Polysporoplasma sparis*, and *Ceratomyxa* sp., isopods, copepods, and monogenean infections among the more problematic parasites.

Athanassopoulou et al. [70] reviewed the drugs used against a variety of parasites and found that amprolium and sanilomycin were the most effective against myxosporans in cultivated breams. Moreover, extracts from oregano revealed anti-myxosporan as well as antibacterial properties. Ivermectin and deltamethrin – drugs used to combat sea lice, have also been tested against copepod and isopod infections in sea bass and were fairly effective, but they tend to become toxic to the fish at fairly low levels.

In order to limit the use of antibiotics and other chemotherapeutants, the European Union established the "Maximum Residue Limit" (MRL) regulation, which monitors the presence of these drugs in all agriculture and aquaculture products and this has had a dramatic effect on the use of therapeutants. Because the MRL differs among countries insofar as which compounds are regulated and which are not, there is a lot of work ahead, but despite this, the trend looks very promising.

Vaccines are one of the preferred measures for prevention of disease outbreaks, however because the Mediterranean finfish market is still fairly small, only a limited number of vaccines have been developed for commercial use. Moreover, consumer concerns and increasing restrictions regarding their use have led the

industry to consider other alternatives to disease "management" [71, 72].

There are other alternatives to the use of chemotherapeutants and vaccines against disease. One of the key factors in the prevention of disease is good husbandry, which focuses on minimizing stress to the farmed stock. This includes proper stocking densities, optimal nutrition, sanitary practices, use of vaccines, and probiotics [66, 73]. The practice of good husbandry ensures fish are healthy and able to resist various disease agents naturally found in their environment. When they become stressed, the dietary requirements of fish for nutrients and vitamins change and a diet that compensates for such needs may optimize the growth of fish in captivity.

In recent years, it has become clear that the integrity of the gastrointestinal tract is essential in defense against pathogen attack as well as in proper endocrine and osmoregulatory activity. In recognition of this, Dimitroglou et al. [74] added the mannan oligosaccharide, Bio-Mos® (Alltech Inc, USA) to the diet of several marine fish including gilthead sea bream and found that this improved the gut morphology.

It is assumed that one of the roles that the mannan plays in protection of the fish is agglutination of pathogenic bacteria, which prevents their colonization of the gut. Indeed, the application of Bio-Mos significantly reduced the bacterial load in fish guts by reducing the biomass of aerobically cultivated bacteria [74]. Torrecillas et al. [75, 76] applied Bio-Mos to sea bass juvenile diets and found that it improved growth rates by 10%. Moreover, challenge trials using *Vibrio alginolyticus* showed that Bio-Mos fed sea bass had fewer of the pathogenic vibrio in their gut.

In recognition of the essential role of healthy gut flora in fish, especially in young fish, the use of immunostimulants, prebiotic, and/or probiotic bacteria have been proposed as a means to reduce gut colonization by pathogens [77], thereby improving the survival of cultured fish. Probiotics involves the addition of nonpathogenic bacteria to the diet and water of fish with the aim of loading the gut with bacteria that will prevent colonization by competing pathogens. The use of prebiotics and immunostimulants focuses on boosting the fish immune system so that the fish may more readily recognize and repel pathogen gut colonization. Although research has been conducted on the use of probiotics in Mediterranean aquaculture, (e.g., [78–80]), this approach has not successfully replaced the use of antibiotics to combat disease. One of the problems related to the use of probiotic bacteria is concern that these may not be as safe as they are supposed to be and their use may lead to other problems rather than a sustainable solution in the battle against disease.

Immunostimulants are commonly used in finfish farming to reduce the risk of disease by stimulating the protective activity of the immune system. The common forms of immunostimulants used in sea bream and sea bass aquaculture include ascorbic acid, a-tocopherol, and glucans [81, 82], which are added to the feed. Their presence appears to enhance antibacterial lysozyme activity and other indicators of disease resistance, but there is considerable discussion about their effectiveness due to the inherently wide range in concentrations and activities of the disease resistance molecules in fish serum.

Another approach to reduce the risk of disease is by means of classical selection/breeding for disease resistance by means of selective breeding programs [83]. The understanding of immune regulatory genes responsible for resistance to finfish pathogens is still in its infancy in Mediterranean aquaculture, but this field is rapidly expanding and it is anticipated that genetically superior lines will dominate the populations of fish reared in intensive aquaculture [84].

Escapes

In addition to problems related to disease and fluctuating profitability of aquaculture operations, fish farmers are also concerned with keeping their fish within the cages so that these can be marketed at the end of the growth cycle. There are many factors that may lead to loss of the farmed stock, including storms that may physically damage the net cages, predators (e.g., sharks, dolphins, bluefish, seals) that may bite the nets in their attempt to eat the enclosed fish, human error (e.g., during replacement of net cages or during harvest), poachers that cut the nets to catch fish, collision of ships with cage farms, etc. All of these generally result in the release of farmed fish to the surrounding environment, involving financial loss to the farmer and potential environmental problems related to genetic and ecological interactions of the escapees with the wild fish. At present, there are an

estimated >1 billion fish; mostly sea bream and sea bass in net cage farms throughout the Mediterranean as compared to much smaller stocks of the wild populations of these species [85], so the potential impact of escapees is considerable. Because many Mediterranean countries do not require farmers to report escapes, there are no reliable data on the frequency of escapes, however, it is assumed that the percentages of escapees are similar to those reported in Norway [86], ranging between 0% and 6%. In addition to genetic "pollution" of the wild-stock gene pool, and potential competition between escapees and wild fish over the same habitat and food resources, there is also concern regarding the spread of disease from farmed fish to wild fish populations [87].

Measures to Reduce Escapes and Damage due to Escapes As the volume of aquaculture production increases in the Mediterranean to match demand, and with the anticipated addition of North Africa to the fish-producing countries, there is a growing need for regulation in order to minimize problems related to escapes. In order to appreciate the scale of escapes from Mediterranean aquaculture, there is a need to legislate reporting of escape events, as is currently done in other parts of the world. Moreover, several new finfish species have been domesticated and their potential effect, as escapees, on wild populations and on the ecosystem need to be assessed. In addition, in order to assess escape impacts, it is useful to be able to track the escaped fish, as described by Triantaphyllidis [88].

There are many measures that may be employed to reduce the risk of escapes from fish cages. Storm damage to farm systems is one of the major causes of escapes and employment of a reliable standard, as practiced in Norway (NS 9415 – requirements for design and operation of marine fish farms) is a promising approach to reduce such risks. Even sturdy, reliable cages are occasionally damaged by especially strong storms, but most of the surface wave energy is concentrated in the upper 10 or 15 m of the water column [85]. Submersible cage systems designed for open sea conditions, such as the Sub-flex system (www.subflex.org) and the Ocean-Spar system (www.oceanspar.com/) are an option to reduce mechanical stress to net cages in high-energy environments. Added advantages of submersible cage systems include the reduced risk of collisions with maritime vessels and the reduced visibility following the "out of sight–out of mind" solution to NIMBYism. Human poachers are a problem that may be reduced by vigilance and by cooperation with the local police or security forces. Marine predators that bite net cages from the outside may be deterred by using stronger materials, though this has financial consequences, or by embedding chemical deterrents in the net material. Several farmed species tend to bite the net material from the inside and this may create holes enabling escapes. The biting may be prevented by using taste deterrents, as described for predators, or stronger material that will be more bite resistant. Moe et al. [86] suggest making the cage environment more "appealing" or stimulating to reduce gnawing on the net mesh which they attribute to boredom.

In addition to reducing the risk and frequency of escapes, there is also a need to reduce the impacts caused by the escaped fish. One direction that is being tested is the development of sterile triploid sea bream and sea bass that will not be able to pass on their genes to wild fish. Another possibility is the recapture of the escaped fish, but this direction is still in early developmental stages. The location of fish farms relative to areas of high ecological sensitivity or to spawning grounds should be one of the major considerations in light of the possibility that some of the stocked fish may escape.

Introduced Exotic Species

Invasive species are probably the cause of the greatest ecological problems identified over the past century, not only in terrestrial but also in aquatic and marine systems [4]. This problem has intensified over the past 20–30 years, as the volume of intercontinental traffic has increased. Aquatic invasive species are a major threat to marine biodiversity and impact human health and the economy [89]. There are numerous examples of the impacts of invasives on human welfare and environmental health, for example, the invasion of the Black Sea by the exotic ctenophore *Mnemiopsis leydi*, which caused the collapse of most of the local fisheries [90]; invasion of the eastern Mediterranean by the Red Sea medusa, *Rhopilema nomadica*, which has

heavily impacted Israeli and Turkish fisheries, tourism and coastal facilities [91].

In the eastern Mediterranean, exotic introductions are mainly channeled through the Suez Canal whereas most of the successful invaders in the western Mediterranean have been introduced by ships and via aquaculture [92].

Species introductions via aquaculture activities may be intentional or accidental, though the consequences are generally similar. Intentional introductions generally include the import of an exotic species and its release into the environment, without the intention that it spreads and dominates its new habitat. Examples include shellfish such as the Japanese oyster that was brought to France and spread rapidly throughout French coastal waters and certain species of sport fish that were intentionally released in northwestern US waters. The majority of introductions are not intentional but rather accidental and may occur in a number of ways. One common example of an accidental introduction is the transfer of a local species of oyster from a hatchery to the coast in a restocking program and the accidental release of an associated seaweed with the oysters. In another case, recreational boaters did not thoroughly wash the bottom of their boat after a holiday in a given bay and when they transported the boat back to their own shore, they brought with them a cryptic gastropod which subsequently invaded the new environment and decimated the local clam population.

Measures to Reduce the Invasion of Exotic Aquatic Species and Associated Damages In order to avoid the various risks involved in the use of exotic species, it is essential to rear/grow native species, as a rule. In many cases, the commercially attractive species are not native and farmers prefer to culture nonnative species. Introduced species may only be considered after taking all required precautions as specified in the ICES *Code of Practices on the Introductions and Transfers of Marine Organisms* [93] and the report on *Alien Species in Aquaculture* by Hewitt et al. [94]. Because the introduced species may escape and invade either local or neighboring environments, with implications for marine biodiversity, there is a need for both regional and international collaboration to address transboundary introductions and invasion issues, as discussed in UNEP [92].

The Mediterranean Aquaculture Market

The dominant species currently reared in the Mediterranean Sea are sea bream and sea bass [95]. These are native species that have been traditionally fished and eaten for centuries in many of the Mediterranean countries. Aquaculture has greatly increased the availability of these fish to the public and as production has increased, the price of the farmed fish has dropped dramatically so that in many cases its profitability is questionable. One of the important elements of a sector's sustainability is its economic performance yet the current trend in the Mediterranean is a plateau in profitability, that is, stagnation due to a glut in production of the two main species and a concurrent drop in their market value.

Alternative Aquaculture Species In order to survive and grow, the Mediterranean aquaculture sector needs to diversify its marine finfish production and include species with high market value. There are many native Mediterranean species that have a market because they are caught and sold by fishers and are suitable for cage culture. These include several species that have already been successfully reared in the eastern Mediterranean, such as Grey mullet (*Mugil cephalus*), Dover sole (*Solea solea*), Meagre (*Argyrosomus regius*), Sharp snout sea bream (*Diplodus puntazzo*), White bream (*Diplodus sargus*), Red porgy (*Pagrus pagrus*), Shi drum (*Umbrina Cirossa*), Striped sea bream (*Lithognathus mormyrus*), Pandora (*Pagellus erythrinus*). Although these fish are commercially available for aquaculture, there are several bottlenecks that prevent large-scale production. These include lack of knowledge regarding their nutritional requirements, lack of farm facilities for production, slow growth rates (may be related to nutrition or other problems), sensitivity to certain pathogens.

Ecosystem Effects

It has been shown that Mediterranean fish farms generally have a local effect, primarily on the underlying benthos, as described above, yet within a short distance from the cages, this effect rapidly dissipates. It has been suggested that the large load of nutrients that pass via the farmed fish into the marine environment are rapidly processed by the biota, yet may exert some

ecosystem effects. This hypothesis was tested by comparing the biological/chemical composition of seawater from fish-farming zones (within 2–3 nautical miles of fish farms) versus nonfarm zones (20 nautical miles of fish farms) in three parts of the Aegean sea in May and in September [49]. The data indicate that there is rapid transfer of nutrients up the food web, from the primary producers, via herbivores [53] to fish [96, 97]. These findings may be interpreted in a number of ways and their ramifications are debatable. If the precautionary approach is adopted, it is not clear what sort of implications these ecosystem-level changes may have and so they should be regarded with caution. On the other hand, if fish farms increase the size of natural fisheries, providing fishermen with an increased catch, this may be regarded as a positive externality of aquaculture (positive socioeconomic impact), which should be encouraged.

Seagrasses

One of the unique features in the Mediterranean Sea is the seagrass meadows of *Posidonia oceanica*. This slow-growing seagrass species occurs exclusively in the Mediterranean and grows best in clear, oligotrophic waters [98]. *P. oceanica* provides many ecosystem services, such as seabed stabilization, provision of a complex habitat to many larval and juvenile animals, oxygen production/release and long-term storage of CO_2 as plant tissue. Due to their slow growth rates, there is concern that these seagrass beds will not manage to recover if damaged and this important ecosystem and the services it provides may be lost. Marine botanists have calculated that some clonal colonies of *P. oceanica* may be 100,000 years old, that is, these are probably the largest and oldest-known living "organisms" on earth (http://en.wikipedia.org/wiki/Posidonia_oceanica). Because of their unique features, important ecological role and relatively low resilience to damage there is a strong movement in many Mediterranean countries to conserve and protect seagrass meadows from pollution, coastal development, trawling, and aquaculture. Recent work indicates that *P. oceanica* meadows located near or under fish farms have sustained considerable loss, including reduced meadow density, high shoot mortality rates (50-Diaz-Almela et al. 2008), increased epiphyte cover [99, 100] and very slow recovery rates following farm removal [101]. An analysis of several variables that may cause the observed damage to *P. oceanica*, in the context of the *MedVeg* project, has identified the deposition of particulate organic matter from the farms onto the seagrasses as the main factor leading to seagrass decline [102].

Measures to Protect Seagrass Meadows A set of recommendations were published by Pergent-Martini et al. [103] for the protection of Posidonia from fish farms, guided by the precautionary principle. These specified that: (a) Fish farms should not be situated directly over *P. oceanica* and *Cymodocea nodosa* (another important seagrass) meadows. (b) If seagrasses grow where a farm is planned, cages should be located at least 200 m from the nearest meadow. (c) Because these seagrasses generally occur at depths shallower than 45 m, farms should be set up at depths of 45–50 m where possible. (d) Environmental Impact Studies that relate to all seagrasses in the region should precede all lease requests to set up a fish farm. (e) If there are *P. oceanica* meadows near fish farms, these should be examined every 4 years to assure they have not been affected by the farming activity. On the basis of more recent findings, Holmer et al. [102] recommended to increase the distance between seagrass beds and fish farms to 400 m and to establish permanent seagrass plots to enable annual monitoring and sampling for seagrass health.

Future Directions

In the early 1990s, finfish aquaculture was generally a novelty in most parts of the Mediterranean, but this has changed radically during the past 20 years, as cage culture has spread throughout the region. Aquaculture is one of the fastest growing sectors worldwide and in the Mediterranean and it has many advantages over other food production industries, but in order to maintain a "green" image, aquaculture production and development must be sustainable. Progress has been made in many aspects of aquaculture technology but there are several areas that require attention and improvements in order to make this industry more environmentally and socioeconomically sustainable. Although numerous projects have focused on understanding the environmental interactions of aquaculture, the calculation of a reliable "carrying capacity" for aquaculture in a given water body is still generally

beyond our means, that is, there is a need for further study of ecological processes on a variety of different scales with respect to fish farms. Because there are so many different types of habitats and ecosystems within the Mediterranean Sea (e.g., hard vs. soft seafloors, Adriatic vs. Levant, etc.), it is essential that the ecological and socioeconomic research address region-specific issues [45].

As aquaculture expands into new areas and new species, there is added urgency to improve the understanding of fish pathology in Mediterranean systems. In addition to bacterial diseases, there is a need for research into antihelminthic treatments, and better understanding of life cycles and early diagnostics for many of the Mediterranean parasites. In view of EU policies concerning reduction of chemical use in the aquatic environment, the prudent and effective use of chemotherapeutants is essential. This may be achieved by combining therapeutic treatments with such health management strategies as breeding of tolerant fish, improving water quality, and vaccination.

Escaped fish may impact wild fish through competition, predation, habitat displacement, gene pool dilution, etc. In an attempt to reduce the numbers of escapees, progress is being made (e.g., in the EU project "Prevent Escape," which includes several partners from Mediterranean countries) in the design of cages that should be more damage resistant and in devising strategies to track the escapees and to reduce migration away from the breeched cages.

A Need for Legislation

One of the areas that urgently requires attention to enable development of the sector is legislation since this aspect is inadequately addressed in many Mediterranean countries. Moreover, in many countries that are active in aquaculture, there is a policy vacuum with regard to this sector. There is a need for clear rules and standards for licensing, planning, environmental impact assessment (EIA), administrative organization, and coordination. In the absence of clarity and transparency in such matters, investors and entrepreneurs will not take the risks involved in establishing aquaculture operations and the development of the industry will be retarded and sluggish. In a review of the legal obstacles to aquaculture, Van Houtte [104] included: (a) the legal status of water used (public or privately owned), the nature of water used (marine, brackish, or freshwater); (b) the legal status and nature of the land used (coastal vs. inland; private vs. public); and (c) the need for government regulation of aquaculture, and related activities. Moreover, the lack of coordination among public and regulatory agencies with regard to the EIA process, planning, etc. complicates the aquaculture application process. To further complicate matters, the permit application process is complex, cumbersome and very time consuming. The number of laws, regulations, rules, and procedures involved in the application process is large and many different authorities are involved at several levels. On top of that, the application requirements vary widely from country to country and in some countries, aquaculture legislation may vary internally on a provincial or regional basis.

One of the most problematic policy issues has to do with site selection and site allocation for aquaculture. As an economic activity that takes place, and has an effect on the littoral, aquaculture competes with many other uses of the coastal zone and needs to be included in Mediterranean coastal planning and management schemes. In recognition of the rapidly growing sector, in 2002 the European Union acknowledged that planning and coastal management would be among the major challenges facing European aquaculture. This was reinforced by the recent EU [105] communication, which emphasizes that "area choice is crucial and spatial planning has a key role to play in providing guidance and reliable data for the location of an economic activity, giving certainty to investors, avoiding conflicts and finding synergies between activities and environments with the ultimate aim of sustainable development" and invites all Member States to "develop marine spatial planning systems, in which they fully recognize the strategic importance of aquaculture."

One of the options chosen by some Mediterranean countries is zoning, that is, allocating a specific area for aquaculture as a means to reduce conflicts between coastal activities. In principle, this sort of approach simplifies things, provided: (a) the criteria used for selection of the aquaculture zones were appropriate and (b) the decisions regarding zoning involved the stakeholders and their interests. It is noteworthy that although there is aquaculture zoning in some

countries, aquaculture jurisdiction generally falls under regional governance, that is, there are no national zoning plans in the Mediterranean [54]. Although zoning is probably one of the better options for site selection, the lack of national coordination regarding the allocation of space for aquaculture will probably increase conflicts with time, thereby jeapordizing the sustainability of the industry. It would therefore be prudent to promote national zoning policy for aquaculture in the Mediterranean.

The conflict over space is fierce in the coastal zone as there are many competing stakeholders and one of the solutions to this is to go offshore [95, 106]. There have been many initiatives over the past few decades promoting offshore or open-ocean aquaculture, including several international conferences in the Mediterranean; however, a number of obstacles have prevented the realization of this concept. These obstacles include (a) economic feasibility of such ventures; (b) engineering and technological solutions for aquaculture in sites exposed to oceanic conditions; (c) international and national (government) support for an offshore aquaculture industry; (d) investors willing to take the risks involved in offshore aquaculture; (e) lack of understanding of the ecological ramifications (water column and benthos; local and regional effects) of large-scale aquaculture in exposed sites; and (f) the biological effects of cultivation in exposed conditions (storms, currents, predators, etc.) on the farmed stock, and other similar issues. At present, there are a few Mediterranean fish farms situated in exposed, offshore sites, but these are the exception rather than the rule, and most farms are situated in protected or semi-sheltered sites. A move away from the coastal zone into offshore waters will probably become a reality rather than an option in the near future and the aquaculture sector stands to benefit if it can accept this and help establish the scientific basis and technology in advance.

Integrated Aquaculture

Another option that makes considerable ecological and economic sense is an integration of different forms of aquaculture within the same farm. By arranging systems for rearing finfish (a form of "fed" aquaculture) adjacent to systems for growing shellfish and/or seaweeds (extractive aquaculture), it may be possible to increase farm sustainability on a number of levels. On the ecological level, shellfish and algae are called "extractive" because they extract their nutrients or food from within the system (autochthonous), and can therefore help reduce the nutrient loads from fish farms. Finfish are usually "fed" with feed that is manufactured from materials that come from outside the system (allochthonous) and the release of wastes and uneaten feed from the farms may affect water and sediment quality and even cause eutrophication. On the social level, cultivation of different products as compared to monoculture will require greater manpower and expertise and create the opportunity for greater employment, both within the farms and in the form of support services. On the economic level, additional crops should increase farm profitability, provided the filtering organisms are able to absorb the nutrients efficiently and they fetch a good price at market. Moreover, by diversifying the cultured stock, the farmer protects himself from risks related to market fluctuations, storms, and disease. Integrated aquaculture is currently practiced in Canada and in China on pilot to commercial scales but it is not clear how this approach will develop with time. In the Mediterranean Sea, there are no commercial integrated aquaculture farms [21] and this is due to the fact that either the secondary crop is a low-value (not profitable) product or the secondary (extractive) crop is not able to grow in the oligotrophic conditions that characterize Mediterranean waters. The potential for integrated Mediterranean aquaculture exists, but it must be both ecologically and economically viable to work.

Herbivorous Fish

One of the major challenges for both global and Mediterranean aquaculture is the limited supply of essential fish oil and fish meal [107]. The artificial diets of many farmed fish, including salmon, sea bream, and sea bass rely heavily on fish meal and fish oil, which places considerable pressure on wild fisheries (the source of fish meal and oil), severely jeapordizing the sustainability of the sector [108]. Several strategies have been proposed to address this problem, including the extraction of oils from fish-processing wastes [109, 110] and from fishery by-catch discards (the noncommercial fish

and animals that are caught by fishermen and subsequently thrown back to sea), and feeding fish with plant oils. There has been some success in the replacement of fish oils with plant oils [107], but many fish species have reduced survival and growth rates when reared without fish oils.

Another solution that has been proposed to address this problem is the rearing of herbivorous fish that do not require fish oils. Although these are generally not the highest value fish, they are nonetheless commercial species that are profitable to rear. The most common farmed herbivore in the Mediterranean is the diadromous gray mullet, *Mugil cephalus* (www.fao.org/fishery/culturedspecies/Mugil_cephalus/en). A lot of the pond rearing technology of this species was developed in Israel [111] and included polyculture. Egypt, the world leader in mullet production, has recently exceeded 1 million t/y. Although this fish is common in some of the southern Mediterranean countries, it does not have a large market in southern Europe and this is a challenge that needs to be overcome to promote herbivores as more sustainable species for aquaculture. Another problem that exists for *M. cephalus* is the absence of commercial hatcheries. Despite recent breakthroughs in spawning induction [112, 113], juvenile mullets are still collected from river mouths for aquaculture purposes thereby jeapordizing natural populations. These problems need to be addressed if this species is to be seriously considered a sustainable alternative to the common Mediterranean carnivores.

Indicators for Sustainable Aquaculture

The Water Framework Directive establishes the Environmental Quality Standards for European waters, and all activities that may affect environmental quality, for example, aquaculture must comply with these standards. Aquaculture lease applications generally include Environmental Impact Assessments (EIA), which assess risks and predict the impacts of aquaculture. Monitoring is an approach to test if EIA predictions were correct, and to establish a feedback system to protect both the environment and the fish farmer. The Modeling-Ongrowing fish farms-Monitoring (MOM) system [114, 115] was developed for salmon farming in Scandinavia, and includes a feedback process of EIA – monitoring – farm adjustment. Although the MOM concept was developed for Scandinavian farms, this approach has been adopted by the operators of several farms in the Mediterranean Sea to monitor their performance and environmental status. Monitoring generally includes measurement of: (a) physical variables, such as hydrography, weather, water temperature, sediment type, etc.; (b) chemical variables, including dissolved oxygen, nutrients, suspended solids, dissolved and particulate organic matter, etc.; and (c) biological attributes, for example, algal pigments, biomass, productivity, macrofauna abundance, diversity, etc. Fernandes et al. [116] reviewed the science underlying aquaculture monitoring in Europe and found that it was generally motivated by research interests rather than by clear environmental objectives. Whereas comprehensive monitoring of marine environments improves the understanding of the functioning of these systems [117], and thus the ability to predict the response of these waters to anthropogenic perturbations, it is often not necessary to include many of the variables that are monitored [102].

The CONSENSUS project recently established a set of 18 indicators (www.euraquaculture.info/index.php?option%20=%20com_content&task%20=%20view&id%20=%20149&Itemid%20=%20118) to promote "European Best Aquaculture Practice." These indicators are currently being evaluated to examine their practicality and suitability for the sector. In a separate project entitled *ECASA* (www.ecasa.org.uk/), a set of indicators to assess aquaculture–environment interactions were evaluated in order to streamline the farm monitoring process. This was done for aquaculture in both northern European and several Mediterranean countries (e.g., [118]) yet despite the advances made in that project, there is still a need to further streamline the list of indicators. The main criteria that should be used as a guideline in the quest for optimal indicators have been described in UNESCO [119] and include: (a) relevance, (b) feasibility (amount of effort, expertise, and cost required to obtain the data), (c) sensitivity (to inform on how the environment is responding), and (d) clarity (how easy it is for stakeholders to understand). Although progress has been made toward developing the final list of such indicators for aquaculture, this work is only partially done and further work is needed to achieve this.

Bibliography

1. FAO (2009) Integrated mariculture: a global review. In: Soto D (ed) FAO fisheries and aquaculture technical paper no 529. FAO, Rome, pp 133–183
2. Zveryaev II, Arkhipkin AV (2008) Structure of climatic variability of the Mediterranean sea surface temperature. Part I. Standard deviations and linear trends. Russ Meteorol Hydrol 33:377–382
3. Thingstad TF, Krom MD, Mantoura RFC, Flaten GAF, Groom S, Herut B, Kress N, Law CS, Pasternak A, Pitta P, Psarra S, Rassoulzadegan F, Tanaka T, Tselepides A, Wassmann P, Woodward EMS, Riser CW, Zodiatis G, Zohary T (2005) Nature of phosphorus limitation in the ultraoligotrophic eastern Mediterranean. Science 309:1068–1071
4. EAA (European Environmental Agency), (2006) Priority issues in the Mediterranean environment. EEA Report 4:88
5. Abdulla A, Gomei M, Maison E, Piante C (2008) Status of marine protected areas in the Mediterranean Sea. IUCN, Malaga, p 152
6. Basurco B (2000) Offshore mariculture in Mediterranean countries. In: Muir J, Basurco B (eds) Mediterranean offshore mariculture. Options Méditerranées: Série B. Etudes et Recherches 30, October 20–24 1997, Zaragoza, Spain
7. Bardach JE, Ryther JH, McLarney WO (1972) Aquaculture: the farming and husbandry of freshwater and marine organisms. Wiley-Interscience, New York, p 868
8. Basurco B, Lovatelli A (2003) The aquaculture situation in the Mediterranean sea. In: International conference on sustainable development of the Mediterranean and Black sea environment, Predictions for the Future, Thessalonica, Greece, May 29–31 2003. http://www.oceandocs.org/bitstream/1834/543/1/Basurco.pdf
9. Nun M (1964) Ancient Jewish fishery (in Hebrew). Hakibbutz Hameuchad, Merkhavia, Israel
10. Raban A, Galili E (1985) Recent maritime archaeological research in Israel-a preliminary report. Int J Naut Archeol 14:321–356
11. Cataudella S (1996) Description of main Mediterranean aquaculture systems. Notes from the TECAM advanced course on food and feeding of farmed fish and shrimp, CIHEAM, FAO, NIOF, Alexandria, Egypt
12. Lovatelli A (2005) Report of the third meeting of the ad hoc GFCM/ICCAT working group on sustainable bluefin tuna farming/fattening practices in the Mediterranean. FAO fisheries report no. 779, FAO, Rome, p 108
13. FAO (1997) FAO technical guidelines for responsible fisheries. Fisheries department, aquaculture development. Report no. 5, FAO, Rome, p 40
14. Fish Site (2006) Farmed vs wild salmon? – a comparison. (http://www.thefishsite.com/articles/107/farmed-vs-wild-salmon-a-comparison)
15. Altintzoglou T, Verbeke W, Vanhonacker F, Luten J (2010) The image of fish from aquaculture among Europeans: impact of exposure to balanced information. J Aquat Food Prod Technol 19(2):103–119
16. Mazur NA, Curtis AL (2006) Risk perceptions, aquaculture, and issues of trust: lessons from Australia. Soc Nat Resour 19:791–808
17. Mazur N, Curtis A (2008) Understanding community perceptions of aquaculture: lessons from Australia. Aquac Int 16:601–621
18. Katrandis S, Nitsi E, Vakrou A (2003) Social acceptability of aquaculture development in coastal areas; the case of two Greek islands. Coast Manage 31:37–53
19. Korchenkov I (2010) Mariculture development: public policy, citizen's attitudes and factors affecting them. MA thesis, University of Haifa, Haifa, p 63
20. Whitmarsh D, Wattage P (2006) Public attitudes towards the environmental impact of salmon aquaculture in Scotland. Europ Environ 16:108–121
21. Angel DL, Freeman S (2009) Integrated aquaculture (INTAQ) as a tool for an ecosystem approach to the marine farming sector in the Mediterranean Sea. In: Soto D (ed) Integrated marine aquaculture: a global review. FAO fisheries and aquaculture technical paper no. 529, FAO, Rome, p 133–183
22. Brown JR, Gowen RJ, McLusky DS (1987) The effect of salmon farming on the benthos of a Scottish sea loch. J Exp Mar Biol Ecol 109:39–51
23. Gowen RJ, Bradbury NB (1987) The ecological impact of salmonid farming in coastal waters: a review. Annu Rev Oceanogr Mar Biol 25:563–575
24. Weston DP (1990) Qualitative examination of macrobenthic community changes along an organic enrichment gradient. Mar Ecol Prog Ser 61:233–244
25. Silvert WL (1992) Assessing environmental impacts of finfish aquaculture in marine waters. Aquaculture 107:67–71
26. Hargrave BT (ed) (1994) Modelling benthic impacts of organic enrichment from marine aquaculture. Canadian technical report, fisheries and aquatic science. Report # 1949, DFO, Canada, p 125
27. Holmer M, Kristensen E (1996) Seasonality of sulfate reduction and pore water solutes in a marine fish farm sediment: the importance of temperature and sedimentary organic matter. Biogeochemistry 32:15–39
28. Pearson TH, Black KD (2001) The environmental impacts of marine fish cage culture. In: Black KD (ed) Environmental impacts of aquaculture. Sheffield Academic, Sheffield, pp 1–31
29. Karakassis I, Eleftheriou A (1997) The continental shelf of Crete: structure of macrobenthic communities. Mar Ecol Prog Ser 160:185–196
30. Karakassis I, Eleftheriou A (1998) The continental shelf of Crete: the benthic environment. PSZNI: Mar Ecol 19:263–277
31. Duineveld GCA, Tselepides A, Witbaard R, Bak RPM, Berghuis EM, Nieuwland G, van der Weele J, Kok A (2000) Benthic-pelagic coupling in the oligotrophic Cretan sea. Prog Oceanogr 46:457–481
32. Karakassis I, Tsapakis M, Hatziyanni E, Papadopoulou K-N, Plaiti W (2000) Impact of cage farming of fish on the seabed in three Mediterranean coastal areas. ICES J Mar Sci 57: 1462–1471

33. Vizzini S, Mazzola A (2004) Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. Mar Pollut Bull 49:61–70
34. Vizzini S, Savona B, Caruso M, Savona A, Mazzola A (2005) Analysis of stable carbon and nitrogen isotopes as a tool for assessing the environmental impact of aquaculture: a case study from the western Mediterranean. Aquac Int 13:157–165
35. Sarà G, Lo Martire M, Buffa G, Mannino AM, Badalamenti F (2007) The fouling community as an indicator of fish farming impact in Mediterranean. Aquac Res 38:66–75
36. Cromey CJ, Black KD (2005) Modelling the impacts of finfish aquaculture. In: Hargrave BT (ed) Environmental effects of marine finfish aquaculture. Springer, Berlin, Heidelberg, pp 129–156
37. Askens A, Izquiredo MS, Robaina L, Vegara JM, Montero D (1997) Influence of fish meal quality and feed pellet on growth, feed efficiency and muscle composition in gilthead sea bream (*Sparus aurata*). Aquaculture 153:251–261
38. Lupatsch I, Kissil GWm, Sklan D (2001) Optimization of feeding regimes for European seabass *Dicentrarchus labrax*: a factorial approach. Aquaculture 202:289–302
39. Clarke S, Smart A, van Barneveld R, Carter C (1997) The development and optimization of manufactured feeds for farmed southern bluefin tuna. Austasia Aquacult 11:59–62
40. McKindsey CW, Thetmeyer H, Landry T, Silvert W (2006) Review of recent carrying capacity models for bivalve culture and recommendations for research and management. Aquaculture 261:451–462
41. Porello S, Lenzi M, Persia E, Tomassetti P, Finoia MG (2003) Reduction of aquaculture wastewater eutrophication by phytotreatment ponds system: I Dissolved and particulate nitrogen and phosphorus. Aquaculture 219:515–529
42. Lenzi M, Gennaro P, Mastroianni A, Mercatali I, Persia E, Roffilli R, Solari D, Tomassetti P, Porrello S (2009) Improvement of a system for treating land-based fish-farm effluents. Chem Ecol 25:247–256
43. Sorokin YI, Sorokin PU, Ravagnan G (2006) Hypereutrophication events in the Ca'Pisani lagoons associated with intensive aquaculture. Hydrobiologia 571:1–15
44. Tett P, Gilpin L, Svendsen H, Erlandsson CP, Larsson U, Kratzer S, Fouilland E, Janzen C, Lee J-Y, Grenz C, Newton A, Ferreira JG, Fernandes T, Scory S (2003) Eutrophication and some European waters of restricted exchange. Cont Shelf Res 23:1635–1671
45. Karakassis I (2001) Aquaculture and coastal marine biodiversity. Oceanis 24:272–286
46. Danovaro R, Gambi C, Luna GM, Mirto S (2004) Sustainable impact of mussel farming in the Adriatic Sea (Mediterranean Sea): evidence from biochemical, microbial and meiofaunal indicators. Mar Pollut Bull 49:325–333
47. Sara G (2007) Ecological effects of aquaculture on living and non-living suspended fractions of the water column: a meta-analysis. Water Res 41:3187–3200
48. Karakassis I, Pitta P, Krom MD (2005) Contribution of fish farming to the nutrient loading of the Mediterranean Sea. Sci Mar 69:313–321
49. Pitta P, Apostolaki ET, Tsagaraki T, Tsapakis M, Karakassis I (2006) Fish farming effects on chemical and microbial variables of the water column: a spatio-temporal study along the Mediterranean Sea. Hydrobiologia 563:99–108
50. Diaz-Almela E, Alvarez E, Santiago R, Marba N, Holmer M, Grau T, Danovaro R, Argyrou M, Karakassis Y, Duarte CM (2008) Benthic input rates predict seagrass (*Posidonia oceanica*) fish farm-induced decline. Mar Pollut Bull 56:1332–1342
51. Apostolaki ET, Marba N, Holmer M, Karakassis I (2009) Fish farming enhances biomass and nutrient loss in *Posidonia oceanica* (L.) Delile. Estuar Coast Shelf Sci 81:390–400
52. Dalsgaard T, Krause-Jensen D (2006) Monitoring nutrient release from fish farms with macroalgal and phytoplankton bioassays. Aquaculture 256:302–310
53. Pitta P, Tsapakis M, Apostolaki ET, Tsagaraki T, Holmer M, Karakassis I (2009) "Ghost nutrients" from fish farms are transferred up the food web by phytoplankton grazers. Mar Ecol Prog Ser 374:1–6
54. IUCN (2009) Aquaculture site selection and site management . Guide for the sustainable development of Mediterranean aquaculture. IUCN Gland, Switzerland and Malaga, Spain, p 303
55. Mourente G, Tocher DR (2009) Tuna nutrition and feeds: current status and future perspectives. Rev Fish Sci 17: 373–390
56. Meyer FP (1991) Aquaculture disease and health management. J Anim Sci 69:4201–4208
57. Bonga SEW (1997) The stress response in fish. Physiol Rev 77:591–625
58. Wedemeyer GA (1997) Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama GK, Pickering AD, Sumpter JD, Schreck CB (eds) Fish stress and health in aquaculture, vol 62, Society for experimental biology seminar, series. Cambridge University Press, Cambridge
59. Pickering AD (1998) Stress responses of farmed fish. In: Black KD, Pickering AD (eds) Biology of farmed fish. CRC, Boca Raton, pp 222–255
60. Varvarigos P (1997) Marine fish diseases in Greece. Fish Farmer 20:10–12
61. Waknitz FW, Tynan TJ, Nash CE, Iwamoto RN, Rutter LG (2002) Review of potential impacts of Atlantic salmon culture on puget sound chinook salmon and hood canal summer-run chum salmon evolutionarily significant units. US Department of Commerce, NOAA technical memoranda, NMFS-NWFSC-53, p 83
62. Diamant A, Colorni A, Ucko M (2007) Parasite and disease transfer between cultured and wild coastal marine fish. In: Briand F (ed) Impact of mariculture on coastal ecosystems, CIESM workshop monographs, Lisbon no. 32, pp 49–54
63. Breuil G, Mouchel O, Fauvel C, Pepin JF (2001) Sea bass *Dicentrarchus labrax* nervous necrosis virus isolates with distinct pathogenicity to sea bass larvae. Dis Aquat Organ 45:25–31

64. Diaz Lopez B (2006) Bottlenose dolphin *Tursiops truncatus* predation on a marine fin fish farm: some underwater observations. Aquat Mamm 32:305–310
65. Diamant A, Colorni A, Ucko M (2007) Parasite and disease transfer between cultured and wild coastal marine fish. Impact of mariculture on coastal ecosystems. CIESM workshop monographs no: 32, Monaco, pp 49–54
66. IUCN (2007) Interactions between aquaculture and the environment. Guide for the sustainable development of Mediterranean aquaculture, Gland, Switzerland and Málaga, Spain, p 110
67. Austin B, Austin DA (1999) Bacterial fish pathogens: disease of farmed and wild fish. Springer-Praxis, Chichester
68. Barnes AC, Hastings TS, Aymes SGB (1995) Aquaculture antibacterials are antagonized by sea-water. J Fish Dis 18:463–465
69. Smith P, Niland T, O'Domhnaill F, O'Tuathaigh G, Hiney M (1996) Influence of marine sediments and divalent cations on the activity of oxytetracycline against *Listonella anguillarum*. Bull Eur Assn Fish P 16:54–57
70. Athanassopoulou F, Pappas IS, Bitchava K (2009) An overview of the treatments for parasitic disease in Mediterranean aquaculture. Options Méditerranéennes 86:65–82
71. Hansen GH, Olafsen JA (1999) Bacterial interactions in early life stages of marine cold water fish. Microb Ecol 38:1–26
72. Verschuere L, Rombaur G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671
73. Lall SP, Lewis-McCrea LM (2007) Role of nutrients in skeletal development in fish – an overview. Aquaculture 267:3–19
74. Dimitroglou A, Davies S, Moate R, Spring P, Sweetman J (2007) The beneficial effect of Bio-Mos on gut integrity and enhancement of fish health. Alltech technical seminar series, Dublin, November 2007
75. Torrecillas S, Makol A, Caballero MJ, Montero D, Robaina L, Real F, Sweetman J, Tort L, Izquierdo MS (2007) Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. Fish Shellfish Immunol 23:969–981
76. Torrecillas S, Caballero MJ, Sweetman J, Makol A, Izquierdo MS (2007b) Effects of feeding Bio- Mos on European sea bass (*Dicentrarchus labrax*) juvenile culture. Alltech technical seminar series, Dublin, November 2007
77. Gatesoupe J (2005) Probiotics and prebiotics for fish culture, at the parting of the ways. Aqua Feeds: Formulation & Beyond 2:1–5
78. Gatesoupe FJ (1999) The use of probiotics in aquaculture. Aquaculture 180:147–165
79. Picchietti S, Mazzini M, Taddei AR, Renna R, Fausto AM, Mulero V, Carnevali O, Cresci A, Abelli L (2007) Effects of administration of probiotic strains on GALT of larval gilthead seabream: immunohistochemical and ultrastructural studies. Fish Shellfish Immunol 22:57–67
80. Avella MA, Olivotto I, Silvi S, Place AR, Carnevali O (2010) Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish. Am J Physiol Regul Integr Comp Physiol 298:359–371
81. Jeney G, Galeotti M, Volpatti D (1994) Effect of immunostimulation on the non specific immune response of sea bass *Dicentrarchus labrax*. In: International symposium on aquatic animal health: program and abstracts, Seattle, Washington DC, September 4–8 1994, p 76
82. Bagni M, Archetti L, Amadori M, Marino G (2000) Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*). J Vet Med 47:745–751
83. Knibb W, Gorshkova G, Gorshkov S (1997) Selection for growth in the gilthead seabream (*Sparus aurata*). Isr J Aquacult/ Bamidgeh 49:57–66
84. Chistiakov DA, Hellemans B, Volckaert FAM (2007) Review on the immunology of European sea bass *Dicentrarchus labrax*. Vet Immunol Immunopathol 117:1–16
85. Dempster T, Moe H, Fredheim A, Jensen Q, Sanchez-Jerez P (2007) Escapes of marine fish from sea-cage aquaculture in the Mediterranean Sea: status and prevention. In: Briand F (ed) Impact of mariculture on coastal ecosystems, CIESM workshop monographs, no. 32, Lisboa, pp 55–60
86. Moe H, Dempster T, Sunde LM, Winther U, Fredheim A (2007) Technological solutions and operational measures to prevent escapes of Atlantic Cod (*Gadus morhua)* from sea-cages. Aquac Res 38:91–99
87. Diamant A (1997) Fish to fish transmission of a marine myxosporean. Dis Aquat Organ 30:99–105
88. Triantafyllidis A (2007) Aquaculture escapes: new DNA based monitoring analyses and application on seabass and seabream. In: Briand F (ed) Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs, no. 32, Lisboa, pp 67–72
89. Carlton JT (2009) Deep invasion ecology and the assembly of communities in historical time. In: Rilov G, Crooks JA (eds) Biological invasions in marine ecosystems. Springer, Berlin, Heidelberg, pp 13–56
90. Shiganova TA (1998) Invasion of the Black Sea by the ctenophore *Mnemiopsis leidyi and* recent changes in pelagic community structure. Fish Oceanogr 7:305–310
91. Galil BS (2007) Seeing red: alien species along the Mediterranean coast of Israel. Aquat Invasions 2:281–312
92. United Nations Environment Programme (UNEP)/ Mediterranean Action Plan (2005) Action plan concerning species introductions and invasive species in the Mediterranean Sea. RAC/SPA, Tunis, p 30
93. ICES (2005) ICES code of practice on the introductions and transfers of marine organisms, p 30
94. Hewitt CL, Campbell ML, Gollasch S (2006) Alien species in aquaculture. Considerations for responsible use. IUCN, Gland, Switzerland and Cambridge, p 32
95. Cardia F, Lovatelli A (2007) A review of cage aquaculture: the Mediterranean Sea. In: Halwart M, Soto D, Arthur JR (eds) Cage aquaculture – regional reviews and global overview. FAO fisheries technical paper no. 498, FAO, Rome, pp 159–190

96. Machias A, Karakassis I, Labropoulou M, Somarakis S, Papadopoulou KN, Papaconstantinou C (2004) Changes in wild fish assemblages after the establishment of a fish farming zone in an oligotrophic marine ecosystem. Estuar Coast Shelf Sci 60:771–779
97. Machias A, Karakassis I, Giannoulaki M, Papadopoulou N, Smith CJ, Somarakis S (2005) Response of demersal fish communities to the presence of fish farms. Mar Ecol Prog Ser 288:241–250
98. Holmer M, Perez M, Duarte CM (2003) Benthic primary producers – a neglected environmental problem in Mediterranean maricultures? Mar Pollut Bull 46:1372–1376
99. Balata D, Nesti U, Piazzi L, Cinelli F (2007) Patterns of spatial variability of seagrass epiphytes in the north-west Mediterranean Sea. Mar Biol 151:2025–2035
100. Pérez M, García T, Ruíz JM, Invers O (2008) Physiological responses of the seagrass *Posidonia oceanica* as indicators of fish farm impact. Mar Pollut Bull 56:869–879
101. Delgado O, Ruiz JM, Pérez M, Romero J, Ballesteros E (1999) Effects of fish farming on seagrass (*Posidonia oceanica*) in a Mediterranean bay: seagrass decline after organic loading cessation. Oceanol Acta 22:109–117
102. Holmer M, Hansen P-K, Karakassis I, Borg JA, Schembri PJ (2008) Monitoring of environmental impacts of marine aquaculture. In: Holmer M, Black K, Duarte CM, Marbà N, Karakassis I (eds) Aquaculture in the ecosystem. Springer, Berlin, pp 47–86
103. Pergent-Martini C, Boudouresque CF, Pasqualini V, Pergent G (2006) Impact of fish farming facilities on *Posidonia oceanica* meadows: a review. Mar Ecol 27:310–319
104. Van Houtte A (2001) Establishing legal, institutional and regulatory framework for aquaculture development and management. In: Subasinghe RP, Bueno P, Phillips MJ, Hough C, McGladdery SE, Arthur JE (eds) Aquaculture in the third millennium. Technical proceedings of the conference on aquaculture in the third millennium, NACA and FAO, Bangkok, Thailand, February 20–25 2000
105. EU (European Union) (2009) Building a sustainable future for aquaculture: a new impetus for the strategy for the sustainable development of European aquaculture. Communication from the Commission to the European Parliament and the Council COM (2009), 162, Brussels, p 13
106. Stickney RR (1997) Offshore Mariculture. In: Bardach JE (ed) Sustainable aquaculture. John Wiley & Sons, New York, pp 53–86
107. Turchini GM, Torstensen BE, Ng W-K (2009) Fish oil replacement in finfish nutrition. Rev Aquacult 1:10–57
108. Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, Forster I, Gatlin DM, Goldburg RJ, Hua K, Nichols PD (2009) Feeding aquaculture in an era of finite resources. Proc Natl Acad Sci 106:15103–15110
109. Raffi SM, (2006) Sustainable Utilisation of Bycatch Resources. In: Kannaiyan S, Balasubramanian T, Ajmalkhan S, Venkataraman K (eds) Biodiversity and Conservation of Marine Bioresources, National Biodiversity Authority, pp 107–113
110. Rubin, S (1993) Fish waste in Japan. Stiftelsen rubin, report no. 007/15, p 22
111. Sarig S (1981) The Mugilidae in polyculture in fresh and brackish water fishponds. In: Oren OH (ed) Aquaculture of grey mullets. Cambridge University Press, Cambridge, UK, pp 391–409
112. De Monbrison D, Tzchori I, Holland MC, Zohar Y, Yaron Z, Elizur A (1997) Acceleration of gonadal development and spawning induction in the Mediterranean grey mullet. Mugil cephalus: preliminary studies. Isr J Aquacult/Bamidgeh 49:214–221
113. Aizen J, Meiri I, Tzchori I, Levavi-Sivan B, Rosenfeld H (2005) Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen Comp Endocrinol 142:212–221
114. Ervik A, Hansen PK, Aure J, Stigebrandt A, Johannessen P, Jahnsen T (1997) Regulating the local environmental impact of intensive marine fish farming I. The concept of the MOM system (Modelling-Ongrowing fish farms-Monitoring). Aquaculture 158:85–94
115. Hansen PK, Ervik A, Schaanning M, Johannessen P, Aure J, Jahnsen T, Stigebrandt A (2001) Regulating the local environmental impact of intensive marine fish farming II. The monitoring programme in the MOM system (Modelling-Ongrowing fish farms-Monitoring). Aquaculture 194: 75–92
116. Fernandes TF, Eleftheriou A, Ackefors H, Eleftheriou M, Ervik A, Sanchez-Mata A, Scanlon T, White P, Cochrane S, Pearson TH, Read PA (2001) The scientific principles underlying the monitoring of the environmental impacts of aquaculture. J Appl Ichthyol 17:181–193
117. Tett P (2008) Fishfarm wastes in the ecosystem. In: Holmer M, Black K, Duarte CM, Marbà N, Karakassis I (eds) Aquaculture in the ecosystem. Springer, Berlin, pp 1–46
118. Borja Á, Germán Rodríguez J, Black K, Bodoy A, Emblow C, Fernandes TF, Forte J, Karakassis I, Muxika I, Nickell TD, Papageorgiou N, Pranovi F, Sevastou K, Tomassetti P, Angel D (2009) Assessing the suitability of a range of benthic indices in the evaluation of environmental impact of fin and shellfish aquaculture located in sites across Europe. Aquaculture 293:231–240
119. UNESCO (2003) A reference guide on the use of indicators for integrated coastal management – integrated coastal area management dossier 1, Intergovernmental Oceanographic Commission Manuals and Guides No. 45.

Marine Fisheries Enhancement, Coming of Age in the New Millennium

Kenneth M. Leber
Center for Fisheries Enhancement, Mote Marine Laboratory, Sarasota, FL, USA

Article Outline

Glossary

Anadromous Species that spawn in freshwater, then their offspring gradually make their way into estuaries or the sea, where they remain during much of the subadult and adult stages of the life cycle, before returning to rivers and streams to spawn.

Catadromous Species whose females release their eggs at sea, then the offspring move as larvae or early juveniles into estuaries, rivers, and streams where they spend the juvenile stage of the life cycle.

Marine Species that spawn in sea water, including those that spend most of their lives at sea and catadromous fishes, which spawn in seawater, then enter freshwater nursery habitats.

Marine fisheries enhancement Release of aquacultured marine organisms into seas and estuaries to increase or restore abundance and fishery yields in the wild.

Outbreeding depression Caused when offspring from crosses between individuals from different populations or subpopulations (stocks) have lower fitness than progeny from crosses between individuals from the same population/stock.

Recruitment The process of joining an existing population. Species *recruit* to the juvenile stages in nursery habitats; juveniles subsequently *recruit* to adult stages in adult habitats. Species *recruit* to a fishery when they reach the minimum size fished.

Reintroduction Temporary release of cultured organisms with the aim of reestablishing a locally extinct population.

Restocking Release of cultured juveniles into wild population(s) to restore severely depleted spawning biomass to a level where it can once again provide regular, substantial yields.

Sea ranching Release of cultured juveniles into unenclosed marine and estuarine environments for harvest at a larger size in "put, grow, and take" operations.

Stock enhancement The release of cultured juveniles into wild populations to augment the natural supply of juveniles and optimize harvests by overcoming limitations in juvenile recruitment.

Supplementation Moderate release of cultured fish into very small and declining populations, with the aim of reducing extinction risk and conserving genetic diversity. Supplementation serves primarily conservation aims and specifically addresses sustainability issues and genetic threats in small and declining populations.

Definition of the Subject

Marine fisheries enhancement (aka "stock enhancement") is the use of hatchery-reared saltwater organisms to increase abundance and fishery yields in the wild. "Conservation hatcheries" also produce and stock depleted, threatened, or endangered organisms – to help preserve species in decline. The practice began in the latter part of the nineteenth century when fish hatcheries were first developed but understanding of the ecology and management of wild stocks into which the hatchery-reared organisms where released was very limited. Early stock enhancement thus has gone through a series of fits and starts and misfires. In the century after its birth, the technologies required for scientific inquiry of the effects and effectiveness of stocking hatchery-reared organisms were lacking. The science needed to guide reliable use of cultured

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

aquatic organisms in conservation and resource management remained undeveloped. Then, at the close of the twentieth century, new mariculture, tagging, and genetic technologies surfaced and rapid advances were made in the science underpinning marine stock enhancement.

As growth in human population size approaches the carrying capacity of the planet in this century, and the world increasingly turns to the oceans to farm and harvest food [1], sustainable fishery yields and conservation of natural resources face unparalleled challenges. Over the past two decades, marine fisheries enhancement has been transformed from a tentative, poorly developed management tool to a maturing science. Some believe research funding for this field would be better spent on traditional fishery management. But today's seafood producers, fishery managers, and "...conservationists need all the tools that biology, ecology, diplomacy and politics can muster if endangered species are to survive beyond the next century," [2] and fisheries are to continue to support a viable seafood industry and sport pastime. This entry traces the emergence and progress of marine fisheries enhancement, and offers a prescription for future direction.

The term *stock enhancement* is originally derived from efforts to augment wild fish sub-populations, or "stocks," by releasing cultured fishes into aquatic environments. Stocking cultured organisms is one of the tools available for managing aquatic natural resources. It has been used with varying degrees of success to help increase abundance of habitat- or recruitment-limited stocks to help restore depleted populations, augment fisheries and help recover threatened or endangered species. There has been much debate over the effectiveness of stock enhancement as a fisheries management tool. However, most of the scientific evaluation of stocking is quite recent [3], as is a code of responsible practices that help guide effective application [4–6], and marine fisheries enhancement is finally poised for effective use.

In the USA, from the 1880s through the early 1950s, stocking hatchery-reared marine fishes was a principal approach used by the US Fish Commission (renamed Bureau of Fisheries in 1903, Bureau of Commercial fisheries in 1956, and later the National Marine Fisheries Service) for maintaining fishery stocks. But by the 1950s the practice of stocking marine fishes to manage US fisheries was curtailed for lack of evidence of its effectiveness in fisheries management [7]. Stocking was replaced by harvest management to control total catch and sustain fisheries. Stocking of freshwater habitats continued (particularly with salmonids into rivers), although the scientific basis for many of the management decisions needed for stocking salmonids was clearly lacking and did not begin to be addressed until the mid-1970s.

In the decade following 1975, scientists began to evaluate survival and fishery contributions of stocked salmon enabled by advances in fish tagging technology [8, 9]. Quantitative evaluation of marine fish stocking began in earnest in the 1980s and 1990s. The science underlying fisheries enhancement has since evolved to the point where, in some situations, stocking can be a useful fishery management tool to help restore depleted stocks and increase abundance in recruitment-limited fisheries [6]. Effective use of enhancement, though, requires full integration with harvest and habitat management, and a good understanding by stakeholders and resource managers of the opportunities where enhancement can be used successfully as well as its limitations [5, 6]. Principles for guiding the successful use of marine fisheries enhancement to help sustain aquatic resources are now being employed to design new enhancements and reform existing efforts. What follows is a brief overview of those principles and progress made in using hatchery-reared organisms to help sustain marine resources.

Introduction

Marine fisheries enhancement is happening around the world and in some countries on a massive scale (e.g., China). However, in many countries the careful assessment of genetic and ecological risks is lagging behind implementation, putting wild stocks, the seafood supply, and sport fisheries at risk. The science of marine enhancement is still in its infancy compared to other fields of fisheries science, but now shows good potential to (1) increase fishery yield beyond that achievable by exploitation of the wild stock alone, (2) help restore depleted stocks, (3) provide protection for endangered species, and (4) provide critical information on the natural ecology, life history and environmental requirements of valuable marine species.

Stock enhancement has often been used as a generic term referring to all forms of hatchery-based fisheries enhancement. Bell et al. [3] and Lorenzen et al. [6] classified the intent of stocking cultured organisms in aquatic ecosystems into various basic objectives. Together, they considered five basic types, listed here from the most production-oriented to the most conservation-oriented:

1. Sea ranching – *recurring release of cultured juveniles into unenclosed marine and estuarine environments for harvest at a larger size in "put, grow, and take" operations*. The intent here is to maximize production for commercial or recreational fisheries. Note that the released animals are not expected to contribute to spawning biomass, although this can occur when harvest size exceeds size at first maturity or when not all the released animals are harvested.
2. Stock enhancement – *recurring release of cultured juveniles into wild population(s) to augment the natural supply of juveniles and optimize harvests by overcoming recruitment limitation in the face of intensive exploitation and/or habitat degradation*. Stock enhancements can increase abundance and fisheries yield, supporting greater total catch than could be sustained by the wild stock alone [10]. However, such increases may be offset, at least in part, by negative ecological, genetic, or harvesting impacts on the wild stock component. Stock enhancements tend to attract greater numbers of fishers, which can offset expected increase in each individual's catch-per-unit-effort (CPUE) [5, 11].
3. Restocking – *time-limited release of cultured juveniles into wild population(s) to restore severely depleted spawning biomass to a level where it can once again provide regular, substantial yields* [12]. Restocking requires release number to be substantial relative to the abundance of the remaining wild stock, and close ecological and genetic integration of wild and cultured stocks, combined with very restricted harvesting [6].
4. Supplementation – *moderate releases of cultured fish into very small and declining populations, with the aim of reducing extinction risk and conserving genetic diversity* [13, 14]. Supplementation serves primarily conservation aims and specifically addresses sustainability issues and genetic threats in small and declining populations [6].
5. Reintroduction – *involves temporary releases with the aim of reestablishing a locally extinct population* [15]. Continued releases should not occur, as they could interfere with natural selection in the newly established population. Fishing should also be restricted to allow the population to increase in abundance rapidly [6].

Scientific development of marine fisheries enhancement was lacking throughout most of the twentieth century. Although stocking cultured marine fishes began in the nineteenth century, the technology was limited to stocking only eggs and larvae. There were no published accounts of the fate of released fish until empirical studies of anadromous salmonids began to be published in the mid-1970s [16, 17], followed by the first studies (published in English) of stocked marine invertebrates in 1983 [18, 19] and marine fishes in 1989 [20].

During the past two decades, the field of marine fisheries enhancement has advanced considerably. Science in this field is rapidly growing, in part because of critical examination and debate about the efficacy of enhancement and the need for quantitative evaluation (e.g., [21, 22]), and in part because of advances made in aquaculture, genetics, tagging, and fishery modeling technologies, which have enabled quantitative studies and predictions of stocking effects. A clear process has emerged for developing, evaluating, and using enhancement [4–6]. Together, this process and the rapid growth of knowledge about enhancement effects should enable responsible and effective use of enhancement in marine fisheries management and ocean conservation.

Scientific Development of Marine Fisheries Enhancement

Scientific and Strategic Development

Since 1989, progress in marine fisheries enhancement has occurred at two levels – scientific advances and adoption of a careful and responsible approach to planning and organizing enhancement programs and manipulating abundance of marine species using aquacultured stocks. Much of the progress made in the 1990s was scientific and involved an expansion of field studies to evaluate survival of released fish

and improve the effectiveness of release strategies. The earliest studies on effectiveness of stocking *marine* fishes, published in English in the scientific literature, were in Japan [20, 23–26] and Norway [27–31], followed by studies in the USA [32–39], and Australia [40]. Progress made with invertebrates is well covered by Bell et al. [12].

Following the initial publications of scientific studies of marine fish enhancement, the number of peer-reviewed publications and symposia in this field began to escalate ([41–52], and see abstracts in [53]). It is now clear that stocking marine organisms can be an effective addition to fishery management strategies, but only when certain conditions are met. For stocking to be productive and economical, and help ensure sustainability of wild stocks, careful attention must be given to several key factors and stocking must be thoroughly integrated with fisheries management [6]. It is clear that stocking can be harmful to wild stocks if not used carefully and responsibly.

Aside from scientific gains in this field, the other level of progress made in the past two decades has been the evolution of a strategic "blueprint" for enhancements, such as the principles discussed in "a responsible approach to marine stock enhancement" [4, 6]. By the early 1990s, salmon enhancement in the US Pacific Northwest, which had been underway for a century, was beginning to incorporate reforms that were needed to improve efficiencies and protect wild stocks from genetic hazards that can lead to loss of genetic diversity and fitness. Concerns had been mounting over uncertainty about the actual effectiveness of salmon hatcheries and impacts on wild stocks. Concerns about wild stock impacts were twofold, including ecological effects of hatchery fish, such as competitive displacement, and genetic issues, such as translocation of salmon stocks, domestication and inbreeding in the hatchery and associated outbreeding depression, and loss of genetic diversity related to hatchery breeding practices (e.g., [54, 55]). Meanwhile, special sessions on marine stock enhancement began appearing at major fisheries and mariculture conferences in the early 1990s [41–44]. These sessions took a sharp turn from past approaches, where the principal focus in conference presentations about stock enhancement had been mainly on Mariculture research topics alone. The conveners of the special sessions on stock enhancement in the 1990s recruited presenters who worked on evaluating the effects and effectiveness of stocking hatchery organisms into the sea and interactions of hatchery and wild stocks. The special sessions focused on the "questions of the day" in marine enhancement and fostered debate in the marine enhancement research community about many of the reform issues being considered in salmon enhancement. The early 1990s was a period of rapid developments in enhancements, characterized by engagement of multiple scientific disciplines in a field that had previously been guided largely by a single discipline – aquaculture.

The salmon experience and reforms underway in salmon enhancement made it clear that a careful and multidisciplinary approach was needed in the development and use of marine enhancement. Many involved in developing new marine fisheries enhancement projects were paying close attention to the debate that had emerged over salmon hatcheries. Following the 1993 special session on "fisheries and aquaculture interactions" held at a mariculture conference in Torremolinos, Spain [44], several of the presenters (including scientists from Japan, Norway, the USA, and Italy [United Nations Food and Agriculture Organization, FAO]) met and formed an "International Working Group on Stock Enhancement," and affiliated the workgroup with the World Aquaculture Society. At that inaugural working group meeting, a decision was made to publish a platform paper to frame the question, "what is a responsible approach to marine stock enhancement?" This paper was presented at the 1994 American Fisheries Society symposium, "Uses and Effects of Cultured Fishes in Aquatic Ecosystems," and published in the 1995 peer-reviewed symposium proceedings [4]. The paper recommended ten principles for developing, evaluating, and managing marine stock enhancement programs. The Responsible Approach paper afforded a model for developing and managing new enhancement programs and refining existing ones. It has also helped frame research questions in the emerging science of marine fisheries enhancement.

The International Working Group on Stock Enhancement (IWGSE) was instrumental in advancing the science of marine fisheries enhancement in the 1990s. The working group focused primarily on

highlighting ongoing stock enhancement research around the world and fostering awareness of the Responsible Approach in their publications and presentations. International awareness and new research in the field was aided by the broad international makeup of the working group. Membership grew and soon included scientists from Australia, Canada, China, Denmark, Ecuador, Italy, Japan, Norway, Philippines, Solomon Islands, Spain, the UK, and the USA. Initially, the primary communication vehicle used by the working group was the special sessions on stock enhancement, which it planned and convened annually in various countries at the international conference of the World Aquaculture Society. The working group promoted a synergy among its members and the influence of the group expanded as members planned additional workshops and symposiums in their own countries and brought IWGSE scientists into the planning process.

The period 1990–1997 was a fertile time that gave birth to a rapid expansion of science in marine fisheries enhancement, which continues to this day, aided since 1997 in large part by the International Symposium on Stock Enhancement and Sea Ranching (ISSESR). The first ISSESR, held in 1997 in Bergen, Norway, was the brainchild of the Norwegian PUSH program (Program for Development and Encouragement of Sea Ranching) and the Norwegian Institute of Marine Research (IMR). In 1995, IMR scientists invited IWGSE scientists to become involved in the International Scientific Committee charged with planning the program for the first ISSESR. The first ISSESR, and the series of follow-up symposia that it launched (see www.SeaRanching.org), have encouraged and brought about fundamental advancements in the field of marine enhancement – by networking the scientists working in this specialized field, highlighting their work at the ISSESR, and publishing their peer-reviewed articles in the symposium proceedings. The 3–5 day ISSESR has now become a regular scientific symposium event, hosted by a different country every 4–5 years. Following the first ISSESR in Bergen [47], subsequent symposiums in the series were held in Kobe, Japan in 2002 [49], in Seattle, USA in 2006 [52], and in Shanghai, China in 2011 [53]. The fifth ISSESR will be held in Sydney, Australia in 2015 or 2016. Inquiries from scientists in different countries interested in hosting the sixth one are already being received by the organizing group. Following the first ISSESR, the IWGSE scientists continued the efforts they started in the working group through their involvement in the International Scientific Committees for the ISSESR and steering committees for other stock enhancement symposia (e.g., [46, 48, 51]). In 2010, a refined and updated version of the Responsible Approach was published [6] and presented at the fourth ISSESR.

As in any new science, lack of a paradigm and consensus on the key issues retard progress. The ISSESR and other marine enhancement symposia and working groups have helped to place scientific focus on critical uncertainties and communicate results of new science in this field at symposiums and in the scientific literature. They have also provided a forum for debate on the issues, and increased networking of scientists, resource managers, students, and educators working in this field worldwide. The focus on key issues is nurturing this new field of science.

Technological and Tactical Constraints

Although marine enhancements do show promise as an important tool in fisheries management, why has this field taken so long to develop and why have marine enhancement programs often failed to achieve their objectives? The scientific development of marine fisheries enhancement has long been impeded by lack of the technologies needed to evaluate effects of stocking cultured fish. Although marine enhancements began in the 1880s, until the advent of the coded-wire tag in the mid-1960s [8], there was no way to identify treatment groups and replicates in experimental releases of juvenile cultured fish [56]; and quantitative marking methods for multiple experimental groups of postlarvae and very small juveniles (<50 mm in length) came much later (e.g., [57]). To make matters worse, scientific development of marine enhancement was also stymied by lack of adequate technology for culturing marine fishes. Rearing methods for larval and juvenile marine fishes, many of which require live feeds during the larval stage, remained undeveloped until the mid- to late 1970s, when breakthroughs finally began to be achieved in rearing a few marine species past metamorphosis [58]. By the mid-1980s mass production of juveniles had been achieved for several

species of marine fishes. Even today, though, many marine fishes cannot yet be cultivated to the juvenile stage in the quantities needed for stocking. Without the availability of juveniles grown to a wide range of sizes, fundamental questions about density dependence, hatchery-wild fish interactions and cost-yield efficiency of size-at-release and other release variables cannot be addressed in field experiments. Thus, even the basic technologies needed to develop and understand the potential of marine enhancement have been unavailable until relatively recent times for some fishes and have yet to be developed for others.

Technology has not been the only constraint to successful development of marine fisheries enhancement. The effective use of stocking cultured marine organisms in fisheries management has been hindered by lack of understanding of the effect of releases on fish population dynamics and a lack of related, quantitative assessment tools [10]. Moreover, there has been a lack of essential governance and fisheries management considerations in planning, designing, implementing, and evaluating enhancement programs [6, 59]. A symptom of this is the relentless concern among stakeholders and hatchery managers alike about the numerical magnitude of fish released, rather than on the effective contribution of the hatchery program to fisheries management goals. Certainly, a hatchery needs to meet some release quotas, but the numbers of fish released is a misleading statistic for gauging success or comparing effectiveness among enhancement programs. Yet, from the very beginning, progress has been judged by the number of eggs, yolk-sac larvae or juveniles stocked, rather than by the number of fish added to the catch or to spawning stock biomass. The thinking behind this approach apparently is "grow and release lots of hatchery fish and of course they'll survive and add to the catch," without realizing the need to optimize release strategies (e.g., [39, 60, 61]) (e.g., to know what size-at-release, release habitat and release magnitude combination has the greatest impact on population size, fishery yields, and economics), or that the impact from stocking could in fact be a negative one on wild stocks (such as replacement of wild fish by hatchery fish) if certain precautions are not taken. This attitude has been pervasive and exists even today among many stakeholders and enhancement administrators. In fact, research now shows that survival and recruitment to the fishery following hatchery releases is a complex issue that requires much greater understanding about the fishery, hatchery fish performance, and biological and ecological factors in the wild than simply "the catch is down, thus releasing large numbers of fish will bring it back up." And quite often large release magnitudes are achieved by releasing millions of postlarvae, rather than fewer but larger juveniles. But releases of postlarvae alone may be effective, yet can also be totally ineffective, depending on conditions at the release site [62].

The key to successful use of stocking is to plan enhancement programs from a fisheries/resource management perspective, using a broad framework and scientific approach [6, 59]. The probability of achieving effective results is greatly increased when stakeholders are engaged from the outset in planning *new* programs, using a framework that is structured, multilayered, participatory, and makes good use of science, to design, implement, and analyze enhancement fisheries systems [6]. Incorporating the key principles in the Responsible Approach into the frameworks of *existing* programs as well is likely to improve performance.

Responsible Approach to Marine Fishery Enhancement

In retrospect, the slow development of marine fish culture (a century behind salmonid aquaculture) has helped marine stock enhancement programs avoid some of the mistakes of the past made with salmon stock enhancement, where lack of understanding of genetic issues during most of the twentieth century led to inadvertent domestication and inbreeding in salmon hatchery populations, leading to reduced fitness in wild stocks. Marine finfish juvenile production technology lagged behind freshwater and anadromous fish culture by a century. Thus, mass release into the sea of juvenile marine fishes large enough to survive and enter the breeding population did not begin until the 1980s. The relatively recent capabilities to conduct marine fisheries enhancement emerged at about the same time that geneticists realized that hatchery practices with salmonids (1) could reduce genetic diversity in the hatchery and ultimately, enhanced wild stocks, owing to inadequate broodstock management, (2) have caused translocations of salmon genes into

environments where they are less fit, and (3) have contributed to loss of local adaptations in the wild population. Today, population genetics is much better understood and broodstock genetics and hatchery practices can be better managed to address these concerns (e.g., [63–65]). Thus, marine enhancement programs need careful guidance from qualified geneticists. The Puget Sound and Coastal Washington Hatchery Reform Project in the USA has been instrumental in reforming salmon enhancements [66]. This group affords a model for managing enhancement hatcheries in the twenty-first century.

As progress was being made in the early 1990s to better understand the genetic structure of stocks and how to manage genetics in hatcheries, realizing the need for reform in approaches to enhancing non-salmonids was just beginning. In the mid-1990s, Cowx [67], for enhancements in freshwater systems, and Blankenship and Leber [4], for enhancements in marine and estuarine systems, published papers calling for a broader, more systematic, reliable, and accountable approach to planning stock enhancement programs. Prompted both by the salmonid hatchery reform movement and by the WAS IWGSE, the ten principles presented in Blankenship and Leber ([4] Table 1) gained widespread acceptance as the "Responsible Approach" to stocking marine organisms and provided a platform for subsequent discussions on planning, conducting, and evaluating marine enhancements (e.g., [6, 12, 22, 51, 52, 68–70]). Since 1995, the awareness of the Responsible Approach has steadily increased and has helped guide hatchery and reform processes for marine enhancements worldwide [11, 36, 37, 39, 60, 62, 69–90].

The Responsible Approach provides a conceptual framework and logical strategy for using aquaculture technology to help conserve and increase natural resources. The approach prescribes several key components as integral parts of developing, evaluating and managing marine fisheries enhancement programs. Each principle is considered essential to manage enhancements in a sustainable fashion and optimize the results obtained [4, 6].

A major development since the publication of the original "Responsible Approach" has been increasing interest from fisheries ecologists in understanding and quantifying the effects of hatchery releases from a fisheries management perspective. This has led to the development of fisheries assessment models that can be used to evaluate stocking as a management option alongside fishing regulations [5, 10]. At the same time, approaches to fisheries governance underwent major changes that allow enhancements to become more integrated into the management framework and in some cases, were driven by interest in enhancement approaches [59].

Walters and Martell [5] discuss four main ways that a marine enhancement program can end up causing more harm than good: (1) the replacement of wild with hatchery recruits, with no net increase in the total stock available for harvest (competition/predation effects); (2) unregulated fishing-effort responses to the

Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 1 The ten principles of a responsible approach to marine stock enhancement [4]

1	Prioritize and select target species for enhancement by ranking and applying criteria for species selection
2	Develop a management plan that identifies how stock enhancement fits with the regional plan for managing stocks
3	Define quantitative measures of success to track progress over time
4	Use genetic resource management to avoid deleterious genetic effects on wild stocks
5	Implement a disease and health management plan
6	Consider ecological, biological, and life history patterns in forming enhancement objectives and tactics; seek to understand behavioral, biological, and ecological requirements of released and wild fish
7	Identify released hatchery fish and assess stocking effects on the fishery and on wild stock abundance
8	Use an empirical process for defining optimal release strategies
9	Identify economic objectives and policy guidelines, and educate stakeholders about the need for a responsible approach and the time frame required to develop a successful enhancement program
10	Use adaptive management to refine production and stocking plans and to control the effectiveness of stocking

Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 2 Code of responsible conduct for marine stock enhancement [5]

• Make certain that management priorities and acceptable trade-offs are absolutely clear
• Do careful stock assessments to show that the target stock is recruitment overfished or can no longer rear successfully in the wild
• Show that enhanced fish can recruit successfully in the wild
• Show that total abundance is at least initially increased by the hatchery fish contribution
• Show that fishery regulations are adequate to prevent continued overfishing of the wild population, unless there has been an explicit decision to "write off" the wild population
• Show that the hatchery production system is actually sustainable over the long run, when it is to be a permanent component of the production system

presence of hatchery fish that cause overfishing of the wild stock; (3) "overexploitation" of the forage resource base for the stocked species, with attendant ecosystem-scale impacts; and (4) genetic impacts on the long-term viability of the wild stock. They stress that it is critical to monitor the impacts of enhancement as the program develops to have evidence in hand if debate about the efficacy of the program does surface. To help guide developing programs, they provide and discuss a "Code of Responsible Conduct" as critical steps in marine fisheries enhancement program design (Table 2).

In 2010, Lorenzen, Leber, and Blankenship [6] published an updated version of the Responsible Approach to refine the original key principles and include five additional ones (Table 3). The key principles added in the updated version bring stakeholders more firmly into the planning process; place much stronger emphasis on a-priori evaluation of the potential impact of enhancements using quantitative models; place marine fishery enhancements more firmly within the context of fishery management systems; emphasize design of appropriate aquaculture rearing systems and practices; and incorporate institutional arrangements for managing enhancements. Lorenzen et al. [6] provide comprehensive discussions for each of the 15 key

Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 3 The updated responsible approach (From [6])

Stage I: Initial appraisal and goal setting	
1	Understand the role of enhancement within the fishery system [*new*]
2	Engage stakeholders and develop a rigorous and accountable decision making process [*new*]
3	Quantitatively assess contributions of enhancement to fisheries management goals
4	Prioritize and select target species and stocks for enhancement
5	Assess economic and social benefits and costs of enhancement
Stage II: Research and technology development including pilot studies	
6	Define enhancement system designs suitable for the fishery and management objectives [*new*]
7	Design appropriate aquaculture systems and rearing practices [*new*]
8	Use genetic resource management to maximize effectiveness of enhancement and avoid deleterious effects on wild populations.
9	Use disease and health management
10	Ensure that released hatchery fish can be identified
11	Use an empirical process for defining optimal release strategies
Stage III: Operational implementation and adaptive management	
12	Devise effective governance arrangements [*new*]
13	Define a management plan with clear goals, measures of success, and decision rules
14	Assess and manage ecological impacts
15	Use adaptive management

principles listed in Table 3. Readers are urged to consult Lorenzen et al. [6] for additional detail, as it is beyond the scope, here, to repeat their discussions of each principle.

The 15 principles in the updated Responsible Approach include the broad range of issues that need to be addressed if enhancements are to be developed or reformed responsibly [6]. Clearly, marine

Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 4 Key areas of expertise needed in marine fisheries enhancement

• Fisheries science
• Fisheries management
• Adaptive management
• Marine aquaculture
• Population genetics
• Aquatic animal health
• Population ecology
• Behavioral ecology
• Community ecology
• Resource economics
• Social science and institutional analysis and design
• Statistics and experimental design
• Tagging technology
• Communications and outreach

enhancement programs are multidisciplinary and their effective use requires specialist knowledge and skills from diverse fields (Table 4). Forming interdisciplinary teams of the various specialists required is an important factor in employing the Responsible Approach in developing, reforming, and executing marine enhancements. For effective design of enhancement programs, specialists in each area of expertise listed in Table 4 should be included in the planning teams.

It should be clear that without a careful monitoring system in place, marine enhancements simply cannot be managed. Monitoring is essential to understand the impacts of enhancement, to manage release strategies so that they are efficient and designed well enough to achieve the goals of the program, to protect against misuse of stocking (as discussed in 5 and 6), resulting in harm to wild stocks, and to document success or failure in meeting enhancement program objectives. Walters and Martel [5] list several key monitoring requirements for managing fishery enhancements well: (1) mark all (or at least a high and known proportion of) fish released from hatcheries; (2) mark as many wild juveniles as possible at the same sizes/locations as hatchery fish are being released; (3) experimentally vary hatchery releases over a wide range from year to year and from area to area, probably in on/off alternation (temporal blocking) so as to break up the confounding of competition/predation effects with shared environmental effects; (4) monitor changes in total recruitment to, production of, and fishing effort in impacted fisheries, not just the percentage contribution of hatchery fish to production; (5) monitor changes in the fishing mortality rates of both wild and hatchery fish directly, through carefully conducted tagging programs that measure short-term probabilities of capture; and (6) monitor reproductive performance of hatchery-origin fish and hatchery-wild hybrid crosses in the wild. Sound management-action design and monitoring is the essence of adaptive management [91] and adaptive management enables refinements, progress, and success in marine enhancement programs [4, 6, 11, 92].

Marine fisheries enhancement is a powerful tool that requires careful and interdisciplinary planning to control its effects. The process of transforming marine enhancement from an idea before its time into an effective resource management and sea ranching tool involves adopting a clear prescription for responsible use. As marine enhancement comes of age in this new millennium, agencies and stakeholders have a growing library of protocols for enhancement at their disposal and the responsibility to use them. The Responsible Approach and Code of Responsible Conduct provide healthy prescriptions for controlling the outcome of enhancements. These principles need to be adopted and used well, in order to increase and ensure the readiness of this tool to aid in conservation and to increase fishery yields when it is needed. Growth in human population size is fast approaching a critical level, and much greater attention will be placed in this century on obtaining food from the sea [1]. It is unwise to not be ready with marine enhancement to help sustain depleted, threatened, and endangered species, help maintain wild stocks in the face of increasing fishing pressure, help sustain sports fisheries, and help increase fishery yields.

Legacy from the Past

Allure of a Quick Fix

Marine enhancement programs are often seen as a "quick fix" for a wide variety of problems in marine

resource management. At best, they may be an important new component of marine ecosystem management; if not implemented responsibly, though, they may lull fishery managers into false confidence and thus lead to inaction and delay in the development of other fisheries management and restoration programs [5, 6].

Although marine fisheries enhancement is certainly not a quick fix, it can be a powerful tool for resource management when conditions warrant the use of this tool and if the time and care needed are taken to develop enhancement programs well. Unfortunately, the allure of a quick fix has often prompted stakeholders and managers to skip or ignore several elements needed to allow those programs to succeed, leading to wholesale failure of such efforts. The field of marine fisheries enhancement is littered with examples of enhancement projects that failed to achieve their potential for lack of a careful enough or quantitative approach (e.g., see accounts discussed in [7, 21, 62, 72, 93–95]). Most of the failures can be traced back to attempts to use enhancements when they were not warranted or failure to consider several, if not most, of the principles now incorporated in the "Responsible Approach" and "Code of Responsible Conduct" for marine fisheries enhancement.

Isolation from the Fisheries Science Community

Historically, marine fisheries enhancements have been conducted more or less isolated from other forms of fisheries management. Enhancement hatcheries have often been promoted by stakeholders and government mandates without the necessary funding or authorization behind them to do much more than produce and release fish without funds for monitoring impacts and adaptive management needed to increase the effectiveness of enhancements. Such programs are often built and implemented from a vantage point within resource management agencies that has little or no connectivity with the existing fishery management process. This has stymied development of this field in two ways – first, by compelling hatcheries to operate within resource management agencies largely independent from stock assessment and fisheries monitoring programs, or even worse, within different agencies altogether. Second, such isolation has fostered development of a production-oriented operational mode, and thwarted development of an enhancement-oriented mode [92].

Part of this isolation from fishery management also stems from the poor track record of the early marine hatcheries as an effective way to recover depleted fish stocks, coupled with the lack of scientific development of marine fisheries enhancement for so long into the twentieth century. This has understandably led to bias against fishery enhancements. Many of today's fishery scientists have been schooled to understand that stock enhancement has not worked, based in part on the lingering legacy from past failures and in part on lack of awareness of new marine fisheries enhancement science, as few citations have yet appeared in fisheries science textbooks. With many of the scientific achievements in fisheries enhancement having occurred only over the past decade or so, this is understandable. But in light of the need to couple fisheries enhancement with fisheries management systems, lack of awareness of progress in this field is an obstacle that may be resolved only by compilation of more and more success stories over time. Thus, it is imperative that existing and developing enhancement programs alike incorporate modern concepts about how to plan and conduct enhancements so they are enabled for success.

Progress in Marine Fisheries Enhancement

Lessons Learned from Marine Enhancement Programs

Much progress has now been made in understanding how to manage enhancement more effectively. Bartley and Bell [96] considered progress made from three decades of stocking initiatives and summarized and discussed lessons learned. These are listed here, below [96], with a brief clarification or caveat on each.

Deciding When and How to Apply the Release of Cultured Juveniles

1. Objective assessment of the need for releases is crucial – and requires an evaluation of the status of the fishery, modeling of stocking impact to determine if stocking can help achieve the goals, coupled with consideration of whether there are recruitment limitations and adequate habitat available for stocking.

2. Releases of cultured juveniles for restocking and stock enhancement need to be made at the scale of self-replenishing populations – releases will not be effective unless the spatial extent of target populations has been identified; thus prior to conducting releases of hatchery organisms, clear identification of genetically discrete stocks should be determined.
3. There are no generic methods for restocking and stock enhancement – largely because of wide variation in life history among different species and variation in ecological conditions among release sites.
4. Very large numbers of juveniles are often needed for effective stock enhancement – this is particularly so for offshore stocks, which can be comprised of a huge number of individuals; more modest releases may suffice for localized enhancement of inshore stocks or those comprised of multiple stocks that occur on relatively small scales.
5. Large areas are needed for stock enhancement of some species – and this can result in user conflict, particularly for sea ranching, where large areas are leased and protected by the enhancement program (e.g., [97]); in other cases, limited dispersal of adults and larvae indicates stocking in smaller areas can be effective, for example, common snook along Florida's Gulf Coast [98].
6. Invertebrates offer good opportunities for restocking and stock enhancement – because invertebrates are often comprised of self-recruiting populations that occur at small scales.

Integrating Interventions with Other Management Measures

7. Problems that caused lower production must be addressed before release of juveniles – particularly in the case of degraded, lost, or insufficient habitat. With better management of the wild resources, the scope for augmentation of total production declines; enhancement becomes a very site specific tool when habitat has been lost, or something needs rebuilding, or there are species of particularly high value [94].
8. Biotechnical research must be integrated with institutional and socio-economic issues – ownership rights and control and use of enhanced stocks need to be well understood by the greater institutional, social, economic, and political environment [99].
9. Successful stock enhancement programs are often run by cooperatives and the private sector – where there is increased incentive in sharing the costs of fisheries enhancement.
10. The costs and time frames involved in restocking programs can be prohibitive – hatchery costs, which can be considerable, are particularly difficult to bear in smaller countries and developing countries.

Monitoring and Evaluation

11. Development of cost-effective tagging methods is critical to efficient evaluation of stock enhancement – refining and monitoring the effects and effectiveness of marine enhancements cannot be done without a way to distinguish hatchery from wild stocks and distinct release groups.
12. Large-scale releases of hatchery-reared juveniles can affect genetic [fitness] of wild populations – genetic hazards can be caused by hatchery-wild fish interactions and these need to be minimized.

Reducing the Cost of Juveniles

13. Costs of stocking programs can be reduced by "piggybacking" production of juveniles for release on existing aquaculture – this could reduce or eliminate the need for expensive new hatchery construction for enhancement programs, as long as appropriate broodstock management protocols are in place for conserving wild-stock genetics.
14. Wild [postlarvae] can provide an abundant, low-cost source of juveniles for stock enhancement programs – this can sometimes be an effective way to reduce costs and eliminate genetic issues; successful scallop enhancement in Japan is based on collection of wild seed stock.
15. The costs of restocking can be reduced greatly for some species by relocating adults to form a viable spawning biomass – rebuilding spawning aggregations by concentrating broodstock can be effective for depleted stocks with limited larval dispersal, but care must be taken to avoid comingling different stocks (i.e., avoid translocation of exogenous genes).

Improving Survival in the Wild

16. Predation is the greatest hurdle to survival of released juveniles – care must be taken to understand ecology of the species and ecosystem at the release site and pilot experiments are needed to develop optimal release strategies to maximize survival.
17. Excessive releases of juveniles cause density-dependent mortality – density has a strong effect on growth and survival in the wild; planning release magnitude must take into account the carrying capacity at release locations. This requires adaptive management and an experimental framework for releases.
18. Small-scale experiments to test methods for releasing juveniles can give misleading results – "commercial scale" releases are needed to test assumptions made from small-scale release experiments.
19. Good survival of released juveniles at one site is no guarantee that the methods can be transferred to other sites – stocking effectiveness will vary with release location and what works at one site may not be effective at another.

Other Manipulations to Increase Abundances

20. Artificial habitats can be used to increase the carrying capacity for target species – and may enable increased production at release sites where there are resource (food, refuge, space) limitations.
21. Yields of some species can be increased by providing suitable settlement habitat and redistributing juveniles from areas of heavy settlement – for example, redistribution can be used to reduce density effects and increase probability of successful recruitment when moved to a location with greater availability of food, refuge, or settlement habitats. But care must be taken to avoid genetic hazards associated with comingling stocks.

Examples of Progress Made in Marine Enhancement

As science and constructive debate have advanced in this field, there are many signs of progress. Some explicit examples of progress made in marine enhancement over the past couple of decades are presented below, ranging in scale from local experimental investigations of release strategies and density-dependent effects on hatchery and wild stocks (e.g., [100]) to documented replenishment impact in large-scale enhancement efforts (e.g., [101, 102]). This is but a sample of examples and is by no means a comprehensive list. There are many more examples in the peer-reviewed proceedings from the ISSESR and other stock enhancement conferences [41–53] and other journal articles.

1. Adoption of a science-based responsible approach to marine stock enhancement has now become widespread, resulting in a much more assessment-driven and precautionary approach than ever before (a few examples include Refs. [4, 6, 10, 12, 20, 22, 27–29, 33, 37–39, 59–61, 68, 69, 72, 75, 84, 86, 87, 89, 96, 103–106]). This has been enabled, in part, by advances in tagging technology (e.g., [8] and see examples in [9, 56]) and in development of new marine aquaculture technologies that can now provide juvenile fishes for marine enhancement research.
2. Networking of Scientists involved in this rapidly advancing field has been fostered by various symposia and working groups, for example, the World Aquaculture Society Working Group on Stock Enhancement and the scientific committees for the International Symposium on Stock Enhancement and Sea Ranching (www.SeaRanching.org).
3. There is a much better appreciation of the importance of managing marine fishery enhancements from a fisheries management perspective (e.g., [6, 59, 107]).
4. New tools are available for modeling stock enhancement effects and effectiveness [10, 82, 108–110].
5. At least two experimental field studies have now been conducted to evaluate density-dependent interactions of stocked hatchery and wild fish; these provide evidence that increased production can be achieved in juvenile nursery habitats without displacing wild fish, but not necessarily without displacing some of the hatchery fish [33, 100].
6. There is now clear evidence and a prescription of techniques for improving post-release survival (often with a doubling effect or more) of stocked marine fishes, and optimizing release strategies to maximize stocking efficiency and control impacts

(e.g., [26, 36, 37, 39, 60–62, 70, 72, 100–115]). There is also ample evidence that in habitats with limited carrying capacity or intense predation, regardless of release strategy used, little can be done to improve survival of hatchery fish and stocking simply cannot increase production [106, 116, 117].

7. It is now fairly clear that marine enhancements may be cost effective only if (a) the supply of recruits is generally limiting, (b) there is adequate habitat to support an increased supply of juveniles, (c) cultured juveniles represent a large portion of recruitment, (d) fishing is regulated appropriately, and (e) other management measures (catch regulations and habitat restoration) are insufficient to restore catch rates [96].
8. Stock enhancement of some species of marine finfish has been successful at the scale of large bays, for example, Hirame flounder and red sea bream in Japan [72, 106] when there is sufficient carrying capacity at release sites. Carrying capacity varies considerably among release sites, and thus must be evaluated and taken into account using monitoring and adaptive management for each release site.
9. Scallop sea ranching has been a large success in Japan, New Zealand, and China, where property rights and large ocean leases have created strong incentives for careful management by fishermen and owners of the sea ranching operations [72, 101, 102, 118]. For example, near Dalian, China, Zhangzidao Fishery Group leases 2,000 km^2 of ocean-bottom-to-ocean-surface for sea ranching. In 2010, Zhangzidao harvested an average of 150 t/day of ocean scallops from their sea ranching operations (over 50,000 t/year) (Wang Qing-yin, personal communication 2011).
10. Property rights have also provided incentives for bivalve culture in the State of Washington, USA, where clam sea ranching operations have remained economically and environmentally sustainable for over three decades [119].
11. Pilot experiments with black bream in an Australian estuary have documented quite good survival and recruitment to the fishery. The latest phase of this project reveals strong rationale for long-term monitoring of enhancement impact [87, 120].
12. Restocking success with red drum in a South Carolina estuary [77, 121]. Pilot experiments revealed surplus productive capacity in the Ashley River in South Carolina, where fishery landings of red drum were doubled over a few years.
13. Pilot experiments to evaluate blue crab enhancement potential in Maryland and Virginia led to improvements in traditional fishery management, with information learned through stocking research [70, 114]. Pilot experiments can be used to provide critical information on the natural ecology, life history, and environmental requirements of valuable marine species [122].
14. Perhaps the largest scale enhancement success for fishes is Japanese chum salmon restocking – a special tool for a circumstance in which the habitat had almost totally been lost [94].

Future Directions

Over the past two decades, there has been a rapid expansion of knowledge about marine fisheries enhancement systems and the effects and effectiveness of stocking a wide variety of marine organisms for sea ranching, stock enhancement and restocking. Many gaps in knowledge have now been filled. Well thought out approaches now provide a roadmap for effective use of enhancements. When models show potential for stocking, efforts to deploy marine enhancements can be successful if the principles in the roadmap are carefully employed. The basic reason that marine enhancement programs do not have more of a track record of success stories yet is that implementing them well is a complex endeavor that demands attention to multiple factors spanning many disciplines. Rarely have these been pulled together in an enhancement program. The Hatchery Reform Project in the Pacific Northwest USA, which includes an independent scientific review panel ("Hatchery Scientific Review Group") is a good example [123]. Because of their efforts, salmonid hatchery reforms now underway are bringing many of the principles of the Responsible Approach into play. The Norwegian PUSH program is another good example. In that case, information gained from quantitative assessments of enhancement showed that stocking would not be an economical way to enhance cod in Norway, thus saving years of wasteful

spending that could have occurred there, had monitoring and adaptive management not been a central part of the enhancement system.

Successful examples of fisheries enhancement are truly group efforts, involving stakeholders, agency officials, and individuals with expertise in the principal sub-disciplines needed. Suffice to say that at this point in time few, if any, marine fisheries enhancement programs have enlisted all of the key elements of the Responsible Approach and Code of Responsible Conduct. But these principles are now well described and laid out in a systematic manner. It is reasonable to expect that if the Responsible Approach is used as the blueprint for planning and executing enhancements, and if the initial appraisal and goal setting stage indicates moving ahead, then there is ample opportunity for success in applying marine fisheries enhancements, as long as dedicated attention is focused on applying each of the key elements.

So how will marine enhancement advance to the next level – emergence of a rapidly growing body of success stories in restocking, stock enhancement, and sea ranching? Listed below are a few factors that are now needed to transition this field to the next level, where marine enhancements are well integrated into resource management systems and used wisely and appropriately.

Enabling Factors for Increasing Successful Marine Enhancements

1. *Greater awareness is needed among all stakeholders of the issues, pitfalls, progress, and opportunities in this field.* The concepts underlying effective enhancements need to be translated into lay language and used to inform stakeholders. This will help all stakeholders recognize the various issues and parameters needed for effective enhancements. Pivotal among stakeholders are public officials who fund enhancement programs, as they need to understand what it takes to develop an effective program or reform existing ones. New enhancement programs that may not be funded well enough to implement all of the key principles in the Responsible Approach would do well to use the results of Stage 1 in Table 3 to document the potential for success, but not proceed beyond Stage 1 until adequate funding is available.
2. *Use of Adaptive management is one of the most important principles for guiding successful enhancement programs.* Active adaptive management [91] is critical for gauging the effectiveness of, improving, and managing fisheries systems in the face of uncertainty. However, it is often dismissed by enhancement programs or given low priority for lack of funding or when enhancement is viewed as a quick fix. But, this important principle is used to optimize release strategies, to identify and deal with ecological or genetic impacts on wild stocks, to refine the enhancement process and identify the results of improvements, to evaluate and improve progress towards goals and objectives, and to monitor and improve economic impact. Active adaptive-management is an essential component of managing enhancement programs; it empowers management teams to understand and control the impacts of enhancements well. Without it, enhancement programs at best rely on hope to achieve their potential (but cannot) and at worst are doomed to failure. Australia is employing active adaptive management principles early in the development stage as part of ongoing work to evaluate enhancement potential for a wide range of species [124].
3. *Adapt the Responsible Approach to local circumstances.* The Responsible Approach is purposely vague on how to implement it. This is partly because not all elements are needed under all situations, but most will be. Fitting the process to particular circumstances is in itself a key part of implementing the Responsible Approach by engaging the various stakeholders in planning [6]. As progress continues in this field, additional principles will emerge that need to be included, for example, to account for needs of regional fishery management plans in response to climate change.
4. *Seek assistance from established workers in the field.* For new and developing enhancement programs, or existing ones seeking to design and implement reforms, there is a broad and expanding network of workers in this field who could be queried for advice on various enhancement issues. The ISSESR website is a good source for identifying individuals with specific kinds of expertise, by perusing presentation abstracts or locating published proceedings from past ISSESR conferences [125]. If researchers

or workers in the field are contacted, but do not have time to provide advice, they usually will help identify others who can.

This entry may help expand awareness among fishery stakeholders, other natural-resource stakeholders, scientists, and fishery managers alike about the pitfalls, challenges, and progress made in using marine hatchery releases as one of the tools in resource management and seafood production. Readers are referred to the articles and symposium proceedings cited herein to gain a better understanding of the issues, lessons learned, and progress.

The debate focused on enhancement is a healthy one, for it is fostering steady improvements and reforms in existing programs, and careful planning and design in new ones. With each advance made, the potential seen by our forefathers to use hatcheries as a tool for recovering depleted stocks, increasing abundance in recruitment-limited stocks, and producing seafood by sea ranching is coming closer to fruition. One of the greatest lessons learned from the past is that the emphasis on expanding hatchery fish production for marine enhancement should not be allowed to take the focus off of the objective – increasing yields in fisheries and recovering stocks in restoration programs. Clearly, marine fisheries enhancement is a strong tool to add to the fishery management toolbox. But only careful analysis of conditions of the wild stock and the fishery will guide when and where it is appropriate to use enhancements in addition to other management options, and when to stop. As Albert Einstein once said, "a perfection of means, and confusion of aims, seems to be our main problem." With the focus shifted to outcomes in marine enhancement programs, the appropriate means should fall into place, aided by healthy debate and prescriptions for a responsible approach to marine fisheries enhancement.

Bibliography

1. Duarte CM, Holmer M, Olsen Y, Soto D, Marbà N, Guiu J, Black K, Karakassis I (2009) Will the oceans help feed humanity? Bioscience 59(11):967–976
2. NOVA (1992) Sex and the single rhinoceros – NOVA examines the high-tech efforts to preserve the world's animal diversity. PBS documentary. NOVA Season 19, Episode 20. Public Broadcasting Service
3. Bell JD, Leber KM, Blankenship HL, Loneragan NR, Masuda R, Vanderhaegen G (eds) (2008) A new era for restocking, stock enhancement and sea ranching of coastal fisheries resources. Special Issue, Rev Fish Sci 16(1–3):402 pp
4. Blankenship HL, Leber KM (1995) A responsible approach to marine stock enhancement. Am Fish Soc Symp 15:167–175
5. Walters CJ, Martell SJD (2004) Fisheries ecology and management. Princeton University Press, Princeton
6. Lorenzen K, Leber KM, Blankenship HL (2010) Responsible approach to marine stock enhancement: an update 2010. Rev Fish Sci 18(2):189–210
7. Richards WJ, Edwards RE (1986) Stocking to restore or enhance marine fisheries. In: Stroud RH (ed) Fish culture in fisheries management. American Fisheries Society, Bethesda, pp 75–80
8. Jefferts KB, Bergman PK, Fiscus HF (1963) A coded-wire identification system for macro-organisms. Nature 198:460–462
9. Blankenship HL, Tipping JM (1993) Evaluation of visible implant and sequentially coded wire tags in sea-run cutthroat trout. North Am J Fish Manag 13:391–394
10. Lorenzen K (2005) Population dynamics and potential of fisheries stock enhancement: practical theory for assessment and policy analysis. Philos Trans R Soc Lond Ser B 260:171–189
11. Leber KM (2004) Marine stock enhancement in the USA: status, trends and needs. In: Leber KM, Kitada S, Blankenship HL, Svåsand T (eds) Stock enhancement and sea ranching: developments, pitfalls and opportunities. Blackwell, Oxford, pp 11–24
12. Bell JD, Rothlisberg PC, Munro JL, Loneragan NR, Nash WJ, Ward RD, Andrew NL (2005) Restocking and stock enhancement of marine invertebrate fisheries. Adv Mar Biol 49:1–370
13. Hedrick PW, Hedgecock D, Hamelberg S, Croci SJ (2000) The impact of supplementation in winter-run Chinook salmon on effective population size. J Hered 91:112–116
14. Hilderbrand RH (2002) Simulating supplementation strategies for restoring and maintaining stream resident cutthroat trout populations. North Am J Fish Manag 22:879–887
15. Reisenbichler RR, Utter FM, Krueger CC (2003) Genetic concepts and uncertainties in restoring fish populations and species. In: Wissmar RC, Bisson PA (eds) Strategies for restoring river ecosystems: sources of variability and uncertainty in natural and managed systems. American Fisheries Society, Bethesda, pp 149–183
16. Hager RC, Noble RE (1976) Relation of size at release of hatchery-reared coho salmon to age, sex, and size composition of returning adults. Progress Fish Cult 38:144–147
17. Bilton HT, Alderdice DF, Schnute JT (1982) Influence of time and size at release of juvenile Coho Salmon (*Oncorhynchus kisutch*) on returns at maturity. Can J Fish Aquat Sci 39:426–447
18. Appledorn RS, Ballentine DL (1983) Field release of cultured queen conchs in Puerto Rico: implications for stock restoration. Proc Gulf Caribb Fish Inst 35:89–98
19. Appeldorn RS (1985) Growth, mortality and dispersion of juvenile laboratory-reared conchs, *Strombus gigas*, and *S. costatus*, released at an offshore site. Bull Mar Sci 37:785–793

20. Tsukamoto K, Kuwada H, Hirokawa J, Oya M, Sekiya S, Fujimoto H, Imaizumi K (1989) Size-dependent mortality of red sea bream *pagrus major* juveniles released with fluorescent otolith-tags in News Bay. Jpn J Fish Biol 35(Supplement A):59–69
21. Peterman RM (1991) Density-dependent marine processes in north Pacific salmonids: lessons for experimental design of large scale manipulations of fish stocks. ICES Mar Sci Symp 192:69–77
22. Hilborn R (1999) Confessions of a reformed hatchery basher. Fisheries 24:30–31
23. Kitada S, Taga Y, Kishino H (1992) Effectiveness of a stock enhancement program evaluated by a two-stage sampling survey of commercial landings. Can J Fish Aquat Sci 49:1573–1582
24. Sudo HT, Goto R, Ikemoto MT, Azeta M (1992) Mortality of reared flounder (*Paralichthys olivaceus*) juveniles released in Shijiki Bay. Bull Seikai Natl Fish Res Inst 70:29–37
25. Fujita T, Mizuta T, Nemoto Y (1993) Stocking effectiveness of Japanese flounder *Paralichthys olivaceus* fingerlings released in the coast of Fukushima Prefecture. Saibai Giken 22:67–73
26. Yamashita Y, Nagahora S, Yamada H, Kitagawa D (1994) Effects of release size on survival and growth of Japanese flounder *Paralichthys olivaceous* in coastal waters off Iwate Prefecture, northeastern Japanese. Mar Ecol Prog Ser 105:269–276
27. Svåsand T, Jorstad T, Kristiansen TS (1990) Enhancement studies of coastal cod in western Norway. Part I. Recruitment of wild and reared cod to a local spawning stock. J Cons Intl Expl Mer 47:5–12
28. Svåsand T, Kristiansen TS (1990) Enhancement studies of coastal cod in western Norway. Part II. Migration of reared coastal cod. J Cons Intl Expl Mer 47:13–22
29. Kristiansen TS, Svåsand T (1990) Enhancement studies of coastal cod in western Norway. Part III. Interrelationships between reared and indigenous cod in a nearly land-locked fjord. J Cons Intl Expl Mer 47:23–29
30. Svåsand T, Kristiansen TS (1990) Enhancement studies of coastal cod in western Norway. Part IV. Mortality of reared cod after release. J Cons Intl Expl Mer 47:30–39
31. Nordheide JT, Salvanes AGV (1991) Observations on reared newly released and wild cod (*Gadus morhua* L.) and their potential predators. ICES Mar Sci Symp 192:139–146
32. Leber KM (1995) Significance of fish size-at-release on enhancement of striped mullet fisheries in Hawaii. J World Aquac Soc 26:143–153
33. Leber KM, Brennan NP, Arce SM (1995) Marine enhancement with striped mullet: are hatchery releases replenishing or displacing wild stocks. Am Fish Soc Symp 15:376–387
34. McEachron LW, McCarty CE, Vega RR (1995) Beneficial uses of marine fish hatcheries: enhancement of red drum in Texas coastal waters. Am Fish Soc Symp 15:161–166
35. Kent DB, Drawbridge MA, Ford RF (1995) Accomplishments and roadblocks of a marine stock enhancement program for white seabass in California. Am Fish Soc Symp 15:492–498
36. Willis SA, Falls WW, Dennis CW, Roberts DE, Whitechurch PG (1995) Assessment of effects of season of release and size at release on recapture rates of hatchery-reared red rum (*Sciaenops ocellatus*) in a marine stock enhancement program in Florida. Am Fish Soc Symp 15:354–365
37. Leber KM, Arce SM, Sterritt DA, Brennan NP (1996) Marine stock-enhancement potential in nursery habitats of striped mullet, *Mugil cephalus*, in Hawaii. Fish Bull US 94:452–471
38. Leber KM, Arce SM (1996) Stock enhancement effect in a commercial mullet *Mugil cephalus* fishery in Hawaii. Fish Manag Ecol 3:261–278
39. Leber KM, Blankenship HL, Arce SM, Brennan NP (1997) Influence of release season on size-dependent survival of cultured striped mullet, *Mugil cephalus*, in a Hawaiian estuary. Fish Bull US 95:267–279
40. Rimmer MA, Russell DJ (1998) Survival of stocked barramundi, *Lates calcarifer* (Bloch), in a coastal river system in far northern Queensland, Australia. Bull Mar Sci 62:325–336
41. Lockwood SJ (1991) Stock enhancement. Special session at the ecology and management aspects of extensive mariculture. In: ICES marine science symposia 192, Nantes. International Council for the Exploration of the Sea, Copenhagen
42. WAS (1991) Enhancement of natural fisheries through aquaculture. In: Special session at 22nd annual conference and exposition, San Juan, Puerto Rico. Programs and abstracts. World Aquaculture Society, San Juan
43. AFS (1993) Emerging marine fish enhancement and evaluation. In: Special session at 123rd annual meeting of the American Fisheries Society, Portland. Book of Abstracts
44. EAS (1993) Fisheries and aquaculture interactions. In: Special session at world aquaculture'93, Torremolinos. Abstracts. Special Publication No. 19. European Aquaculture Society, Gent
45. Schramm HL Jr, Piper RG (eds) (1995) Uses and effects of cultured fishes in aquatic ecosystems, vol 15, American fisheries society symposium. American Fisheries Society, Bethesda, 608 pp
46. Travis J, Coleman FC, Grimes CB, Conover D, Bert TM, Tringali M (1998) Critically assessing stock enhancement: an introduction to the Mote symposium. Bull Mar Sci 62(2):305–311
47. Howell BR, Moksness E, Svåsand T (eds) (1999) Stock enhancement and sea ranching. Fishing News Books/Blackwell, Oxford
48. Nakamura Y, McVey JP, Leber KM, Neidig C, Fox S, Churchill K (eds) (2003) Ecology of aquaculture species and enhancement of stocks. In: Proceedings of the thirtieth U.S.-Japan meeting on aquaculture, Sarasota, 3–4 Dec 2001. UJNR Technical Report No. 30
49. Leber KM, Kitada S, Blankenship HL, Svåsand T (eds) (2004) Stock enhancement and sea ranching: developments, pitfalls and opportunities. Blackwell, Oxford, 606 pp
50. Nickum M, Mazik PM, Nickum JG, MacKinlay DD (eds) (2004) Propagated fish in resource management, vol 44, American Fisheries Society symposium. American Fisheries Society, Bethesda, 644 pp
51. Bell JD, Bartley DM, Lorenzen K, Loneragan NR (2006) Restocking and stock enhancement of coastal fisheries: potential, problems and progress. Fish Res 80:1–8

52. Bell JD, Leber KM, Blankenship HL, Loneragan NR, Masuda R (2008) A new era for restocking, stock enhancement and sea ranching of coastal fisheries resources. Rev Fish Sci 16(1–3):1–9
53. Loneragan N, Abraham I (2011) The fourth international symposium on stock enhancement and sea ranching, part of the 9th Asian fisheries and aquaculture forum, Shanghai Ocean University, 21–23 April 2011. Book of Abstracts for Oral and Poster presentations, Shanghai. http://www.SeaRanching4.org/documents/4thISSESR2011.pdf. Accessed Aug 2011
54. Allendorf FW, Phelps SR (1980) Loss of genetic variation in a hatchery stock of cutthroat trout. Trans Am Fish Soc 109:537–543
55. Busac CA, Currens KP (1995) Genetic risks and hazards in hatchery operations: fundamental concepts and issues. Am Fish Soc Symp 15:71–80
56. Leber KM, Blankenship HL (2011) How advances in tagging technology improved progress in a new science: marine stock enhancement. In: McKenzie J, Phelps Q, Kopf R, Mesa M, Parsons B, Seitz A (eds) Advances in fish tagging and marking technology, vol 76, American fisheries society symposium. American Fisheries Society, Bethesda
57. Tringali MD (2006) A Bayesian approach for genetic tracking of cultured and released individuals. Fish Res 77:159–172
58. Kirk R (1987) A history of marine fish culture in Europe and North America. Fishing News Books, Farnham, 192 pp
59. Lorenzen K (2008) Understanding and managing enhancement fisheries systems. Rev Fish Sci 16:10–23
60. Leber KM, Brennan NP, Arce SM (1998) Recruitment patterns of cultured juvenile Pacific threadfin, *Polydactylus sexfilis* (Polynemidae), released along sandy marine shores in Hawaii. Bull Mar Sci 62(2):389–408
61. Leber KM, Cantrell RN, Leung PS (2005) Optimizing cost-effectiveness of size at release in stock enhancement programs. North Am J Fish Manag 25:1596–1608
62. Tringali MD, Leber KM, Halstead WG, McMichael R, O'Hop J, Winner B, Cody R, Young C, Neidig C, Wolfe H, Forstchen A, Barbieri L (2008) Marine stock enhancement in Florida: a multi-disciplinary, stakeholder-supported, accountability-based approach. Rev Fish Sci 16(1–3):51–57
63. Waples RS (1999) Dispelling some myths about hatcheries. Fisheries 26(2):12–21
64. Tringali MD, Leber KM (1999) Genetic considerations during the experimental and expanded phases of snook stock enhancement. Bull Natl Res Inst Aquac Suppl 1:109–119
65. Lorenzen K, Beveridge MCM, Mangel M. Cultured fish: integrative biology and management of domestication and interactions with wild fish. Biol Rev (in press)
66. HRP (2011) US Hatchery Reform Program. http://www.HatcheryReform.us. Accessed Aug 2011
67. Cowx IG (1994) Stocking strategies. Fish Manag Ecol 1:15–31
68. Munro JL, Bell JD (1997) Enhancement of marine fisheries resources. Rev Fish Sci 5:185–222
69. Taylor MD, Palmer PJ, Fielder DS, Suthers IM (2005) Responsible estuarine finfish stock enhancement: an Australian perspective. J Fish Biol 67:299–331
70. Zohar Y, Hines AH, Zmora O, Johnson EG, Lipcius RN, Seitz RD, Eggleston DB, Place AR, Schott EJ, Stubblefield JD, Chung JS (2008) The Chesapeake Bay blue crab (*Callinectes sapidus*): a multidisciplinary approach to responsible stock replenishment. Rev Fish Sci 16:24–34
71. Bartley DM, Kent DB, Drawbridge MA (1995) Conservation of genetic diversity in a white seabass hatchery enhancement program in southern California. Am Fish Soc Symp 15:249–258
72. Masuda R, Tsukamoto K (1998) Stock enhancement in Japan: review and perspective. Bull Mar Sci 62(2):337–358
73. Kitada S (1999) Effectiveness of Japan's stock enhancement programmes: current perspectives. In: Howell BR, Moksness E, Svåsand T (eds) Stock enhancement and sea ranching. Fishing News Books/Blackwell, Oxford, pp 103–131
74. Blaylock RB, Leber KM, Lotz JM, Ziemann DA (2000) The U.S. Gulf of Mexico marine stock enhancement program (USGMSEP): the use of aquaculture technology in "responsible" stock enhancement. Bull Aquac Assoc Can 100:16–22
75. Kuwada H, Masuda R, Kobayashi T, Shiozawa S, Kogane T, Imaizumi K, Tsukamoto K (2000) Effects of fish size, handling stresses and training procedure on the swimming behaviour of hatchery-reared striped jack: implications for stock enhancement. Aquaculture 185:245–256
76. Friedlander AM, Ziemann DA (2003) Impact of hatchery releases on the recreational fishery for Pacific threadfin (*Polydactylus sexfilis*) in Hawaii. Fish Bull 101:32–43
77. Smith TIJ, Jenkins WE, Denson MR, Collins MR (2003) Stock enhancement research with anadromous and marine fishes in South Carolina. In: Nakamura Y, McVey JP, Fox S, Churchill K, Neidig C, Leber K (eds) Ecology of aquaculture species and enhancement of stocks. Proceedings of the thirtieth U.S.–Japan meeting on aquaculture, Sarasota, 3–4 Dec 2001. UJNR Technical Report No. 30. Mote Marine Laboratory, Sarasota, pp 175–190
78. Woodward AG (2003) Red drum stock enhancement in Georgia: a responsible approach. Coastal Resources Division, Georgia Department of Natural Resources, Brunswick. http://www.peachstatereds.org/approach.pdf. Accessed Oct 2010
79. Jenkins WE, Denson MR, Bridgham CB, Collins MR, Smith TIJ (2004) Year-class component, growth, and movement of juvenile red drum stocked seasonally in a South Carolina estuary. North Am J Fish Manag 24:636–647
80. Kuwada H, Masuda R, Kobayashi T, Kogane T, Miyazaki T, Imaizumi K, Tsukamoto K (2004) Releasing technique in striped jack marine ranching: pre-release acclimation and presence of decoys to improve recapture rates. In: Leber KM, Kitada S, Blankenship HL, Svåsand T (eds) Stock enhancement and Sea ranching: developments, pitfalls and opportunities. Blackwell, Oxford, pp 106–116
81. Fairchild EA, Fleck J, Howell WH (2005) Determining an optimal release site for juvenile winter flounder *Pseudopleuronectes americanus* (Walbaum) in the Great Bay estuary, NH, USA. Aquac Res 36:1374–1383

82. Mobrand LE, Barr J, Blankenship L, Campton DE, Evelyn TTP, Flagg TA, Mahnken CVW, Seeb LW, Seidel PR, Smoker WW (2005) Hatchery reform in Washington State. Fisheries 30:11–23
83. Eggleston DB, Johnson EG, Kellison GT, Plaia GR, Huggett CL (2008) Pilot evaluation of early juvenile blue crab stock enhancement using a replicated BACI design. Rev Fish Sci 16:91–100
84. Gardner C, Van Putten EI (2008) The economic feasibility of translocating rock lobsters in increase yield. Rev Fish Sci 16:154–163
85. Karlsson S, Saillant E, Bumguardner BW, Vega RR, Gold JR (2008) Genetic identification of hatchery-released red drum in Texas bays and estuaries. North Am J Fish Manag 28:1294–1304
86. Le Vay L, Lebata MJH, Walton M, Primavera J, Quinitio E, Lavilla-Pitogo C, Parado-Estepa F, Rodriguez E, Ut VN, Nghia TT, Sorgeloos P, Wille M (2008) Approaches to stock enhancement in mangrove-associated crab fisheries. Rev Fish Sci 16:72–80
87. Potter IC, French DJW, Jenkins GI, Hesp SA, Hall NG, de Lestang S (2008) Comparisons of growth and gonadal development of otolith-stained cultured black bream, *Acanthopagrus butcheri*, in an estuary with those of its wild stock. Rev Fish Sci 16:303–316
88. Purcell SW, Simutoga M (2008) Spatio-temporal and size-dependent variation in the success of releasing cultured sea cucumbers in the wild. Rev Fish Sci 16:204–214
89. Støttrup JG, Overton JL, Paulsen H, Mollmann C, Tomkiewicz J, Pedersen PB, Lauesen P (2008) Rationale for restocking the Eastern Baltic cod stock. Rev Fish Sci 16:58–64
90. Taylor MD, Suthers IM (2008) A predatory impact model and targeted stock enhancement approach for optimal release of mulloway (*Argyrosomus japonicus*). Rev Fish Sci 16:125–134
91. Walters CJ, Hilborn R (1978) Ecological optimization and adaptive management. Ann Rev Ecol Syst 9:157–188
92. Leber KM (2002) Advances in marine stock enhancement: shifting emphasis to theory and accountability. In: Stickney RR, McVey JP (eds) Responsible marine aquaculture. CABI Publishing, New York, pp 79–90
93. Grimes CB (1998) Marine stock enhancement: sound management or techno-arrogance? Fisheries 23(9):18–23
94. Hilborn R (1998) The economic performance of marine stock enhancement projects. Bull Mar Sci 62:661–674
95. Serafy JE, Ault JS, Capo TR, Schultz DR (1999) Red drum, *Sciaenops ocellatus*, stock enhancement in Biscayne Bay, FL, USA: assessment of releasing unmarked early juveniles. Aquac Res 30:737–750
96. Bartley DM, Bell JD (2008) Restocking, stock enhancement, and sea ranching: arenas of progress. Rev Fish Sci 16:357–364
97. Arbuckle M, Metzger M (2000) Food for thought. A brief history of the future of fisheries management. Challenger Scallop Enhancement Company, Nelson
98. Tringali MD, Seyoum S, Wallace EM, Higham M, Taylor RG, Trotter AA, Whittington JA (2008) Limits to the use of contemporary genetic analyses in delineating biological populations for restocking and stock enhancement. Rev Fish Sci 16:111–116
99. Garaway CJ, Arthur RI, Chamsingh B, Homekingkeo P, Lorenzen K, Saengvilaikham B, Sidavong K (2006) A social science perspective on stock enhancement outcomes: lessons learned from inland fisheries in southern LAO PDR. Fish Res 80:37–45
100. Brennan NP, Walters CJ, Leber KM (2008) Manipulations of stocking magnitude: addressing density-dependence in a juvenile cohort of common Snook (*Centropomus undecimalis*). Rev Fish Sci 16:215–227
101. Drummond K (2004) The role of stock enhancement in the management framework for New Zealand's southern scallop fishery. In: Leber KM, Kitada S, Blankenship HL, Svåsand T (eds) Stock enhancement and Sea ranching: developments, pitfalls and opportunities. Blackwell, Oxford, pp 397–411
102. Uki N (2006) Stock enhancement of the Japanese scallop *Patinopecten yessoensis* in Hokkaido. Fish Res 80:62–66
103. Stoner AW (1994) Significance of habitat and stock re-testing for enhancement of natural fisheries: experimental analyses with queen conch *Strombus gigas*. J World Aquac Soc 25:155–165
104. Leber KM (1999) Rationale for an experimental approach to stock enhancement. In: Howell BR, Moksness E, Svasand T (eds) Stock enhancement and sea ranching. Blackwell, Oxford, pp 63–75
105. Agnalt AL, Jørstad KE, Kristiansen T, Nøstvold E, Farestveit E, Næss H, Paulsen LI, Svåsand T (2004) Enhancing the European lobster (*Homarus gammarus*) stock at Kvitsoy Islands: perspectives on rebuilding Norwegian stocks. In: Leber KM, Kitada S, Blankenship HL, Svåsand T (eds) Stock enhancement and sea ranching: developments, pitfalls and opportunities. Blackwell, Oxford, pp 415–426
106. Kitada S, Kishino H (2006) Lessons learned from Japanese marine finfish stock enhancement programs. Fish Res 80:101–112
107. Bartley DM (1999) Marine ranching: a global perspective. In: Howell BR, Moksness E, Svasand T (eds) Stock enhancement and sea ranching. Blackwell, Oxford, pp 79–90
108. Lorenzen K (2006) Population management in fisheries enhancement: gaining key information from release experiments through use of a size-dependent mortality model. Fish Res 80:19–27
109. Medley PAH, Lorenzen K (2006) EnhanceFish: a decision support tool for aquaculture-based fisheries enhancement. Imperial College, London. http://www.aquaticresources.org/enhancefish.html. Accessed Aug 2011
110. Ye Y, Loneragan N, Die DJ, Watson R, Harch B (2005) Bioeconomic modeling and risk assessment of tiger prawn (*Penaeus esculentus*) stock enhancement in Exmouth Gulf, Australia. Fish Res 73:231–249
111. Yamashita Y, Yamada H (1999) Release strategy for Japanese flounder fry in stock enhancement programmes. In: Howell BR, Moksness E, Svasand T (eds) Stock enhancement and sea ranching. Blackwell, Oxford, pp 191–204
112. Tsukamoto K, Kuwada H, Uchida K, Masuda R, Sakakura Y (1999) Fish quality and stocking effectiveness: behavioral

approach. In: Howell BR, Moksness E, Svasand T (eds) Stock enhancement and sea ranching. Blackwell, Oxford, pp 205–218
113. Brennan NP, Darcy MC, Leber KM (2006) Predator-free enclosures improve post-release survival of stocked common snook. J Exp Mar Biol Ecol 335:302–311
114. Lipcius RN, Eggleston DB, Schreiber SJ, Seitz RD, Shen J, Sisson M, Stockhausen WT, Wang HV (2008) Importance of metapopulation connectivity to restocking and restoration of marine species. Rev Fish Sci 16:101–110
115. Hervas S, Lorenzen K, Shane MA, Drawbridge MA (2010) Quantitative assessment of a white seabass (*Atractoscion nobilis*) stock enhancement program in California: post-release dispersal, growth and survival. Fish Res 105:237–243
116. Smedstad OM, Salvanes AGV, Fosså JH, Nordeide JT (1994) Enhancement of cod, *Gadus morhua* L., in Masfjorden: an overview. Aquac Fish Manag 25:117–128
117. Otterå H, Kristiansen TS, Svåsand T, Nødtvedt M, Borge A (1999) Sea ranching of Atlantic cod (*Gadus morhua* L.): effects of release strategy on survival. In: Howell BR, Moksness E, Svåsand T (eds) Stock enhancement and sea ranching. Fishing News Books/Blackwell, Oxford, pp 293–305
118. Wang Q, Wu H, Liu H, Wang S (2011) Ecosystem based sea ranching in Zhangzidao in northern yellow sea. In: Fourth international symposium on stock enhancement and sea ranching, Shanghai. Abstract, available within pdf file. http://www.SeaRanching4.org/documents/4thISSESR2011.pdf. Accessed Aug 2011
119. Becker P, Barringer C, Marelli DC (2008) Thirty years of sea ranching Manila clams (*Venerupis philippinarum*): successful techniques and lessons learned. Rev Fish Sci 16:44–50
120. Chaplin J, Hesp A, Gardner M, Cottingham A, Phillips N, Potter I, Jenkins G (2011) Biological performance and genetics of restocked and wild black sea bream in an Australian estuary. In: Fourth international symposium on stock enhancement and sea ranching, Shanghai. Abstract, available within pdf file. http://www.SeaRanching4.org/documents/4thISSESR2011.pdf. Accessed Aug 2011
121. Jenkins WE, Smith TIJ, Denson MR (2004) Stocking red drum: lessons learned. Am Fish Soc Symp 44:45–56
122. Miller JM, Walters CJ (2004) Experimental ecological tests with stocked marine fish. In: Leber KM, Kitada S, Blankenship HL, Svåsand T (eds) Stock enhancement and sea ranching: developments, pitfalls and opportunities. Blackwell, Oxford, pp 142–152
123. HSRG (2011) Hatchery scientific review group, puget sound and coastal Washington hatchery reform project: applying the principles of reform to Western Washington's hatcheries. http://www.lltk.org/improving-management/hatchery-reform/hrp/hsrg. Accessed Aug 2011
124. Loneragan N, Jenkins G, Taylor M (2011) Stock enhancement and restocking in Australia and opportunities for finfish, particularly in Western Australia. In: Fourth international symposium on stock enhancement and sea ranching, Shanghai. Abstract, available within pdf file. http://www.SeaRanching4.org/documents/4thISSESR2011.pdf. Accessed Aug 2011
125. ISSESR (2011) The international symposium on stock enhancement and sea ranching, Shanghai. http://www.SeaRanching.org. Accessed Aug 2011

Marker-Assisted Breeding in Crops

Roberto Tuberosa
Department of Agroenvironmental Science and Technology, University of Bologna, Bologna, Italy

Article Outline

Glossary

Backcross Procedure used by plant breeders to introgress an allele at a locus of interest (e.g., disease resistance) from a donor parent to a recurrent parent, usually a successful cultivar. The recurrent parent is crossed several times to the original cross and selection is performed at each cycle to recover the plants with the desired allele and the largest portion of the genome of the recurrent parent.

Candidate gene A coding sequence that is supposed to be causally related to the trait under selection. The candidate-gene approach is best applied with simple biochemical traits when a clear cause-effect relationship can be established between the gene function and the target trait.

Epistasis The interaction between two or more genes to control a single phenotype. Interaction between two or more loci that control the same trait. The presence of epistatic loci makes it more difficult to predict the phenotypic value of progeny derived either from crosses or from selfing.

Forward genetics Approaches to dissect the genetic makeup of traits starting from the observation of the phenotype. QTL mapping and positional cloning are examples of forward genetics to investigate quantitative traits.

Haplotype Chromosome fragment of varying length carrying a common set of marker alleles in close linkage at adjacent loci. When using haplotypes in association mapping studies, the information of several linked bi-allelic markers is combined as a single, multi-locus informative marker.

Heritability The portion (from 0% to 100%) of phenotypic variability that is genetically determined. The additive portion (i.e., not due to dominance) of variability is inherited from one generation to the next and is the main determinant of the gain from selection. Heritability is specific to a particular population in a particular environment.

Introgression library lines (ILLs) A collection of lines (ca. 80–100) obtained by subsequent backcrosses of a recurrent parent (usually an elite cultivar) with a donor parent, usually a line highly diversified from the recurrent parent for one or more traits. Each ILL carries a fragment (from ca. 20 to 40 cM) of the donor genome different from that carried by the other lines. Collectively, the fragments of all ILLs cover the entire genome with partial overlap. ILLs are ideal for the fine mapping and cloning of major loci and to investigate epistatic interactions.

Linkage disequilibrium (LD) The level of nonrandom assortment of alleles at different loci. The level of LD varies greatly according to the species and the mode of reproduction.

Linkage drag The negative phenotypic effects (e.g., lower yield) on the recurrent parent associated with the loci of the donor parent tightly linked to the locus of interest being backcrossed.

Logarithm of the odds ratio (LOD) A logarithmic value (base 10) of the ratio between the probability of the presence of a QTL vs. its absence. A LOD value of 3.0 indicates that the probability of the presence of the QTL is 1,000-fold higher than its absence.

Metanalysis A comprehensive analysis based on the data of several mapping populations of the same species. The objective is to obtain a better resolution of the LOD profile of the QTLs for the traits of interest.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Near isogenic lines (NILs) A set of two or more inbred lines that share most of the genome except for a small portion that contains functionally different alleles at the target locus. NILs are commonly used for the positional cloning of a locus of interest.

Phenotypic selection Selection based on the observation of the phenotype at different levels of functional organization based on the target trait(s). If the selected trait is highly influenced by environmental conditions and has low heritability, the effectiveness of phenotypic selection quickly decreases.

Pleiotropy Condition where a single locus controls more than one trait. It is more common for biochemical traits.

Positional cloning A series of procedures to clone a locus of interest. Positional cloning is based on the joint analysis of phenotypic data and genotyping profiles of near isogenic material with recombination events at the target region.

Quantitative trait locus A portion of DNA that influences the expression of a quantitative trait. The presence of QTLs is determined through appropriate statistical analysis of phenotypic and molecular data of a mapping population (e.g., linkage mapping) or a collection of unrelated genotypes (e.g., association mapping).

Recombinant inbred lines A collection of homozygous lines (usually from 150 up to 400) obtained following subsequent selfings (usually four or five) of an equivalent number of randomly chosen F_2 plants.

Reverse genetics An approach for discovering the function of a locus by analyzing the phenotypic effects of specific sequences obtained by DNA sequencing. Reverse genetics attempts to connect a given genetic sequence with specific effects on the organism.

Synteny The physical colocalization of linked loci on the same chromosome among different species. Study of synteny can show how the genome of phylogenetically related species has evolved from a common ancestor (e.g., rice for cereals) through rearrangements of the genome (e.g., translocations, inversions, duplications, etc.) in the course of evolution.

Definition of the Subject

Attaining global food security by means of increased crop productivity will require an increase in gains from selection achieved through conventional breeding. To this end, the identification of molecular markers associated with loci controlling traits of agronomic interest coupled with the exploitation of marker-assisted breeding (MAB) approaches provides the opportunity to accelerate gain from selection. In particular, marker-assisted selection (MAS) and marker-assisted backcrossing have been widely adopted to improve resistance to diseases and other relatively simple traits. Notwithstanding these remarkable achievements, the improvement of yield and other complex quantitative traits via MAB has been marginal, mainly due to the difficulty in identifying major quantitative trait loci (QTLs) with an adequately stable effect across environments and genetic backgrounds. Additionally, the effect of most QTLs affecting yield is too small to be detected with either biparental mapping or association mapping. Genomic selection (GS) circumvents this problem by using an index for the selection of unmapped QTLs of small individual effects but with otherwise sizable effect at the whole plant level when selected together. GS is already having a positive impact on the improvement of crop yield, mainly in the private sector where high-throughput infrastructures allow breeders to handle the large number of molecular datapoints that are required for effectively deploying GS. Ultimately, an effective exploitation of MAB to enhance crop performance will rely on a closer integration between molecular approaches and conventional breeding.

Introduction: Global Food Security and Plant Genomics

During the past century, plant breeders have been very successful in constantly raising crop yields to a level sufficient to meet the global demand in food, feed, and fiber. For wheat and rice, the two most important staples of humankind, the so-called Green Revolution spearheaded by Norman Borlaug, awarded the Nobel Peace Prize in 1970, provides the most spectacular example of the contribution of science toward an improved food security [1, 2]. Similar progress has been achieved also in maize, particularly following the

introduction of hybrids [3]. This notwithstanding, during the past decade, the rate of increase in yield in cereals, especially wheat and rice, has not met the global demand [4] as shown by the substantial decrease in the amount of global cereal reserves. Additionally, during the past two decades the number of chronically hungry people has increased and is fast approaching one billion. A number of reasons have contributed to this worrisome scenario that has already sparked food riots (e.g., during the 2007–2008 food crisis and also in 2009) and social unrest in a number of less-developed countries. An even bleaker picture looms on the horizon, when mankind will reach a projected nine billion in 2050. Consequently, an acceleration in the rate of gain in crop yields is a must in order to keep up with the need of a burgeoning population that increasingly seeks a protein-enriched, nutritionally balanced diet. The challenge faced by modern breeders is even more daunting in view of (1) global warming and the consequent increased frequency of drought, floods, high temperatures, etc., (2) the decreased availability of natural resources (e.g., water, fertilizers, arable land, etc.), (3) the increasing cost of fuels, (4) the necessity to safeguard the remaining biodiversity, and (5) the increased societal awareness of the critical need to improve the long-term sustainability of agricultural practices and decrease its impact on the environment. More simply, agriculture will need to produce more with fewer resources and more sustainably.

In this daunting scenario, genomics has ushered in a new breeding paradigm based on molecular approaches and platforms that in some cases have already contributed to accelerate the yield gain commonly achieved through conventional breeding practices [5–13]. However, a more widespread adoption of genomics-assisted selection will require the definition of new strategies based on a more effective integration of conventional and nonconventional breeding approaches as well as agronomic practices [14]. Clearly, a better knowledge of the genetic factors that determine yield and its variability from season to season will be instrumental in devising effective marker-assisted breeding (MAB) strategies for enhancing crop performance under a broad range of environmental conditions. As compared to conventional breeding approaches, MAB approaches offer unprecedented opportunities to dissect the genetic control of traits, particularly those that are quantitatively inherited, such as biomass production, yield, and many other agronomic traits selected by breeders.

Molecular Dissection of the Genetic Control of Traits Governing Crop Performance

The first step for the dissection of the genetic control of traits that govern crop performance is the assembly of a linkage (genetic) map based upon the data of the molecular profiles of the marker loci – from as few as 100 up to several thousand – surveyed in a mapping population, usually comprised of ca. 150–200 genotypes such as F_2 plants, F_3 families, recombinant inbred lines (RILs), doubled haploids (DHs), etc., usually derived from the cross of two parental lines differing for the trait(s) of interest. The assembly of a genetic map is based on the level of linkage disequilibrium (LD, i.e., the level of nonrandom assortment of alleles at different loci) among adjacent marker loci on the same chromosome. Accordingly, mapping the loci that control the target trait is also based on the LD between the locus and nearby markers.

The estimated genetic distance between loci (markers or genes) is a function of the average number of recombination events (i.e., crossing-overs) between them at meiosis. The measuring unit used for expressing the distances among loci along a genetic map is the centimorgan (cM), which defines the interval along which one recombination event is expected to occur per 100 gametes produced at each meiotic cycle (i.e., at each sexually reproduced generation). Because a density of one marker per ca. 10–15 cM is usually sufficient to detect the presence of a functionally polymorphic locus with a major effect on the phenotypic variability of a mapping population, the number of well-spaced markers required to adequately sample the targeted species varies from as little as 100–120 as in the case of rice – one of the crops with the smallest genome size (ca. 0.45 billion bp) – to well over 300 for large genomes such as in bread wheat (ca. 16 billion bp). The desired level of genetic resolution will depend on the objective being pursued and the type of genetic materials being used.

For breeding purposes, a density of one marker every 5–10 cM is sufficient for most applications when dealing with elite cultivars. Nonetheless, for the

introgression of a particular gene (e.g., a locus for disease resistance) from a wild relative of the crop to the crop itself, a high resolution is desirable in order to avoid the negative effects of the so-called linkage drag caused by negative effects of wild alleles at the loci closely linked to the one being targeted for introgression. A much higher genetic resolution is required when the goal is the cloning of the sequence that affects the target trait. In this case, the screening of several thousands of individuals is required to reach the desired level of resolution.

Cloning the loci that govern a particular trait can be achieved via either forward- or reverse-genetics approaches, or their combination. While forward genetics focuses on the phenotype as starting point, reverse-genetics approaches rely on sequence and functional information of candidate sequences (e.g., expressed sequence tags: ESTs) that are postulated to play a role in the expression of the target trait [15]. Although most results in the dissection of the genetic basis of crop performance and agronomic traits have been obtained via forward genetics, the use of reverse-genetics approaches in *Arabidopsis* and other model species (e.g., resurrection plants, rice, *Brachypodium*, etc.) has been instrumental to elucidate the genetic networks of the signaling pathways that regulate the adaptive response of plants to abiotic and biotic constraints [16–18]. Notably, the spectacular decrease in sequencing costs [19] and the increased availability of sequence information in public databases make the reverse-genetics approach increasingly attractive and feasible.

Following the assembly of the first genetic maps based on the molecular profiling of RFLPs (restriction fragment length polymorphisms; [20, 21]), the introduction of AFLPs (amplified fragment length polymorphisms; [22]), SSRs (simple sequence repeats; [23]), and DArT (diversity array technology; [24]) markers improved substantially the assembly of genetic maps. More recently, high-throughput platforms based on SNPs (single nucleotide polymorphisms), the most frequent polymorphism in living organisms, have enabled a quantum leap in saturating maps with thousands of markers [25–29]. Notably, the spectacular advances obtained with next-generation sequencing (NGS) technology will soon allow for the resequencing of entire mapping populations and association mapping panels of species for which a template sequence is available, thus providing an almost endless supply of markers [30–34].

Once all the molecular and phenotypic data are available, statistical tests will be applied to verify whether the means of the trait values of the genotypes carrying different alleles at a particular marker are significantly different. A test statistic larger than a threshold value rejects the "null hypothesis" (i.e., the mean is independent of the genotype at a specific marker locus) and implies a significant association between the investigated marker and a linked locus that affects the phenotypic value of the target trait. The exploitation of syntenic relationships among phylogenetically related crops has greatly contributed to the identification of additional markers at target regions [35–37] and, most importantly, candidates for the investigated traits, particularly when the genome sequence of one or more of the syntenic species becomes available. This is the case of cereals, where the annotated sequence for rice, *Brachypodium*, sorghum, and maize has allowed for the identification of conserved orthologous set (COS) markers from ESTs that have maintained their microlinearity throughout evolution and speciation [37]. These markers are particularly valuable to assess the possible role of candidate genes in species not yet sequenced (e.g., wheat) and to identify orthologous sequences that have maintained their functions and colinearity across species. Thus, a good understanding of the syntenic relationships at regions underlining a QTL for rather simple traits can provide excellent clues to pinpoint the most likely candidate.

Notably, mapping loci controlling the target traits allows breeders to implement marker-assisted selection (MAS) on the basis of the polymorphic molecular markers flanking the relevant loci. Traits are usually categorized as monogenic (qualitative or Mendelian traits controlled by a single locus) and polygenic (or quantitative; controlled by many loci), the latter being highly influenced by environmental conditions and considerably more difficult to improve consequent to their lower heritability, [38]. Quantitative traits (e.g., flowering time, plant height, biomass production, yield, etc.) are particularly important for breeding purposes. Although the genetic dissection of both qualitative and quantitative traits relies on similar principles, the latter requires more extensive phenotyping and much larger mapping populations.

The prevailing assumption in the field of quantitative genetics has been that continuous variation in trait performance is caused by the segregation and action of multiple genes with a rather similar effect on the phenotype, together with a major influence of the environment which acts like some sort of "statistical fog" that blurs and limits our capacity to identify the genes that control the target trait. These genes, also referred to as polygenes, are known as quantitative trait loci (QTLs; [39]). Although the original concept – but not the acronym – of QTL mapping was first suggested in 1923 [40], the dissection of quantitative traits became eventually possible in the 1980s and the 1990s with the introduction of molecular marker platforms that allowed for genome profiling with the needed level of genetic resolution [41–45]. Two decades of dedicated experiments indicate that most QTL effects are of small magnitude as originally predicted by the so-called infinitesimal model [38, 46, 47]. This notwithstanding, a limited number of so-called major QTLs have shown a rather large effect and, in a number of cases, have been cloned [48, 49]. Once a QTL has been cloned, both genomics and genetic engineering offer additional opportunities for tailoring improved cultivars and crossing reproductive barriers among species, thus expanding the repertoire of genes available to breeders. In view of the importance of quantitative traits in breeding activities and crop performance, particular attention should be devoted to QTL mapping and the implementation of MAB for this category of traits.

Biparental Linkage Mapping

The early studies in QTL mapping were conducted based on the analysis of the means at single markers using simple test statistics, such as linear regression, *t*-test, and analysis of variance. Because a genome-wide survey typically involves a large number of markers, the probability of detecting one or more false positives at the whole-genome level quickly increases unless the threshold of significance is adequately readjusted according to the number of tested markers [50]. Typically, a threshold level of $P_{0.05}$ entails a false-positive discovery rate (i.e., declaring the presence of a locus able to affect the target trait when actually there is no locus) of approximately 5%. Consequently, a mapping experiment based on 100 markers tested at $P_{0.05}$ will identify, on average, five markers putatively associated with loci even when no real locus segregates in the population. In order to avoid this problem, the significance threshold is corrected accordingly through a multiple test adjustment (e.g., Bonferroni's or Tukey's) that will adjust the *P* level according to the number of independent statistical tests that are performed. This notwithstanding, a much more critical shortcoming of this single-marker approach is that no information is provided on the most likely position of the locus and its effects on the phenotype. Due to these major limitations, single-marker analysis was quickly replaced by interval mapping and similar methods based on the estimated linear order of markers on a genetic map. In comparison to single-marker analysis, interval mapping provides a much more accurate estimate of the position and genetic effects of each locus [51–53]. In interval mapping, statistical methods are applied to test for the likelihood of the presence of a QTL. The result of the likelihood tests carried out at regular intervals across the ordered markers is expressed as LOD (Logarithm of the ODds ratio) scores, computed as the $\log_{10}$ of the ratio between the chance of a real QTL being present given the phenotypic effect measured at that position, divided by the chance of having a similar effect when no QTL is present. Thus, LOD values of 2.0 and 3.0 indicate that the presence of the QTL is 100- and 1,000-fold more likely than its absence, respectively. The graphical output is an LOD profile that allows one to compute an empirical confidence interval (usually computed as LOD – 1) around the QTL peak. In order to avoid declaring false-positive QTLs (i.e., declaring the presence of a QTL when the QTL is actually absent), a reasonably high threshold value for the LOD score should be set (usually > 2.5). Iterative software based upon resampling procedures provides a more accurate estimate of threshold values according to the size of the mapping population and the number of markers [54].

Epistasis can greatly influence the outcome of interval mapping. This problem can be partially overcome with the use of composite interval mapping, a statistical procedure that can account for the effects of other QTLs inherited independently from the interval (i.e., chromosome region) being considered, thus reducing the possibility of detecting "ghost" (i.e., false) QTLs. Compared to single-QTL interval mapping, statistical

approaches for locating multiple QTLs are more powerful because they can differentiate between linked and/or interacting QTLs that will otherwise go undetected when using single QTL interval mapping. Given the potential impact of epistasis on the response to selection, quantifying its influence on target traits is an important component for designing and organizing any MAS strategy [55]. It is likely that the incorporation of epistatic interactions into more properly devised statistical models will play a relevant role in explaining complex regulatory networks governing the expression of quantitative traits.

A major shortcoming of QTL studies is the low accuracy in detecting the real number of QTLs affecting the genetic variation of the investigated traits, particularly with populations of less than 150–200 families, which is the case in the majority of QTL studies reported so far. A simulation study applied to experimental data showed that with populations of ca. 100–200 families only a modest fraction of QTLs was identified; furthermore, the effect of each single QTL was usually overestimated [56]. Another study showed that detection of QTLs of small effect is very difficult with mapping populations with less than 500 families [44]. These predictions were supported in experiments carried out with maize mapping populations large enough (>400 families) to allow for a meaningful subsampling [57, 58]. Therefore, the chance of detecting a QTL in several environments is small even in the absence of QTL × Environment (QTL × E) interaction. Accordingly, inconsistency of QTL detection across environments has been repeatedly reported [59, 60].

Association Mapping

In the past decade, as an alternative to linkage mapping with biparental populations, association mapping based on the evaluation of panels of unrelated accessions (ca. 150 or more) has been adopted as an additional option for trait dissection [61–65]. The assumption underlying the use of association mapping to detect the presence of loci influencing the target trait is that alleles at two closely linked loci share a historical ancestor, and this original co-occurrence will gradually decay in the population due to recombination events during subsequent meioses. Consequently, the relative allele distributions of an unknown gene and that of a closely linked marker will be nonrandom because the two are in LD. A major factor to be considered for a correct application of association mapping is the presence of population structure, which will significantly bias the results and inflate spurious marker-trait associations (i.e., declaring false positives). Algorithms and methods are being developed to correct for these effects. An important advantage of association mapping is that the linkage is evaluated over the large number of historic meiosis, which in turn entails a much lower LD and higher genetic resolution as compared to linkage mapping with biparental populations. Another advantage is that the genetic variability explored by a large panel of unrelated accessions is much larger than that present in a segregating population derived from two parental lines. Conversely, a major shortcoming of association mapping is that it does not allow for the detection of the effect that a rare, but otherwise agronomically valuable, allele may have on the target trait. In fact, the statistical procedures used for revealing the effects associated to a particular locus/haplotype consider only alleles with a frequency higher than 10% over the entire population; alleles with a frequency lower than 10% are considered rare and as such, are discarded. The cutoff threshold of 10% has been introduced to reduce the ascertainment bias that a small sample (i.e., less than 10%) of accessions would inevitably introduce, being unable to correctly represent the effect of that particular allele at the level of the entire population [62]. Clearly, this is not an issue when dealing with mapping populations where allelic frequencies are expected to be equal to ca. 50%, barring the presence of genetic factors that might influence the transmission of gametes carrying the different parental alleles. In association mapping, the procedure of discarding the individuals carrying rare alleles inevitably reduces the statistical power to identify the role of such loci in controlling the variability measured for the target trait. An example of this has recently been reported in durum wheat, where a locus with a large effect on yield in a biparental cross [162] showed no appreciable effect in a parallel association mapping study where only one of the parental alleles was considered, due to the fact that the other parental allele was present in low frequency [65].

The main factors to be carefully considered for optimizing the effectiveness of association mapping are the level of LD among the investigated accessions and the presence of population structure that could greatly increase the false-discovery rate (i.e., type-I error). Closely related to the concept of LD is the concept of "haplotype," which can be defined as the chromosome fragment carrying a common set of marker alleles in close linkage at adjacent loci [66]. When using haplotypes in association studies, the information of several linked bi-allelic markers is combined as a single, multi-locus informative marker. Haplotypes can be generated *in silico* from sequences deposited in the database, by resequencing target loci (sequence haplotypes) or genetic maps (marker haplotypes). Therefore, haplotypes will extend according to the level of LD, the value of which varies greatly (up to 100-fold or even more) not only among species, but also within a single species according to the frequency of crossing-over events in each chromosome region. As an example, centromeric regions are characterized by very low recombination if compared to subtelomeric, gene-rich regions. Populations characterized by high LD (i.e., extending for $>$ 1 cM, corresponding to several million base pairs (bp) depending on the ratio of the genetic and physical distance) are best suited for a genome-wide search [65]. Alternatively, the utilization of panels with a low LD (i.e., extending $<$ 10,000 bp, typically a small fraction of 1 cM), a condition that is typical of allogamous species like maize [67], allows for a much higher level of genetic resolution and for the validation of a candidate sequence. Clearly, the level of LD influences the number of markers/cM required to obtain meaningful information. As compared to a low LD condition, a high LD level is associated with a proportionally longer haplotype, hence requiring a lower number of markers to conduct meaningful genome-wide surveys. This feature is more prominent in elite materials that have undergone high selection pressure as a result of modern breeding practices, which in most cases has led to a reduction of haplotype diversity as compared to locally grown landraces and, more notably, wild relatives of crops that have not gone through the domestication bottleneck. As an example, LD in wheat – a selfing species that has undergone a very stringent selection mostly due to the importance of quality parameters required by the food industry – extends up to 5–10 cM [65], while in outcrossing species like maize LD is usually below a fraction of cM or even less than 10,000 bp [68]. An example of the high level of genetic resolution made possible through association mapping is shown by the fine mapping and, in one case, positional cloning of QTLs for flowering time in maize [67, 68]. In particular, association mapping revealed that the most important QTL for flowering time per se (i.e., independently from photoperiod sensitivity) in maize corresponds to a 2.3 kb, noncoding, long-distance enhancer region located 70 kb upstream of a gene known to regulate flowering time also in *Arabidopsis* [49]. Another remarkable example in which the functional polymorphism responsible for phenotypic variability was assigned to a noncoding region far (ca. 5,000 bp) from the structural gene has been reported in sorghum through the cloning of a major QTL for aluminum tolerance [69]. Clearly, only a positional cloning approach is able to unequivocally highlight the role of noncoding regions in controlling the level of expression of a particular gene and the resulting phenotype. To what extent noncoding, long-distance enhancers might be involved in regulating the expression of quantitative traits is presently unknown. Notwithstanding the importance of this issue for a more complete understanding of the regulation of gene expression, this level of genetic dissection is certainly not required from a breeding standpoint, since both MAS and genetic engineering would still allow breeders to fully exploit the beneficial effects linked to either natural allelic variation or the ectopic expression of the structural locus encoding for the target trait.

Despite the clear advantages of association mapping on biparental linkage mapping (e.g., multiallelism, higher genetic variability and genetic resolution, no need to assemble a mapping population, shorter time required to identify relevant loci, etc.), a major limitation of the former is represented by the high rate of false positives (i.e., Type-I error rate), hence spurious association, due to the presence of hidden population structure among the accessions being evaluated [62]. An additional constraint to a more widespread utilization of association mapping for the dissection of physiologically complex traits may derive from factors other than statistical issues.

For highly integrative and functionally complex traits such as yield, particularly under adverse conditions, association mapping may quickly lose its effectiveness as the level of functional complexity of the target trait increases. In this case, similar phenotypic values in different genotypes can result from the action of different gene networks and/or trait compensation (e.g., yield components), thus undermining the identification of significant marker-trait association across a broad range of genotypes such are those usually present in the panels used for association mapping. Although a similar limitation also pertains to a mapping population developed from the cross of two divergent lines, its relevance in the case of association mapping for complex traits is greatly increased by the much wider functional variability explored with association mapping. This is particularly the case whenever the investigated trait (e.g., yield under drought conditions) is strongly influenced by differences in phenology, mainly flowering time and/or plant height; in this case, the overwhelming effects on yield of phenological traits will inevitably overshadow the effects due to the action of loci controlling yield per se, i.e., irrespectively of flowering time and plant height.

Comparative QTL Mapping and Metanalysis

A major shortcoming in QTL mapping is the limited accuracy in identifying the most likely position of each single QTL on the chromosome. Unless highly isogenic materials are evaluated, the confidence interval in assigning a QTL is rarely shorter than 10 cM, an interval likely to contain several hundred genes. The availability of QTL data for two or more mapping populations of the same species allows for the comparison of the position of QTLs by means of a metanalysis carried out with dedicated software [70]. This, in turn, provides a better genetic resolution of the QTL interval and reduces the confidence interval around the peak of the LOD profile. This exercise is particularly useful when a reference map with hundreds of well-spaced markers is available and contains "anchor markers" (usually RFLPs, SSRs, and/or SNPs) also used to investigate other mapping populations of the same species. An additional advantage of a reference map is that it allows one to compare the map position of QTLs with that of mutants for the same trait, thus contributing relevant information for the identification of possible candidate genes causally affecting the investigated trait. Accordingly, Robertson [71] suggested that a mutant phenotype may be caused by an allele with a much more drastic effect in comparison to that of QTL alleles at the same locus, a hypothesis that has been validated in maize for a QTL for plant height colocalized with the mutant *dwarf3* [72]. These results indicate that no real boundary exists between Mendelian and quantitative genetics, while suggesting that loci can be classified in either category based upon the magnitude and heritability of the effect of the alleles being considered. It follows that the information provided by mutants is of great value for QTL studies and breeding applications.

Isogenic Materials for Mapping and Cloning QTLs

A valuable opportunity for investigating the effects of a particular QTL and eventually isolate the functionally polymorphic sequence responsible for its effects is offered by the analysis of pairs of isogenic materials (e.g., near isogenic lines: NILs) contrasted for the parental chromosome regions (usually ca. 10–30 cM long) present at the target QTL. NILs can be obtained through repeated selfings of F_3-F_5 individuals heterozygous at the QTL region prior to isolating the homozygotes for each one of the two parental segments carrying the functionally contrasting QTL alleles [73]. Alternatively, each parental line of the mapping population originally evaluated for discovering the QTL can be used as recurrent parent in a backcross scheme in which a single genotype heterozygous at the QTL in question is utilized as donor of the alternative QTL alleles; in this case, the congenic lines are identified as backcrossed-derived lines [74]. With NILs, it is thus possible to "mendelize" major QTLs characterized by a sizable additive effect. Unlike genome-wide QTL studies where more than 100–150 genotypes are usually screened, experiments conducted with NILs involve few genotypes (two as a minimum), thus allowing for a much more refined and detailed phenotypic evaluation of the effects of the QTL [74, 75]. However, it should always be appreciated that the results of NIL-based studies could to a certain extent be biased by the action of one or more closely linked genes affecting the investigated traits, a particularly likely event when the region flanking the QTL extends for several cM.

A more systematic search of QTLs is made possible with the use of a series of isogenic lines obtained through the introgression, via backcrossing, of a small portion (ca. 20–30 cM) of the genome of a donor line into a common recurrent line, usually an elite cultivar [76]. The final objective is to assemble a collection of so-called introgression library lines (ILLs; at least 70–80 or more lines for each cross), basically a collection of NILs, each one differing for the introgressed chromosome portion and collectively representing the entire donor genome [76]. A major advantage of ILLs is the rapid progress that they allow for the fine mapping and positional cloning of major QTLs [48, 77]. Besides the well-documented effectiveness of ILLs for the mapping and cloning of QTLs in tomato [77, 78], ILLs have been instrumental for mapping drought-adaptive QTLs in rice [79] and maize [163]. Once ILLs are made available and major loci for the target traits are identified, testing for epistasis becomes particularly feasible using a small number of genotypes, unlike with mapping populations, where an accurate testing for epistasis will require the evaluation of at least 200 families.

The availability of NILs for a major QTL is an important prerequisite for undertaking the cloning of the sequence underlying the trait being targeted. Besides contributing to a better understanding of the functional basis of quantitative traits [68, 80], QTL cloning provides an essential opportunity for more effectively mining and exploiting the allelic diversity present in germplasm collections [49, 82]. Recent advances in high-throughput profiling and sequencing of both the genome and transcriptome coupled with reverse-genetics approaches/platforms (e.g., collections of knockout mutants, TILLING, RNAi, etc.) have streamlined the procedures and markedly reduced the time required to identify the sequences governing variation in quantitative traits. Until now, the molecular dissection of a candidate locus has been prevailingly achieved through positional cloning and association mapping. Both approaches exploit LD to identify the most promising candidate gene(s) and benefit from the map information of candidate genes and mutants in the species under investigation and in closely related ones. As sequence information accumulates and our understanding of biochemical pathways improves, QTL cloning via the candidate-gene approach becomes an attractive alternative to positional cloning, particularly for traits underlined by a known metabolic pathway [83, 84].

Modeling QTL Effects

QTL-based modeling holds promise to allow for a more effective design of "molecular ideotypes" on the basis of estimated QTL effects for growth parameters of response curves to environmental factors revealed by exposing mapping populations to such environmental factors [85–87]. Additionally, crop modeling provides useful clues to unravel the genetic basis of G × E interactions and toward a better understanding of traits' plasticity [88], a feature of increasing importance in view of the effects on crop growth and yield due to the enhanced vagaries in weather conditions consequent to global warming. An accurate estimate of the consistency of QTL effects in a particular genetic background can be obtained through extensive testing of the genetic materials under different environmental conditions as to level of irrigation, nutrients, temperature, etc.

In maize, an ecophysiological model and QTL analysis have been integrated to investigate the genetic basis of leaf growth in response to drought and predict leaf elongation rate as a function of estimated QTL effects at varying air humidity, temperature, and soil water status (Tardieu 2003). QTLs with a limited QTL × E interaction and with a linear response to a particular environmental factor will provide more predictable opportunities to improve crops' performance through MAS. An important issue rarely addressed in view of the inherent difficulty in doing so from an experimental standpoint under field conditions is that crop performance is often constrained by more than one environmental factor (e.g., drought and heat) occurring simultaneously, a condition which greatly undermines the prediction of QTL effects, particularly when considering multiple QTLs.

Marker-Assisted Breeding to Improve Crop Performance

The improvement of crop performance through conventional breeding has for the most part been achieved with little or no knowledge of the genetic basis of the selected traits, particularly yield and its underlying

morphophysiological determinants. The main obstacle to raising crop yield via conventional breeding by means of phenotypic selection is represented by the low heritability of yield, particularly under marginal conditions and low-input agriculture (e.g., low supply of nutrients and/or water). As an alternative to phenotypic selection, MAB can be applied to more effectively improve crop performance. The ultimate goal of MAB is to increase the cost-effectiveness of the selection gain per unit time. Although the costs entailed by MAB are still quite high when compared to conventional breeding practices, the sizable reduction in the time required to release an improved cultivar made possible through MAB can justify its application once agronomically valuable alleles at target loci (genes or QTLs) are identified. The convenience of adopting MAB to improve the efficiency of the selection process should be carefully evaluated on a case-by-case basis. The success of MAB will depend on the identification of the agronomically beneficial alleles at target loci, their effect in the different elite genetic backgrounds prevalently grown by farmers and their pyramiding in the correct combinations. MAB could thus be regarded as an extension and evolution of the so-called ideotype breeding, an approach based on phenotypic selection for an ideotype characterized by those morphophysiological features deemed necessary to maximize yield. As compared to ideotype breeding, MAB allows us to dissect the genetic basis of key traits and to piece back together the best alleles in a sort of molecular jigsaw puzzle, the main limitation being that only a very small number of the jigsaw tassels (i.e., genes and QTLs) have been identified. This approach, referred to as "breeding by design" [89], extends the concept of "graphical genotypes" first introduced by Young and Tanksley [90] to portray the parental origin and allelic contribution of each genotype on a genome-wide basis. Although a breeding-by-design approach is technically applicable to all major crops, its impact has been much more tangible for traits with a simple genetic control (e.g., quality, disease resistance; [91–95]) as compared to more complex quantitative traits, such as yield under adverse environmental conditions [60], a result mainly due to our rudimental understanding of the genetic basis of the latter category of traits, their interaction with environmental factors and, most importantly, the difficulty in predicting the phenotypic value of a new genotype tailored through MAB for several QTLs. Along this line, it should be underlined that the effects of QTL alleles for complex traits (e.g., yield) characterized by a large G × E interaction can drastically change according to the conditions (e.g., water availability along the crop life cycle) present in the environment being targeted.

The molecular profiles obtained with molecular markers provide the basic information required to identify the haplotype of each individual plant at a target locus. Haplotype profiling of collections of elite cultivars released during the past decades and derived from a limited number of founders (i.e., genotypes that in view of their positive features have been frequently used by breeders as parental lines) provides a means to identify the chromosome regions that have been preferentially retained throughout the breeding activities carried out during such time period. It is plausible to hypothesize that these chromosomal regions harbor loci (genes or QTLs) important for the selection of improved cultivars.

The strategies deployed to improve crop performance based on molecular information can be categorized according to the level of knowledge and understanding of the loci that underline the phenotypic traits under selection. While MAS and marker-assisted recurrent selection (MARS) during the past two decades have deployed allelic variation at mapped loci often characterized by a rather large effect on the phenotype, the new paradigm ushered in by genomic selection (GS) via high-throughput profiling has emphasized the selection of unmapped, uncharacterized loci with rather limited individual effects but with otherwise sizable effects when selected together. The next sections will critically analyze some of the main features of these rather different approaches that should not be regarded as antagonistic, but rather complementary.

Marker-Assisted Selection

Once loci are mapped and their effects characterized, the two most common applications of MAS in crop breeding are to (1) accelerate the backcross (BC) procedures required to transfer beneficial alleles at one or more loci into an elite cultivar and (2) facilitate the selection of one or more target traits within

a segregating population. The former application is the one that so far has been most frequently adopted in breeding programs and is usually referred to as marker-assisted backcross (MABC). MAS has also been deployed frequently to create isogenic lines (e.g., NILs, introgression libraries, etc.). These materials are used to identify and map genes/QTLs and, as such, usually do not impact directly on the outcome of breeding practices and the release of improved cultivars.

As compared to the conventional BC procedure, MABC based on the use of markers uniformly spaced along the genome (ca. 20–25 cM apart) can save three to four BCs in recovering most of the genome of the recurrent parent, thus reducing the time required for the release via BC of the improved version of the recurrent parent [96]. The advantage is greater for the incorporation via BC of recessive resistance genes, the phenotypic detection of which is only possible for the homozygous individuals carrying recessive alleles at both loci. In this case, phenotypic selection takes twice longer as compared to dominant alleles, since a selfing generation is required after each BC for the phenotypic identification of the homozygous recessive resistant plants to be used for the next BC. The utilization of codominant markers (e.g., SSRs) allows for the identification of heterozygous plants carrying the resistance-encoding allele directly in F_1, thereby saving one generation for each BC cycle. During the past two decades, MABC has been routinely deployed by seed companies to introgress beneficial alleles from unadapted accessions (e.g., landraces or wild, sexually compatible relatives of crops) and particularly to introgress transgenes into elite materials [9, 97, 98]. At each generation, individuals heterozygous at the region flanking the target locus are identified based on the results of molecular profiling. In comparison to conventional BC, MABC provides additional, distinct advantages such as (1) avoiding the vagaries in phenotyping when the conditions do not allow an accurate classification of the progeny segregating for the target trait (e.g., absence of the pathogen when backcrossing an allele for resistance to the disease), (2) reducing the number of plants to be screened in each selection cycle, and (3) identifying plants with the shortest possible chromosome segment introgressed from the donor line. The latter factor is particularly important when the donor is a wild accession of the recurrent, elite line being backcrossed. In this case, the introgressed chromosome segment flanking the target locus is likely to contain many alleles with a detrimental effect on quality and yield. Therefore, it is necessary to select individuals with the shortest possible chromosomal fragment contributed by the donor parent. An additional benefit is when the phenotyping of the trait under transfer is expensive and/or cumbersome like in the case of genes affecting tolerance to diseases/pests that require artificial inoculation in order to correctly identify those plants carrying the tolerant alleles (e.g., resistance to nematodes; [99]). Other cases where MABC provides a distinct temporal advantage as compared to conventional procedures is when the phenotypic evaluation of the target trait is destructive or when the trait is expressed after flowering. Selection before flowering greatly reduces the number of plants to be selfed or crossed, thus reducing the operating costs, particularly with species with a long life cycle.

During backcrossing, different rates of recovery of the recipient genome are expected at the target region and the nontarget chromosomes. Because each BC reduces by half the percentage of the donor genome at nontarget regions, at least six or seven BCs are required for a satisfactory recovery (ca. 99%) of the recipient genome. However, the number of BCs is frequently higher due to residual linkage drag around the target locus and it is not uncommon that up to nine or ten BCs are implemented before the improved cultivar is finally released. Clearly, the longer the time required to complete the BC procedures, the lower the probability of success of the new cultivar, since other improved, competing cultivars will be released in the meantime. Simulation and practice have both shown that in a moderately sized population of a species with a relatively small genome (<500 million bp, such as rice) using more than two to three well-spaced markers per chromosome arm hardly brings any additional benefit. For a species with large chromosomes (e.g., wheat, ca. 16 billion bp), a larger number of markers in each chromosome are beneficial. With an increasing genome size, more independent recombination events are needed to reduce the contribution of the donor parent, which in turn requires a larger population size. To what extent the contribution of the donor

parent should be reduced will depend on the type of alleles carried by such fragments and, most importantly, the genetic distance between the donor parent and the recurrent parent. Nowadays, the availability of large number of SNPs in most of the major crops facilitates the screening of the BC individuals to verify in great detail to what extent the genome of the donor parent has been retained.

Formulas are available to compute the level of concordance between the allelic state at the target locus and the flanking markers during the BC procedures [81]. These formulas values indicate that the level of control made possible with only one marker is insufficient to keep the risk of losing the target allele below 5% throughout five cycles of BC. Conversely, the level of control possible with two flanking markers is considerably higher even when the markers are not tightly linked to the target locus. If the BC procedure targets a QTL instead of a Mendelian locus, the uncertainty about the exact position of the sequence underlining the QTL introduces further complexity. Because the quantity of donor genes on the carrier chromosomes decreases much more slowly in comparison to the noncarrier chromosomes, after six BCs the majority of heterozygous loci with undesirable donor alleles will be on the carrier chromosome, with the vast majority included in the intact fragment flanking the target locus.

At the chromosomes not targeted by the BC procedure, it is expected that after "n" BCs, the probability that any locus remains heterozygous between the donor and the recipient is $(0.5)^n$, which means that each BC halves the residual level of heterozygosity. Consequently, six BCs ensure a level of similarity with the recurrent parent above 99%. Results in different species have shown that there may be a significant deviation from the 75% genomic portion of the recurrent parent expected in the BC_1 generation [100, 101], thus demonstrating the usefulness of genotype-based selection to identify plants with the highest possible portion of the genome from the recurrent parent.

Pyramiding Beneficial Alleles at Multiple Loci

The possibility to rapidly introgress and pyramid into existing cultivars a suite of beneficial alleles allows breeders to more quickly release improved cultivars to farmers. The best examples are in the area of disease resistance. Monogenic (Mendelian) resistance based on a single major gene is usually nondurable due to the high mutation rate in plant pathogens, which can lead to the selection of new virulent strains able to overcome the physiological barrier of an individual resistance gene. Consequently, the durability of disease resistance can be increased by screening for new sources of resistance followed by marker tagging of the relevant genes and their incorporation in elite cultivars. Pyramiding identifies the procedure for stacking the beneficial resistance alleles in a single line or cultivar, which provides a more durable resistance to pathogens as compared to monogenic resistance based on a single major gene. The advantage of pyramiding multiple alleles for resistance is particularly evident with diseases that require repeated inoculation and when phenotypic selection alone is too cumbersome and fails altogether to detect and combine multiple resistance genes in a single genotype.

Direct disease screening based on phenotypic observations is not always desirable due to a number of factors: quarantine restrictions, lack of routine screening methods and informative pathogen races for discriminating specific resistance genes, host escapes, and/or the inability to identify specific genes or gene combinations due to the occurrence of race or pathogen mixtures in the field. In these cases, MAS of race-specific genes offers a viable alternative for stacking beneficial alleles in improved genotypes which will eventually turn into novel cultivars characterized by more durable resistance to rapidly changing pathogen populations. Along this line, the constant changes in pathogen populations in different environments underline the potential value of previously defeated resistance genes. In this case, MAS offers the only practical solution to maintain such genes in current cultivars since they are masked by the epistatic effects of other resistance genes that are still effective.

In all major crops, the availability of markers tightly linked to resistance loci now allows breeders to tailor new cultivars with a suite of resistance genes able to enhance durable disease resistance to highly variable pathogens [102]. In broader terms, pyramiding is also implemented for combining beneficial alleles at loci (Mendelian or QTLs) that control traits other than disease resistance. In wheat, alleles at major loci that

influence quality (e.g., semolina color, protein content, micronutrient concentration, etc.) and tolerance to abiotic stress (e.g., aluminum, boron, salinity, etc.) are routinely introgressed via MABC [94].

When multiple loci are targeted in a BC program, the minimum population size to be considered increases considerably and rapidly becomes a major limiting factor when more than three or four loci are targeted, a number that can be increased to five or six when Mendelian loci are considered. When the targeted loci are QTLs, the uncertainty of the exact location of each selected QTL adds further constraints and reduces the number of loci that can be selected with a population of manageable size. When different lines contribute the beneficial alleles, the easiest strategy is to cross them to produce recombinant progenies and select the desired individuals. Multiple crosses might be required to pyramid all the desired alleles in one single genotype. A more general framework and the underlying theory to optimize breeding schemes for gene pyramiding have been described [103].

Marker-Assisted Selection in a Segregating Population

MAS has been extensively used for the selection of single genes conferring tolerance to diseases/pests [91, 94, 102, 104–106]. Although early simulation studies suggested the effectiveness of MAS for the improvement of biparental populations segregating for moderately complex traits [107], the first applications of MAS in maize were disappointing [57, 108]. Sweet corn is the only exception, the main reason being its much narrower genetic basis as compared to maize used for feed production, a feature that increases the reliability of predicted gains from selection and extrapolation of the effects of different loci to different populations [109]. Another feature that makes the application of MAS particularly attractive in sweet corn is the high costs associated to conventional phenotyping, in view of the large amount of grain that needs to be processed in order to obtain an accurate estimate of the phenotypic values of the progeny to be selected. MAS applications have been more widespread in the private sector as compared to public institutions, most likely owing to a lack in the latter of the infrastructure required for an effective exploitation of MAS.

Notwithstanding the remarkable progress in identifying and in some cases cloning major loci regulating agronomically valuable traits [48, 49], more limited success has been reported for MAS of quantitative traits [110], mainly due to the difficulty in identifying major QTLs with a sufficiently large and stable effect for justifying their deployment via MAS. While true QTL $\times$ E interaction due to variable expression of a trait may cause lack of consistency in QTL detection particularly with traits characterized by low to moderate heritability, the interaction between a mapping population of small size – hence with limited power in QTL detection – with variable environments is probably an equally important factor causing inconsistency in QTL detection. This is particularly evident for the improvement of crop yield under drought conditions, one of the most difficult traits to improve not only via MAS [14, 60, 111–113] but also through conventional breeding.

Marker-Assisted Recurrent Selection

Although marker-assisted recurrent selection (MARS) was first proposed in the early 1990s [114], only recently its adoption has provided a tangible contribution to crop improvement, mostly due the difficulty in identifying multiple loci characterized by limited G $\times$ E interaction and reasonably consistent effects in different genetic backgrounds other than that in which they were originally identified. The goal of MARS is pretty much similar to that pursued in pyramiding alleles at multiple loci, i.e., accumulating the beneficial alleles at as many as possible, preferably all, loci being targeted. Pyramiding alleles at many loci (e.g., >10) is best achieved with a recurrent selection strategy [115]. In this case, simulation showed that with 50 QTLs and a population of 200 plants the frequency of favorable alleles reached 100% after ten cycles when markers cosegregated with the QTL (i.e., they coincided), but only 92% when the marker-QTL interval was equal to 5 cM, hence increasing the possibility of losing the desired QTL allele due to recombination. In practice, with a higher number of loci under selection the occurrence of plants carrying the desired ideal combination becomes increasingly unlikely and basically impossible when more than 20 loci are targeted simultaneously. This problem can be partially mitigated through

successive cycles of crossing individuals carrying complementary combinations of the desired alleles [89]. This concept can be extrapolated to crosses with multiple parents.

MARS can start irrespectively of knowing the map position of the desired loci, which instead can be identified during the selection process. Simulation has clearly shown the superiority of MARS over phenotypic selection (from 5% to 20%), particularly when the selected population is highly heterozygous [116]. In maize, MARS has been applied rather extensively for improving relatively complex traits such as disease resistance, tolerance to abiotic stress, and also grain yield [111, 117–119].

The outcome of both MAS and MARS within a segregating population can be influenced by the genetic makeup of the targeted genetic background in terms of alleles present at other loci that interact epistatically with the target locus, an aspect which becomes particularly relevant for quantitative traits in view of the high number of loci involved in their control. Accordingly, since most evaluations of QTL effects and MAS strategies assume that QTLs act independently [55], it has been argued that MAS has little if any power over traditional phenotypic selection [46]. With maize as a model species, computer simulation showed that gene information is most useful in selection when few loci (<10) control the trait, while with many loci (>50) the least squares estimates of gene effects become imprecise. Based on these results, the typical reductionist approach pursued through QTL discovery strongly limits the outcome of MAS carried out for traits controlled by many QTLs [46].

Genomic Selection

In genomic selection (GS), genetic markers in number sufficient to cover the entire genome according to the level of LD are used so that most QTLs controlling the trait being selected are in LD with at least one neighboring marker. Unlike in MAS, in GS the individual plants are chosen without mapping the underlying QTLs that remain unknown along the entire process. Originally devised for animal breeding, only recently has GS been adopted for improving crop performance [120–122]. This was due to the fact that only in the past few years its application has become technically feasible in plants thanks to the introduction of SNP profiling with a level of genome saturation sufficient to detect the cumulative effects of the plethora of minor QTLs affecting quantitative traits which, on a single basis, will inevitably remain undetected in a biparental mapping population.

In GS, the breeding values of all the markers distributed across the genome are fitted as random effects in a linear model. The trait values are then predicted as the sum of the breeding values of each individual genotype across all the profiled markers and selection is based on these genome-wide predictions. A simulation study showed that across different numbers of QTLs (from 20 to 100) and levels of heritability, the response to GS was from 18% to 43% higher as compared to MARS. The number of markers that are used to predict the breeding values usually varies from a minimum of ca. 200 up to 500. A higher number of markers are required as the functional complexity of the targeted trait increases and LD decreases. Notably, GS is most effective for complex, low-heritable traits controlled by a large number of QTLs.

Implementation of GS is already having a major impact on the improvement of yield and other complex traits, mainly in the private sector where high-throughput infrastructures and robots allow for the routine creation and handling of millions of datapoints. Clearly, GS is not antagonistic to either MAS or MARS. Rather, they should be deployed in a complementary fashion on a case-by-case basis and according to the availability of mapped major QTLs, the accurate evaluation of their effect, and the frequency of the agronomically desirable alleles in the germplasm under selection.

Integrating Marker-Assisted Breeding in Conventional Breeding Projects

Among other factors, a broader application of MAB in conventional breeding projects will depend on the cost of molecular profiling [123, 124]. SNP markers are ideally suited for this role. In maize, the cost-effectiveness of MAS for the introgression of a single dominant allele into an elite line was compared with that of conventional breeding [125]. In this particular case, neither method showed clear superiority in terms of both cost and speed: Conventional breeding schemes

were found to be less expensive while MAS-based breeding schemes were shown to be faster. High-throughput genotyping based on the scoring of markers that do not need the use of gels [126–128] coupled with quick DNA extraction protocols are needed to streamline MAS and lower its cost.

An important factor to be carefully considered prior to embarking in any MAS activity targeting specific loci is the robustness of the marker-locus association and their genetic distance. Clearly, the level of LD of the genetic materials used to investigate the genetic makeup of the target traits plays a pivotal role in determining the level of genetic resolution. Accordingly, biparental F_2 populations have the maximum amount of LD, hence the lowest level of genetic resolution. Although this feature is advantageous for the initial QTL mapping studies in view of the limited number of markers that are required, it clearly limits the accuracy of MAS and usually does not allow us to resolve tightly linked QTLs from pleiotropic ones [129]. This problem can be circumvented by deploying genetic materials that capture a higher recombinational level, either historically (e.g., panels of unrelated genotypes suitable for association mapping; [67, 130]) or through subsequent random matings of the individuals of the original mapping population [131]. Increasing the genetic resolution not only enhances the reliability of MAS but also reduces the list of the possible candidates, an important prerequisite in identifying the sequence responsible for the phenotype of interest. Therefore, prior to undertaking an association mapping study, it is important to acquire a good understanding of the LD patterns in the set of genetic materials to be evaluated. In fact, LD can be caused by factors other than linkage. Spurious associations in a collection of germplasm accessions can be due to LD between unlinked genomic regions (i.e., >50 cM apart) on the same chromosome and/or between genomic regions located on different chromosomes. Dedicated softwares are available to reduce the frequency of false-positive associations due to the bias introduced by preexisting population structure.

One of the most critical steps in any breeding program is the choice of suitable parental lines to create the new segregating populations that will undergo selection. Ideally, such parental lines will contribute beneficial alleles at the loci most critical for the target traits and, more in general, crop performance and its quality. Molecular profiling can contribute in two major ways to expedite the selection process and increase the response to selection. In autogamous crops, MAS is applied to choose the parental lines that are crossed to generate new mapping populations (mostly biparental) and then to select during the subsequent generations the recombinant progeny that carry the desired alleles at the targeted loci. In wheat, MAS is being deployed in a number of breeding programs both in the public and private sectors [94]. In particular, more than 30 traits have been targeted, mainly for disease resistance, quality, and abiotic stress tolerance. In allogamous crops (e.g., maize) where the populations used to extract new parental lines routinely undergo recurrent selection, MARS can be applied at each selection cycle to increase the frequency of the beneficial alleles within the population until the best performing alleles are fixed within the population and, as such, no longer require selection. By increasing the frequency of beneficial alleles in a breeding population, the probability of recovering a genotype with the combination of desired alleles is increased. As an example, increasing the favorable allele frequency from 0.50 to 0.96 will increase the probability of recovering the ideal genotype for 20 independent regions from one in a trillion to one in five [9]. This change in allele frequency will improve the mean performance for the selected trait of the population and any line derived from it. Breeders can deploy different MARS schemes depending on the selection model and the desired genetic structure (e.g., inbreeding level) of the population obtained after MARS. The MARS schemes require optimization for best managing field and laboratory resources, hence containing the costs, as well as for expediting the selection process, hence the accumulation of favorable allele frequency. When several traits and loci are targeted simultaneously, a multiple trait index is used to combine the values of each individual trait into a single index and different weights are assigned according to the perceived importance of each trait. The output of this process is an estimated genotypic value calculated for each progeny being considered for selection. MARS can also be applied to autogamous crops (e.g., soybean) in order to enhance the performance of the breeding populations used to select improved genotypes that will hopefully outperform the existing cultivars.

As compared to conventional breeding practices, the outcome of MARS has clearly indicated its superiority for improving yield in maize, sunflower, and soybean [9]. Of utmost importance for the successful implementation of MARS is that breeders perform phenotypic selection on the lines per se that will be utilized for MARS. Additionally, phenotypic evaluation and selection among and within derived lines should continue after MARS.

Systematic profiling of parental lines is now routinely applied with a different level of genetic resolution, hence according to the level of LD of the target species. SSR profiling is rapidly being replaced by SNP profiling, much more effective than the former to define haplotype structure and much cheaper and amenable to high-throughput profiling. SNP platforms are particularly suited to the high-throughput profiling required by GS.

Once the template sequence of a crop becomes available, resequencing of lines can be used to obtain a far deeper understanding of their genomic architecture, allelic composition, and ultimate haplotype [132–134]. The spectacular reduction in cost that followed the introduction of second-generation sequencers makes resequencing of single genotypes a rather attractive and affordable option [135–137]. Additional progress in sequencing will further reduce the costs in as much direct resequencing of entire mapping populations may soon become more affordable than SNP profiling.

Mining Beneficial Alleles in Wild Relatives of Crops

As compared to their wild counterparts, the domestication bottleneck that all crops went through coupled with the strong selection first empirically practiced by farmers and then more systematically by modern breeders have markedly reduced the level of genetic variability within cultivated species, an aspect even more relevant for traits playing a substantial role in survival under natural conditions [82]. This limitation can be overcome through the implementation of advanced backcross QTL (AB-QTL) analysis [138], an approach that allows breeders to quickly discover and exploit beneficial QTL alleles present in wild germplasm but otherwise absent from elite germplasm. The AB-QTL approach relies on the evaluation of BC_2 families between an elite cultivar utilized as recurrent parent and a donor accession, usually a wild species that is sexually compatible with the crop. Usually, QTL analysis is delayed until the BC_2 generation and after selection in BC_1 against features known to affect negatively yield (e.g., ear shattering in small-grain cereals). The effectiveness of the AB-QTL approach has been proven in tomato [138, 139], rice [140], and barley [141]. These results are encouraging for using AB-QTL as a germplasm enhancement strategy for identifying wild alleles capable of improving the yield of the related crop, particularly under low-input agriculture and marginal environments where wild alleles may prove more beneficial, particularly for yield per se and disease resistance. An essential prerequisite is that the introgression of such beneficial alleles should bear no negative consequences when crops are grown under more favorable and high-yielding conditions.

Wild relatives of crop species can contribute to the identification of novel alleles for agronomically relevant traits by focusing on those loci that molecular evidence indicates as having been targeted by selection during both domestication and modern breeding [142]. To this end, the comparison of the allelic diversity present in elite accessions, landraces, and the undomesticated wild relatives of each crop allows for the identification of loci devoid of genetic variation within the elite germplasm, most likely as a result of domestication and subsequent man-made selection. The underlying assumption is that the loss of genetic diversity observed from the wild parent to the cultivated crop highlights the strong man-made selection at loci that control the expression of agronomically important traits, particularly those relevant for adaptation to abiotic stress. Therefore, both this "diversity screen" approach and the AB-QTL approach allow for the identification of valuable loci which would otherwise go undetected due to a lack of allelic diversity in the cultivated gene pool. An additional advantage of the diversity screen approach is that it allows for the identification of candidate genes of potential agronomic importance even without prior knowledge of gene function.

Leveraging the "-Omics" Platforms

During the past decade, a number of technologically sophisticated platforms have become available to

collect a large amount of data on the dynamics of the transcriptome, proteome, and metabolome. The availability of these "-omics" profiling data facilitates the identification of candidate genes and provides us with a more holistic picture of the molecular events characterizing functions at the cellular, organ, and plant levels and how these are influenced by environmental cues [84, 143–146].

Unlike from the classical QTL positional cloning approach in which an adequately large mapping population is basically "interrogated" in order to identify the genetic determinants of QTLs, the candidate-gene approach capitalizes on gathering experimental evidence to support and validate the causal role of a coding sequence (e.g., glutamine synthetase gene) in governing variation for the putative target trait (e.g., nitrogen-use efficiency). The major advantage of the candidate-gene approach is that it bypasses the tedious and expensive procedures required by positional cloning. Identifying suitable candidate genes and elucidating their function can be expedited by combining different approaches and high-throughput -omics platforms applied to target crops and/or to model species. From a technical standpoint, combining laser-capture microdissection with the -omics platforms offers an unprecedented level of functional resolution at the tissue level, down to a single-cell layer [145]. Among the different platforms available for the mass-scale profiling of the transcriptome, microarrays have been more frequently utilized to investigate the changes in gene expression, particularly in plants exposed to adverse conditions [147–150]. Nonetheless, microarray platforms are quickly being replaced by high-throughput transcriptome sequencing by means of second-generation sequencing platforms [151].

Additional information on the changes in cellular metabolism is provided by the profiling of the proteome [152] and metabolome [153, 154] that, as compared to the transcriptome, are functionally closer to the phenotype, thus reporting also on variability due to posttranscriptional and posttranslational regulation. However, it should be appreciated that both proteomics and metabolomics report changes for a rather limited portion (ca. 5%) of the expressed genes; additionally, proteomics is often unable to detect the changes in gene products (e.g., transcription factors) that despite their low level are more likely to play an important role in pivotal functions (e.g., signal transduction in response to biotic and abiotic stress) and consequently, to underline QTLs.

Metabolome profiling can also be used to identify loci regulating the level of a particular metabolite and verify its coincidence with QTLs for yield and/or genes involved in metabolic pathways. With the present technology, up to ca. 2,000 different metabolites can be profiled in a single sample [155]. In maize, QTLs for invertase activity have been identified in a population subjected to drought stress [156]. The number of QTLs for invertase activity detected under drought was more than twice the number detected under well-watered conditions, an indirect indication of the important role of this enzyme under drought conditions. One QTL common to both treatments was located near *Ivr2*, an invertase-encoding gene. The colocation reported between the activities of three enzymes (invertase, sucrose-P synthase, and ADP-glucose pyrophosphorylase) involved in sucrose and starch metabolism and a corresponding structural gene suggests its role as a candidate gene for explaining part of the variability in enzyme activity [157]. These studies indicate that invertase activity is an important limiting factor for grain yield in maize exposed to drought during the reproductive phase [158].

The candidate-gene approach is particularly effective when a clear cause-effect relationship can be unequivocally established between the gene product and the target trait. An example of this approach is the cloning of a QTL for cell-wall beta-glucans in barley grains based on a synteny analysis between barley and rice that revealed the presence in the syntenic portion of the rice genome of a cellulose synthase-like *CslF* gene that genetic engineering unequivocally showed to influence beta-glucans content in barley grains as well as in other species, including also *Arabidopsis* [83]. This notwithstanding, identifying suitable candidates for functionally complex traits such as yield and yield components is a much more daunting undertaking given the large number of genes that influence these traits.

Future Directions

The first comprehensive report of DNA-based markers (RFLPs; [20]) in a crop species was published in 1986.

Since then, an almost countless number of studies have shed light on the genetic control of plant growth and functions, and, most importantly crop yield. One clear take-home message that has emerged from these studies is the existence of a continuum between Mendelian and quantitative traits that will eventually help in identifying the functional polymorphisms, either of genetic or epigenetic origin that underlie quantitative trait variation. In this respect, QTL cloning will become a more routine and easier practice thanks also to the massive resequencing of mutant collections. This, in turn, will facilitate the identification of the best performing QTL alleles, their pyramiding through MAS, and the identification of novel alleles via TILLING [159] or by means of site-directed mutagenesis at the key functional domains of the encoded proteins. It is under this QTL cloning paradigm that the molecular basis of quantitative traits will be dissected in order to advance our understanding of the genetic makeup of this category of traits and to more accurately tailor crop morphology and productivity with beneficial alleles.

From an applicative standpoint, although conventional selection based on phenotypic evaluation will likely remain the mainstay for most breeding programs, particularly in the public domain, MAB and its applications will increasingly be adopted and will in some cases become prevalent as compared to conventional practices. As the twenty-first century unfolds, a multitude of genomics and postgenomics platforms are at hand to expand our understanding of the genetic basis of crop performance and to improve the efficiency of selection procedures for the release of new, improved cultivars. Resequencing will revolutionize the way breeders deal with their germplasm and will provide unsurpassed opportunities for a deeper mining of allelic diversity and harnessing its full potential. Nonetheless, our understanding of the functional basis of yield and other quantitative traits is likely to remain rudimental. The elusive nature of the QTLs that govern yield and yield stability is a formidable hurdle toward a more effective selection targeting specific loci and a better understanding of quantitative traits. Notably, GS can and will be applied irrespective of our degree of understanding of the genetic architecture of quantitative traits. Importantly, MAS and GS should be considered as complementary rather than alternative approaches, the utilization of which should be determined on a case-by-case basis. Bioinformatics and user-friendly databases will play a pivotal role for handling and managing the deluge of data produced by the molecular and phenotypic platforms.

In terms of experimental materials utilized for QTL studies, a growing attention will be devoted to the exploitation of multiparental crosses and mini-core collections of germplasm accessions with varying LD levels. In the mapping populations so far utilized for QTL discovery, most QTLs go undetected owing to the small size of the population, the presence of functionally monomorphic alleles and the small effects of many of such QTLs. Along this line, nested-association mapping (NAM) populations provide an interesting option to take advantage of both biparental (linkage) mapping and association mapping [160]. On a finer scale, high-throughput proteome and metabolome profiling will accelerate the identification of the causative mechanisms contributing to adaptive responses to adverse environmental conditions (e.g., drought, flooding, heat, etc.) whose frequency and intensity are expected to increase due to global warming. Nonetheless, the deluge of information originated through the molecular approaches and the -omics platforms will not automatically translate into novel cultivars. A "systems biology"-like approach will be instrumental for optimizing the accurate integration and exploitation in breeding terms of all the -omics information.

From an applicative standpoint, accurate phenotyping often remains the main limiting factor for identifying novel loci [161]. Semiautomated, high-throughput phenotyping under both controlled conditions and in the field promises to streamline gene discovery and narrowing the genotype-phenotype gap that hampers a more widespread deployment of MAB in crop improvement [87]. Along this line, it is important to emphasize that any molecular approach aiming to discover genes/QTLs and test their effects should preferably be carried out in an experimental context whose results are as relevant as possible and readily applicable to the conditions prevailing in farmers' fields [150]. An effective exploitation of genomics approaches to enhance crop performance will depend on their integration with conventional breeding. Although it is not possible to predict to what extent and how quickly the latter will be replaced by MAB, the

future release of improved cultivars will be expedited and made more cost effective through a systematic marker-based manipulation of the loci that govern crop performance and the desired features targeted by breeders.

Bibliography

Primary Literature

1. Borlaug NE, Dowswell CR (2005) Feeding a world of ten billion people: a 21st century challenge. In: Tuberosa R, Phillips RL, Gale M (eds) In the wake of the double helix: from the green revolution to the gene revolution, proceedings of the international congress, 27–31 May 2003, Bologna, pp 3–23
2. Borlaug NE (2007) Sixty-two years of fighting hunger: personal recollections. Euphytica 157:287–297
3. Duvick DN (2005) The contribution of breeding to yield advances in maize (*Zea mays* L.). Adv Agron 86:83–145
4. Tester M, Langridge P (2010) Breeding technologies to increase crop production in a changing world. Science 327:818–822
5. Lande R, Thompson R (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743–756
6. Lee M (1995) DNA markers and plant breeding programs. Adv Agron 55:265–344
7. Morgante M, Salamini F (2003) From plant genomics to breeding practice. Curr Opin Biotechnol 14:214–219
8. Varshney RK, Graner A, Sorrells ME (2005) Genomics-assisted breeding for crop improvement. Trends Plant Sci 10:621–630
9. Eathington SR, Crosbie TM, Edwards MD, Reiter R, Bull JK (2007) Molecular markers in a commercial breeding program. Crop Sci 47:S154–S163
10. Yano M, Tuberosa R (2009) Genome studies and molecular genetics-from sequence to crops: genomics comes of age. Curr Opin Plant Biol 12:103–106
11. Flavell R (2010) Knowledge and technologies for sustainable intensification of food production. New Biotechnol 27:505–516
12. Wei XJ, Liu LL, Xu JF, Jiang L, Zhang WW, Wang JK et al (2010) Breeding strategies for optimum heading date using genotypic information in rice. Mol Breeding 25:287–298
13. Schneeberger K, Weigel D (2011) Fast-forward genetics enabled by new sequencing technologies. Trends Plant Sci 16:282–288
14. Xu YB, Crouch JH (2008) Marker-assisted selection in plant breeding: from publications to practice. Crop Sci 48:391–407
15. Raju NL, Gnanesh BN, Lekha P, Jayashree B, Pande S, Hiremath PJ et al (2010) The first set of EST resource for gene discovery and marker development in pigeonpea (*Cajanus cajan* L.). BMC Plant Biol 10:45
16. Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227
17. Fujii H, Zhu JK (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc Natl Acad Sci USA 106:8380–8385
18. Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J et al (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. Plant J 61:672–685
19. Service RF (2006) Gene sequencing – the race for the $1000 genome. Science 311:1544–1546
20. Helentjaris T, Slocum M, Wright S, Schaefer A, Nienhuis J (1986) Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor Appl Genet 72:761–769
21. Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. BioTechnology 7:257–264
22. Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M et al (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414
23. Senior ML, Chin ECL, Lee M, Smith JSC, Stuber CW (1996) Simple sequence repeat markers developed from maize sequences found in the GENBANK database: map construction. Crop Sci 36:1676–1683
24. Kilian A (2005) The fast and the cheap: SNP and DArT-based whole genome profiling for crop improvement. In: Tuberosa R, Gale M, Phillips RL (eds) In the wake of the double helix: from the green revolution to the gene revolution, proceedings of the international congress, 27–31 May 2003, Bologna, pp 443–461
25. Rafalski A (2002) Applications of single nucleotide polymorphisms in crop genetics. Curr Opin Plant Biol 5:94–100
26. Barbazuk WB, Emrich SJ, Chen HD, Li L, Schnable PS (2007) SNP discovery via 454 transcriptome sequencing. Plant J 51:910–918
27. Akhunov E, Nicolet C, Dvorak J (2009) Single nucleotide polymorphism genotyping in polyploid wheat with the Illumina GoldenGate assay. Theor Appl Genet 119:507–517
28. Close TJ, Bhat PR, Lonardi S, Wu YH, Rostoks N, Ramsay L et al (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582
29. McCouch SR, Zhao KY, Wright M, Tung CW, Ebana K, Thomson M et al (2010) Development of genome-wide SNP assays for rice. Breeding Sci 60:524–535
30. Bentley DR (2006) Whole-genome re-sequencing. Curr Opin Genet Dev 16:545–552
31. Nordborg M, Weigel D (2008) Next-generation genetics in plants. Nature 456:720–723
32. Schnable PS, Ware D, Fulton RS, Stein JC, Wei FS, Pasternak S et al (2009) The B73 maize genome: complexity, diversity, and dynamics. Science 326:1112–1115
33. Varshney RK, Nayak SN, May GD, Jackson SA (2009) Next-generation sequencing technologies and their implications for crop genetics and breeding. Trends Biotechnol 27:522–530

34. Deschamps S, Campbell MA (2010) Utilization of next-generation sequencing platforms in plant genomics and genetic variant discovery. Mol Breeding 25:553–570
35. Moore G, Foote T, Helentjaris T, Devos K, Kurata N, Gale M (1995) Was there a single ancestral cereal chromosome? Trends Genet 11:81–82
36. Gale MD, Devos KM (1998) Plant comparative genetics after 10 years. Science 282:656–659
37. Bolot S, Abrouk M, Masood-Quraishi U, Stein N, Messing J, Feuillet C et al (2009) The 'inner circle' of the cereal genomes. Curr Opin Plant Biol 12:119–125
38. Falconer DS (1981) Introduction to quantitative genetics. Longman, London
39. Geldermann H (1975) Investigation on inheritance of quantitative characters in animals by gene markers. I. Methods. Theor Appl Genet 46:319–330
40. Sax K (1923) The association of size differences with seed-coat patterns and pigmentation in *Phaseolus vulgaris*. Genetics 8:552–560
41. Edwards MD, Stuber CW, Wendel JF (1987) Molecular-marker-facilitated investigations of quantitative-trait loci in maize. I. Numbers, genomic distribution and types of gene-action. Genetics 116:113–125
42. Stuber CW, Edwards MD, Wendel JF (1987) Molecular marker facilitated investigations of quantitative trait loci in maize. II. Factors influencing yield and its component traits. Crop Sci 27:639–648
43. Tanksley SD (1993) Mapping polygenes. Annu Rev Genet 27:205–233
44. Beavis WD (1998) QTL analysis: power, precision, and accuracy. In: Molecular dissection of complex traits. CRC Press, Boca Raton, pp 145–162
45. Ribaut JM, Hoisington D (1998) Marker-assisted selection: new tools and strategies. Trends Plant Sci 3:236–239
46. Bernardo R (2001) What if we knew all the genes for a quantitative trait in hybrid crops? Crop Sci 41:1–4
47. Bernardo R (2008) Molecular markers and selection for complex traits in plants: learning from the last 20 years. Crop Sci 48:1649–1664
48. Frary A, Nesbitt TC, Grandillo S, Knaap E, Cong B, Liu J et al (2000) fw2.2: a quantitative trait locus key to the evolution of tomato fruit size. Science 289:85–88
49. Salvi S, Tuberosa R (2007) Cloning QTLs in Plants. In: Varshney RK, Tuberosa R (eds) Genomics-assisted crop improvement-volume 1: genomics approaches and platforms. Springer, Dordrecht, pp 207–226
50. Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971
51. Lander ES, Botstein D (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199
52. Darvasi A, Weinreb A, Minke V, Weller JI, Soller M (1993) Detecting marker-QTL linkage and estimating QTL gene effect and map location using a saturated genetic map. Genetics 134:943–951
53. Kao CH, Zeng ZB, Teasdale RD (1999) Multiple interval mapping for quantitative trait loci. Genetics 152:1203–1216
54. Doerge RW (2002) Mapping and analysis of quantitative trait loci in experimental populations. Nat Rev Genet 3:43–52
55. Podlich DW, Winkler CR, Cooper M (2004) Mapping as you go: an effective approach for marker-assisted selection of complex traits. Crop Sci 44:1560–1571
56. Beavis WD (1994) The power and deceit of QTL experiments: lessons from comparative QTL studies. In: Proceedings of the forty-ninth annual corn and sorghum research conference, Washington, DC, pp 250–266
57. Openshaw S, Frascaroli E (1997) QTL detection and marker-assisted selection for complex traits in maize. In: Annual corn and sorghum research conference American seed trade association. Washington, DC, pp 44–53
58. Melchinger AE, Utz HF, Schon CC (1998) Quantitative trait locus (QTL) mapping using different testers and independent population samples in maize reveals low power of QTL detection and large bias in estimates of QTL effects. Genetics 149:383–403
59. Abiola O, Angel JM, Avner P, Bachmanov AA, Belknap JK, Bennett B et al (2003) The nature and identification of quantitative trait loci: a community's view. Nat Rev Genet 4:911–916
60. Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? Plant Physiol 147:469–486
61. Buckler ESI, Thornsberry JM (2002) Plant molecular diversity and applications to genomics. Curr Opin Plant Biol 5:107–111
62. Ersoz ES, Yu J, Buckler ES (2007) Applications of linkage disequilibrium and association mapping. In: Varshney RK, Tuberosa R (eds) Genomics-assisted crop improvement-volume 1: genomics approaches and platforms. Springer, Dordrecht, pp 97–120
63. Clark RM (2010) Genome-wide association studies coming of age in rice. Nat Genet 42:926–927
64. Rafalski A (2010) Association genetics in crop improvement. Curr Opin Plant Biol 13:174–180
65. Maccaferri M, Sanguineti MC, Demontis A, El-Ahmed A, del Moral LG, Maalouf F et al (2011) Association mapping in durum wheat grown across a broad range of water regimes. J Exp Bot 62:409–438
66. Buntjer JB, Sorensen AP, Peleman JD (2005) Haplotype diversity: the link between statistical and biological association. Trends Plant Sci 10:466–471
67. Buckler ES, Holland JB, Bradbury PJ, Acharya CB, Brown PJ, Browne C et al (2009) The genetic architecture of maize flowering time. Science 325:714–718
68. Salvi S, Sponza G, Morgante M, Tomes D, Niu X, Fengler KA et al (2007) Conserved noncoding genomic sequences associated with a flowering-time quantitative trait locus in maize. Proc Natl Acad Sci USA 104:11376–11381
69. Magalhaes JV, Liu J, Guimaraes CT, Lana UGP, Alves VMC, Wang YH et al (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nat Genet 39:1156–1161

70. Salvi S, Castelletti S, Tuberosa R (2009) An updated consensus map for flowering time QTLs in maize. Maydica 54:501–512
71. Robertson DS (1985) A possible technique for isolating genic DNA for quantitative traits in plants. J Theor Biol 117:1–10
72. Touzet P, Winkler RG, Helentjaris T (1995) Combined genetic and physiological analysis of a locus contributing to quantitative variation. Theor Appl Genet 91:200–205
73. Tuinstra MR, Ejeta G, Goldsbrough PB (1997) Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci. Theor Appl Genet 95:1005–1011
74. Landi P, Sanguineti MC, Salvi S, Giuliani S, Bellotti M, Maccaferri M et al (2005) Validation and characterization of a major QTL affecting leaf ABA concentration in maize. Mol Breeding 15:291–303
75. Landi P, Giuliani S, Salvi S, Ferri M, Tuberosa R, Sanguineti MC (2010) Characterization of root-yield-1.06, a major constitutive QTL for root and agronomic traits in maize across water regimes. J Exp Bot 61:3553–3562
76. Zamir D (2001) Improving plant breeding with exotic genetic libraries. Nat Rev Genet 2:983–989
77. Paran I, Zamir D (2003) Quantitative traits in plants: beyond the QTL. Trends Genet 19:303–306
78. Eshed Y, Zamir D (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141:1147–1162
79. Li ZK, Fu BY, Gao YM, Xu JL, Ali J, Lafitte HR et al (2005) Genome-wide introgression lines and their use in genetic and molecular dissection of complex phenotypes in rice (*Oryza sativa* L.). Plant Mol Biol 59:33–52
80. Takahashi Y, Shomura A, Sasaki T, Yano M (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the a subunit of protein kinase CK2. Proc Natl Acad Sci USA 98:7922–7927
81. Hospital F (2003) Marker-assisted breeding. In: Newbury HI (ed.) Plant molecular breeding. Blackwell Publishing and CRC Press, Oxford and Boca Raton, pp 30–59
82. Feuillet C, Langridge P, Waugh R (2008) Cereal breeding takes a walk on the wild side. Trends Genet 24:24–32
83. Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Stone BA et al (2006) Cellulose synthase-like *CslF* genes mediate the synthesis of cell wall (1,3;1,4)-β-D-glucans. Science 311:1940–1942
84. Belo A, Zheng PZ, Luck S, Shen B, Meyer DJ, Li BL et al (2008) Whole genome scan detects an allelic variant of *fad2* associated with increased oleic acid levels in maize. Mol Genet Genomics 279:1–10
85. Yin XY, Stam P, Kropff MJ, Schapendonk AH (2003) Crop modeling, QTL mapping, and their complementary role in plant breeding. Agron J 95:90–98
86. Hammer G, Cooper M, Tardieu F, Welch S, Walsh B, van Eeuwijk F et al (2006) Models for navigating biological complexity in breeding improved crop plants. Trends Plant Sci 11:587–593
87. Tardieu F, Tuberosa R (2010) Dissection and modeling of abiotic stress tolerance in plants. Curr Opin Plant Biol 13:206–212
88. Chapman SC (2008) Use of crop models to understand genotype by environment interactions for drought in real-world and simulated plant breeding trials. Euphytica 161:195–208
89. Peleman JD, van der Voort JR (2003) Breeding by design. Trends Plant Sci 8:330–334
90. Young ND, Tanksley SD (1989) Restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor Appl Genet 77:95–101
91. Young ND (1999) A cautiously optimistic vision for marker-assisted breeding. Mol Breeding 5:505–510
92. Willcox MC, Khairallah MM, Bergvinson D, Crossa J, Deutsch JA, Edmeades GO et al (2002) Selection for resistance to southwestern corn borer using marker-assisted and conventional backcrossing. Crop Sci 42:1516–1528
93. Sørensen AP, Stuurman J, van der Voort JR, Peleman J (2007) Molecular breeding: maximizing the exploitation of genetic. In: Varshney RK, Tuberosa R (eds) Genomics-assisted crop improvement-volume 1: genomics approaches and platforms. Springer, Dordrecht, pp 31–56
94. Gupta PK, Langridge P, Mir RR (2010) Marker-assisted wheat breeding: present status and future possibilities. Mol Breeding 26:145–161
95. Xue SL, Li GQ, Jia HY, Lin F, Cao Y, Xu F et al (2010) Marker-assisted development and evaluation of near-isogenic lines for scab resistance QTLs of wheat. Mol Breeding 25:397–405
96. Randhawa HS, Mutti JS, Kidwell K, Morris CF, Chen XM, Gill KS (2009) Rapid and targeted introgression of genes into popular wheat cultivars using marker-assisted background selection. PLoS One 4:e5752
97. Hospital F, Charcosset A (1997) Marker-assisted introgression of quantitative trait loci. Genetics 147:1469–1485
98. Concibido VC, La Vallee B, McLaird P, Pineda N, Meyer J, Hummel L et al (2003) Introgression of a quantitative trait locus for yield from *Glycine soja* into commercial soybean cultivars. Theor Appl Genet 106:575–582
99. Zwart RS, Thompson JP, Milgate AW, Bansal UK, Williamson PM, Raman H et al (2010) QTL mapping of multiple foliar disease and root-lesion nematode resistances in wheat. Mol Breeding 26:107–124
100. Frisch M, Bohn M, Melchinger AE (1999) Comparison of selection strategies for marker-assisted backcrossing of a gene. Crop Sci 39:1295–1301
101. Frisch M, Bohn M, Melchinger AE (1999) Minimum sample size and optimal positioning of flanking markers in marker-assisted backcrossing for transfer of a target gene. Crop Sci 39:967–975
102. Maccaferri M, Mantovani P, Tuberosa R, DeAmbrogio E, Giuliani S, Demontis A et al (2008) A major QTL for durable leaf rust resistance widely exploited in durum wheat breeding programs maps on the distal region of chromosome arm 7BL. Theor Appl Genet 117:1225–1240

103. Servin B, Martin OC, Mezard M, Hospital F (2004) Toward a theory of marker-assisted gene pyramiding. Genetics 168: 513–523
104. Knapp SJ (1998) Marker-assisted selection as a strategy for increasing the probability of selecting superior genotypes. Crop Sci 38:1164–1174
105. Gebhardt C, Bellin D, Henselewski H, Lehmann W, Schwarzfischer J, Valkonen JPT (2006) Marker-assisted combination of major genes for pathogen resistance in potato. Theor Appl Genet 112:1458–1464
106. Ejeta G, Knoll JE (2007) Marker-assisted selection in sorghum. In: Varshney RK, Tuberosa R (eds) Genomics-assisted crop improvement-volume 2: genomics applications in crops. Springer, Dordrecht, pp 187–206
107. Edwards MD, Johnson L (1994a) RFLPs for rapid recurrent selection. In: Proceedings joint plant breeding symposium series. American Society Horticulture and Crop Science Society America, Corvallis
108. Moreau L, Charcosset A, Gallais A (2004) Experimental evaluation of several cycles of marker-assisted selection in maize. Euphytica 137:111–118
109. Yousef GG, Juvik JA (2001) Comparison of phenotypic and marker-assisted selection for quantitative traits in sweet corn. Crop Sci 41:645–655
110. Chen LM, Zhao ZG, Liu X, Liu LL, Jiang L, Liu SJ et al (2011) Marker-assisted breeding of a photoperiod-sensitive male sterile *japonica* rice with high cross-compatibility with *indica* rice. Mol Breeding 27:247–258
111. Ribaut JM, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations, and alternatives. J Exp Bot 58:351–360
112. Reynolds M, Tuberosa R (2008) Translational research impacting on crop productivity in drought-prone environments. Curr Opin Plant Biol 11:171–179
113. Herve P, Serraj R (2009) Gene technology and drought: a simple solution for a complex trait? Afr J Biotechnol 8:1740–1749
114. Edwards MD, Page NJ (1994) Evaluation of marker-assisted selection through computer simulation. Theor Appl Genet 88:376–382
115. Charmet G, Robert N, Perretant MR, Gay G, Sourdille P, Groos C et al (1999) Marker-assisted recurrent selection for cumulating additive and interactive QTLs in recombinant inbred lines. Theor Appl Genet 99:1143–1148
116. van Berloo R, Stam P (2001) Simultaneous marker-assisted selection for multiple traits in autogamous crops. Theor Appl Genet 102:1107–1112
117. Eathington SR, Dudley JW, Rufener GK (1997) Usefulness of marker-QTL associations in early generation selection. Crop Sci 37:1686–1693
118. Eathington SR (2005) Practical applications of molecular technology in the development of commercial maize hybrids. In: Proceedings of the 60th annual corn and sorghum seed research conferences. American Seed Trade Association, Washington, DC
119. Crosbie T, Eathington S, Johnson G, Edwards M, Reiter R, Stark S, et al (2006) Plant breeding: past, present, and future. In: Lamkey KR, Lee M (eds) Plant breeding: the Arnel R. Hallauer international symposium. Blackwell, Ames, pp 3–50
120. Heffner EL, Sorrells ME, Jannink JL (2009) Genomic selection for crop improvement. Crop Sci 49:1–12
121. Bernardo R (2010) Genomewide selection with minimal crossing in self-pollinated crops. Crop Sci 50:624–627
122. Lorenz AJ, Chao SM, Asoro FG, Heffner EL, Hayashi T, Iwata H et al (2011) Genomic selection in plant breeding: knowledge and prospects. Adv Agron 110:77–123
123. Moreau L, Lemarie S, Charcosset A, Gallais A (2000) Economic efficiency of one cycle of marker-assisted selection. Crop Sci 40:329–337
124. Kuchel H, Fox R, Reinheimer J, Mosionek L, Willey N, Bariana H et al (2007) The successful application of a marker-assisted wheat breeding strategy. Mol Breeding 20:295–308
125. Morris M, Dreher K, Ribaut JM, Khairallah M (2003) Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Mol Breeding 11:235–247
126. Salvi S, Tuberosa R, Phillips RL (2001) Development of PCR-based assays for allelic discrimination in maize by using the 5′-nuclease procedure. Mol Breeding 8:169–176
127. Canovas A, Rincon G, Islas-Trejo A, Wickramasinghe S, Medrano JF (2010) SNP discovery in the bovine milk transcriptome using RNA-Seq technology. Mamm Genome 21:592–598
128. Mondini L, Nachit MM, Porceddu E, Pagnotta MA (2011) HRM technology for the identification and characterization of INDEL and SNP mutations in genes involved in drought and salt tolerance of durum wheat. Plant Genetic Resources-Characterization and Utilization 9:166–169
129. Graham GI, Wolff DW, Stuber CW (1997) Characterization of a yield quantitative trait locus on chromosome five of maize by fine mapping. Crop Sci 37:1601–1610
130. Darvasi A, Soller M (1995) Advanced intercross lines, an experimental population for fine genetic-mapping. Genetics 141:1199–1207
131. Lee M, Sharopova N, Beavis WD, Grant D, Katt M, Blair D et al (2002) Expanding the genetic map of maize with the intermated B73 × Mo17 (IBM) population. Plant Mol Biol 48:453–461
132. Atwell S, Huang YS, Vilhjalmsson BJ, Willems G, Horton M, Li Y et al (2010) Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. Nature 465:627–631
133. Bergelson J, Roux F (2010) Towards identifying genes underlying ecologically relevant traits in *Arabidopsis thaliana*. Nat Rev Genet 11:867–879
134. Huang XH, Wei XH, Sang T, Zhao QA, Feng Q, Zhao Y et al (2010) Genome-wide association studies of 14 agronomic traits in rice landraces. Nat Genet 42:961–976
135. Gupta PK (2008) Ultrafast and low-cost DNA sequencing methods for applied genomics research. Proc Natl Acad Sci India 78:91–102

136. Huang XH, Feng Q, Qian Q, Zhao Q, Wang L, Wang AH et al (2009) High-throughput genotyping by whole-genome resequencing. Genome Res 19:1068–1076
137. Lam HM, Xu X, Liu X, Chen WB, Yang GH, Wong FL et al (2010) Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection. Nat Genet 42:1053–1059
138. Tanksley SD, Grandillo S, Fulton TM, Zamir D, Eshed Y, Petiard V et al (1996) Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium*. Theor Appl Genet 92: 213–224
139. Bernacchi D, Beck-Bunn T, Eshed Y, Inai S, Lopez J, Petiard V et al (1998) Advanced backcross QTL analysis of tomato. II. Evaluation of near-isogenic lines carrying single-donor introgressions for desirable wild QTL-alleles derived from *Lycopersicon hirsutum* and *L. pimpinellifolium*. Theor Appl Genet 97:170–180
140. Xiao J, Li J, Grandillo S, Ahn SN, Yuan L, Tanksley SD et al (1998) Identification of trait-improving quantitative trait loci alleles from a wild rice relative, *Oryza rufipogon*. Genetics 150:899–909
141. Talame V, Sanguineti MC, Chiapparino E, Bahri H, Ben Salem M, Forster BP et al (2004) Identification of *Hordeum spontaneum* QTL alleles improving field performance of barley grown under rainfed conditions. Ann Appl Biol 144:309–319
142. Yamasaki M, Tenaillon MI, Bi IV, Schroeder SG, Sanchez-Villeda H, Doebley JF et al (2005) A large-scale screen for artificial selection in maize identifies candidate agronomic loci for domestication and crop improvement. Plant Cell 17:2859–2872
143. Tuberosa R, Gill BS, Quarrie SA (2002) Cereal genomics: ushering in a brave new world. Plant Mol Biol 48:445–449
144. Druka A, Druka I, Centeno AG, Li H, Sun Z, Thomas WTB et al (2008) Towards systems genetic analyses in barley: integration of phenotypic, expression and genotype data into GeneNetwork. BMC Genet 9:73
145. Hochholdinger F, Tuberosa R (2009) Genetic and genomic dissection of maize root development and architecture. Curr Opin Plant Biol 12:172–177
146. Urano K, Kurihara Y, Seki M, Shinozaki K (2010) 'Omics' analyses of regulatory networks in plant abiotic stress responses. Curr Opin Plant Biol 13:132–138
147. Ozturk ZN, Talamè V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N et al (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol 48:551–573
148. Zinselmeier C, Sun YJ, Helentjaris T, Beatty M, Yang S, Smith H et al (2002) The use of gene expression profiling to dissect the stress sensitivity of reproductive development in maize. Field Crop Res 75:111–121
149. Schnable PS, Hochholdinger F, Nakazono M (2004) Global expression profiling applied to plant development. Curr Opin Plant Biol 7:50–56
150. Talame V, Ozturk NZ, Bohnert HJ, Tuberosa R (2007) Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. J Exp Bot 58:229–240
151. Kofler R, Torres TT, Lelley T, Schlotterer C (2009) PanGEA: identification of allele specific gene expression using the 454 technology. BMC Bioinformatics 10:143
152. Hochholdinger F, Woll K, Guo L, Schnable PS (2005) The accumulation of abundant soluble proteins changes early in the development of the primary roots of maize (*Zea mays* L.). Proteomics 5:4885–4893
153. Fernie AR, Schauer N (2009) Metabolomics-assisted breeding: a viable option for crop improvement? Trends Genet 25:39–48
154. Saito K, Matsuda F (2010) Metabolomics for functional genomics, systems biology, and biotechnology. Annu Rev Plant Biol 61:463–489
155. Sakurai N, Shibata D (2006) KaPPA-view for integrating quantitative transcriptomic and metabolomic data on plant metabolic pathway maps. J Pestic Sci 31:293–295
156. Pelleschi S, Guy S, Kim JY, Pointe C, Mahe A, Barthes L et al (1999) Ivr2, a candidate gene for a QTL of vacuolar invertase activity in maize leaves. Gene-specific expression under water stress. Plant Mol Biol 39:373–380
157. Pelleschi S, Leonardi A, Rocher JP, Cornic G, de Vienne D, Thévenot C et al (2006) Analysis of the relationships between growth, photosynthesis and carbohydrate metabolism using quantitative trait loci (QTLs) in young maize plants subjected to water deprivation. Mol Breeding 17:21–39
158. Boyer JS, McLaughlin JE (2007) Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. J Exp Bot 58:267–277
159. Talame V, Bovina R, Sanguineti MC, Tuberosa R, Lundqvist U, Salvi S (2008) TILLMore, a resource for the discovery of chemically induced mutants in barley. Plant Biotechnol J 6:477–485
160. McMullen MD, Kresovich S, Villeda HS, Bradbury P, Li HH, Sun Q et al (2009) Genetic properties of the maize nested association mapping population. Science 325:737–740
161. Zhou M (2011) Accurate phenotyping reveals better QTL for waterlogging tolerance in barley. Plant Breeding 130:203–208
162. Maccaferri M, Sanguineti MC, Corneti S, Ortega JLA, Ben Salem M, Bort J et al (2008) Quantitative trait loci for grain yield and adaptation of durum wheat (*Triticum durum* Desf.) across a wide range of water availability. Genetics 178:489–511
163. Salvi S, Corneti S, Bellotti M, Carraro N, Sanguineti MC, Castelletti S, Tuberosa R (2011) Genetic dissection of maize phenology using an intraspecific introgression library. BMC Plant Biol 11:4

Books and Reviews

Bernardo R (ed) (2010) Breeding for quantitative traits in plants, 2nd edn. Stemma Press, Woodbury, p 400
Costa de Oliveira A, Varshney RK (eds) (2011) Root genomics. Springer, Berlin/Heidelberg, p 318

Guimarães E, Ruane J, Scherf B, Sonnino A, Dargie J (eds) (2007) Marker-assisted selection. current status and future perspectives in crops, livestock, forestry and fish. Food and Agriculture Organization, Rome, p 492

Kole C (ed) (2006) Genome mapping and molecular breeding in plants – cereals and millets. Springer, Berlin/Heidelberg, p 349

Lamkey KR, Lee M (eds) (2006) Plant breeding: the Arnel R. Hallauer international symposium. Blackwell, Ames, p 379

Liu BH (ed) (1998) Statistical genomics: linkage, mapping, and QTL analysis. CRC Press, Boca Raton, p 611

Rao DC, Gu CC (eds) (2008) Genetic dissection of complex traits, 2nd edn. Academic, New York, p 760

Varshney RK, Tuberosa R (eds) (2007) Genomics-assisted crop improvement, volume 1: genomics approaches and platforms. Springer, Dordrecht, p 386

Varshney RK, Tuberosa R (eds) (2007) Genomic-assisted crop improvement, volume 2: genomics applications in crops. Springer, Dordrecht, p 509

Xu Y (ed) (2010) Molecular plant breeding. CABI, Wallingford, p 734

Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures

Suvi T. Häkkinen, Anneli Ritala, Heiko Rischer, K.-M. Oksman-Caldentey
VTT Technical Research Centre of Finland, Espoo, Finland

Article Outline

Glossary

Bioreactor A fermentor in which plant cell cultures can be cultivated in sterile, controlled, and contained condition for biotechnological production of cell biomass and/or particular protein or small molecule.

Medicinal plants Plants that are used for medicinal purposes; whole plants or specific plant organs or compounds derived thereof can be utilized.

Metabolic engineering A process to understand metabolic pathways; a targeted alteration of metabolic pathways with the aim of improved yield, quality, and/or spectrum of produced metabolites.

Plant cell culture Process where plant cells are cultivated under controlled conditions; may consist of differentiated tissues or organs (e.g., shoots, roots, embryos, stems) or undifferentiated cells (e.g., callus, suspension cultures).

Secondary metabolites Low molecular weight compounds with enormous chemical diversity often found in plants in small amounts essential for plants' defense system; many secondary metabolites are used as pharmaceuticals, dyes, flavors, and fragrances by humans.

Transgene A gene that has been transferred from one organism to another.

Definition of the Subject

Plants are the most excellent designers and producers of a variety of small compounds that are beneficial to mankind as foods, medicines, and industrial raw materials. The use of medicinal plants for human health dates back to ancient history of mankind. The first written document of the use of medicinal plants can be found in Papyrus Ebers (1800 BC). Even if the use of certain medicinal plants was known to treat certain diseases – often using the trial-and-error approach – it is only less than 200 years ago the isolation of the first active chemical constituent (secondary metabolite) responsible for its pharmacological effect occurred. Today, many plant-derived compounds are used in pharmaceutical industry, and plants also serve as an important source for new lead compounds.

Many plants containing high-value secondary metabolites are difficult to cultivate or are becoming endangered because of the overharvesting. Furthermore, the chemical synthesis of plant-derived compounds is often not economically feasible due to their highly complex structures and the specific stereochemical requirements of the compounds. The biotechnological production of valuable secondary metabolites in plant cell or organ cultures is an attractive alternative to the extraction of whole plant material. However, the use of plant cell or organ cultures has had only limited commercial success so far. This is explained by the empirical nature of selecting high-yielding, stable cultures and the lack of understanding of how secondary metabolites are synthesized or how their synthesis is regulated.

Introduction

It has been estimated that there are at least 400,000 higher plant species in the world of which only about 10% are characterized chemically to certain extent [1]. There is no doubt that the chemical diversity of plants is much greater than any chemical library made by humans, and thus the plant kingdom represents an enormous reservoir of pharmacologically valuable molecules waiting to be discovered. Plants are thus excellent organic chemists in nature and constantly respond to environmental changes by adjusting their capacity to produce natural products. Functional genomics may open entirely new avenues to screen

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

unexplored medicinal plant species for their pharmacological value. Many pharmaceutical companies have now renewed their interest on plant-derived compounds due to too high expectations on combinatorial chemistry or computational drug design to obtain new drug leads during the past decades [2, 3].

Many secondary metabolites of industrial value are complex in their structures making chemical synthesis very challenging and expensive. Moreover, plants contain usually very low contents of these compounds, and therefore other production processes are essential to be developed. Biotechnological production using plant cells as real green factories is a very promising technology, but currently there are still many limiting factors, mainly related to our poor understanding how the plants synthesize these high-value compounds and how the synthesis is regulated.

In the following sections, an overview is given how secondary metabolites are produced in plant and tissue cultures, how the production can be enhanced by classical optimization methods, and what metabolic engineering has to offer today and in the future. Spectacular advances in plant genomics and metabolite profiling offer unprecedented possibilities to explore the extraordinary complexity of the plant biochemical capacity. State-of-the-art genomics tools can be used to engineer the enhanced production of known target metabolites or to synthesize entire novel compounds by the so-called combinatorial biochemistry in cultivated plant cells. Finally, some future perspectives are given for novel techniques and tools that are just now emerging.

High-Value Products from Medicinal Plants

Medicinal Plants

Many plants such as crops play a central role in our everyday diet. The nutritional value of edible plants and their constituents has been studied for decades. Besides the edible plants, there is a huge variety of toxic plants in the plant kingdom. These include, for example, many alkaloid or terpene containing medicinal plants such as *Atropa belladonna*, *Camptotheca acuminata*, *Capsicum annuum*, *Catharanthus roseus*, *Erythroxylum coca*, *Papaver somniferum*, *Cannabis sativa*, *Artemisia annua*, and *Taxus* species – just to name a couple of them. These plants have been and still are an important source of pharmaceuticals. Molecules derived from medicinal plants make up a sizable proportion of known drugs currently available on the market. These include compounds such as morphine, codeine, and several anticancer drugs such as paclitaxel, vincristine, and vinblastine, the monetary value of which is very high. In Western medicine, over 25% of prescription drugs sold in pharmacies contain at least one active principle which is directly or indirectly (via semi-synthesis) a natural product. This number does not include the over-the-counter sold drugs or pure phytopharmaceuticals.

According to WHO, 11% of the current 252 drugs considered essential for humans are exclusively derived from flowering plants. Furthermore, plants are also important source of new drug lead compounds. During the past 25 years, 1,010 new drug entities (NDEs) were introduced to the market; 27% of them were either natural products or derived from natural products as semi-synthetic derivatives [3]. In addition, 15% of the drugs were synthesized after the molecule was first discovered from natural resources. Table 1 shows the origin of the 458 NDEs representing the four major therapy groups with anti-infectives (antibacterial, antiviral, antifungal, and antiparasitic), anticancer, antihypertensive, or anti-inflammatory activities discovered between 1981 and 2006. It is remarkable that over 68% of all antibacterial compounds and 51% of all anticancer drugs were directly or indirectly derived from natural resources. Natural sources will undoubtedly continue to play a prominent role in the discovery of pharmaceuticals in the future.

Secondary Metabolism in Plants

Secondary metabolites are low molecular weight compounds found in small quantities throughout the whole plant kingdom. They exhibit many biological functions vital for the survival of the plant such as responding to stress, mediating pollination, or acting as defense compounds. In plant cell, they are accumulated often in the vacuoles. Besides the importance for the plant itself, secondary metabolites have always been of interest to humans as flavors, fragrances, dyes, pesticides, and pharmaceuticals. However, for most of the secondary metabolites, the exact function in plants still remains unknown.

Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Table 1 Number of new drug entities (NDEs) discovered during 1981–2006 belonging to the four most important therapy groups (modified from [3])

Therapy group	Total	N	ND	NS	B	S	V	N+D+NS	%
Antimicrobial	230	12	74	34	13	60	37	120	52.2
Anti-bacterial	109	10	64	1	0	23	11	75	68.8
Anti-fungal	29	0	3	0	1	25	0	3	10.3
Anti-viral	78	0	2	31	12	8	25	33	42.3
Anti-parasitic	14	2	5	2	0	4	1	9	64.3
Anti-cancer	100	9	25	17	17	30	2	51	51.0
Anti-hypertensive	77	0	2	34	0	41	0	36	46.8
Anti-inflammatory	51	0	13	0	1	37	0	13	25.5
Total	458	21	114	85	31	168	39	220	48.0

N natural product, *ND* natural product derivative, *NS* product is synthesized but the original molecule is discovered from natural sources, *B* biotechnologically produced compound (often a large molecule, protein), *S* synthetic molecule, *V* vaccine

More than 200,000 secondary metabolites have hitherto been discovered from the plant kingdom, but only half of them are structurally fully elucidated [4–6]. They are characterized by an enormous chemical diversity, and every plant has its own characteristic set of secondary metabolites. The production of specific alkaloids is often strongly restricted to certain plant families, whereas, for example, flavonoids are abundant in many plant species. Based on their biosynthetic origins, plant secondary metabolites can be structurally divided into five major groups: polyketides, isoprenoids (e.g., terpenoids), alkaloids, phenylpropanoids, and flavonoids [7]. The polyketides are produced via the acetate-mevalonate pathway; the isoprenoids (terpenoids and steroids) are derived from the five-carbon precursor, isopentenyl diphosphate (IPP) produced via the classical mevalonate pathway, or the novel MEP pathway (see the details in section "Targeting the Metabolic Enzymes"); the alkaloids are synthesized from various amino acids; phenylpropanoids are derived from aromatic amino acids phenylalanine or tyrosine; and the flavonoids are synthesized by the combination of phenylpropanoids and polyketides [8].

Since the discovery of the opium alkaloid morphine almost two centuries ago, alkaloids are still one of the most studied groups of plant secondary metabolites although terpenoids are the largest chemical family of secondary metabolites. It is somehow surprising that such an extensive array of different nitrogen-containing organic molecules are known in higher plants even though only 2% of the plant dry weight is composed of the element nitrogen. The largest requirement of nitrogen is the synthesis of amino acids which function as building blocks of proteins as well as precursors to many secondary metabolites. Alkaloids are thus the most prominent nitrogenous compounds with diverse, complex structures and often possessing strong physiological properties leading their wide use as pharmaceuticals. Human use of them dates back to more than 3,000 years. Currently, more than 12,000 alkaloids are known and they are classified into several subclasses based on the amino acids from which they are derived and according to their chemical structures [9].

At the present time, small amounts of plant compounds including alkaloids, for example, morphine, scopolamine, and vincristine are isolated with often some difficulties from natural vegetation or cultivated plants which explain the high price of the raw material. Numerous secondary metabolites have also served as models for modern synthetic pharmaceuticals [3]. However, the biosynthetic pathways leading to their formation in plants are often long, complex multistep events catalyzed by various enzymes, and are still largely unknown in enzymatic and genetic level.

The best characterized pathways after the decades' intensive classical biochemical research are the biosynthesis of opium and terpenoid indole alkaloids.

Besides the low quantities of the compounds in plants, the production rates may vary from year to year and secondary metabolites often accumulate in specific plant organs in particular time of the vegetative stage of the plant. Some substances can only be isolated from extremely rare plants which is not a choice for sustainable production. Therefore, alternative production systems for plant-derived compounds are needed. The biotechnological production, that is, producing the plant secondary metabolites in cultured plant cells in large bioreactors may offer an attractive alternative approach.

Biotechnological Production Options

The production of a secondary metabolite of interest for industrial needs is often a challenge. As explained above, these compounds accumulate in plants in small quantities. The biotechnological production of high-value plant secondary metabolites therefore is a viable option to isolation processes from the intact plants or to the total chemical synthesis.

Biotechnology focuses on the exploitation of metabolic properties of living organisms for the production of valuable products of a very different structural and organizational level for the benefit of humans. The organisms vary from microbes (bacteria, fungi, yeast) to plants and animals. Over the decades, many laboratories all over the world have studied the possibilities to produce desired secondary metabolites using plant cell or tissue cultures. Cell cultures have been established from many plants, but often they do not produce sufficient amounts of the required secondary metabolites or the production is unstable. Various classical optimization tools have been applied (see in detail section "Enhancing the Production by Classical Optimization"), but very few success stories exist contrary to many good examples using microbial production systems.

Molecular biology of plants has emerged enormously during the past decades, but still the plant metabolic engineering has met only limited success, again in sharp contrast to microorganisms. This is due to our limited knowledge on complex biosynthesis of secondary metabolites. Despite the rapid development of not only plant genomics but also of analytical tools, genetic maps of biosynthetic pathways are far from complete. Furthermore, regulation of the individual steps leading to the desired end product is poorly understood (section "Metabolic Engineering").

Plant Cell Cultures Plant cell culture is a method where plant cells are cultivated under sterile conditions *in vitro*. Commonly, cell cultures are established from callus tissues by cultivating callus in liquid medium, and cell aggregates are broken by either mechanically or by orbital shaking in the cultivation vessel. Plant cells are biosynthetically totipotent, which means that each cell in culture retains its complete genetic information and thus is able to produce the same metabolites as the parent plant. Plant cell cultures have been extensively exploited for various biotechnological applications as an alternative to the traditional agricultural cultivation of plants. The use of cell culture systems offers advantages to produce metabolites in a controlled environment, independent of climatic conditions and under conditions in which the different production parameters can be optimized. Plant cell cultures can be categorized in two main classes, differentiated and undifferentiated cell cultures. The former consists of, for example, organs like shoots, roots, or embryos, whereas callus and cell suspension cultures are referred to as undifferentiated cell cultures. Since the first gene transfers in plants in 1983, achieved by four independently working groups [10–13], a number of efficient gene transfer techniques have been developed for genetic engineering of plants. In addition to so-called direct gene transfer techniques (e.g., particle bombardment, electroporation, microinjection), *Agrobacterium*-mediated gene transfer has been the most commonly used method for gene delivery to plants.

Hairy Root Cultures *Agrobacterium* (Rhizobiaceae) is a soil bacterium, which is able to deliver its own plasmid-DNA into the nuclear genome of the plant cell. The bacterium attaches into the wound site of the plant tissue and recognizes certain wound substances, for example, acetosyringone, secreted by the plant [14]. As a result, the *vir* (virulence) region of the plasmid becomes activated and processing of the T-DNA (transferred DNA) for the gene transfer starts [14, 15].

After successful integration of the bacterial DNA into the host plant genome, the tumor formation in the wound site begins as well as the production of low molecular weight tumor substances called opines. The opines are used as a nutrient for the bacterium [16]. The host range of *Agrobacterium* is perhaps broader than that of any other plant pathogenic bacterium, although a number of cultivated monocotyledonous plants and legumes are not natural hosts for this bacterium. The molecular mechanism of the resistance to *Agrobacterium* is not known, although the production of antimicrobial metabolites [17], a lack of *vir* gene inducers [18], inefficient T-DNA integration [19], and *Agrobacterium*-induced programmed cell death [20] have all been suggested. Successful gene transfer in monocot plants via *Agrobacterium* has been performed with maize, rice, wheat, and barley [21].

Hairy root is a plant disease caused by the infection of *Agrobacterium rhizogenes* carrying Ri (root-inducing) plasmid. During infection of the plant, the T-DNA of the Ri-plasmid is transferred and integrated in the nuclear genome of the host. As a result of the transformation, hairy roots appear at the infection site [22]. In the T-DNA, there are four genetic loci, called *rol*A, *rol*B, *rol*C, and *rol*D, which are responsible for the hairy root phenotype. These genes were shown to positively affect the secondary metabolite production in *Nicotiana* [23] and in *Atropa* [24]. Hairy roots are able to grow without externally supplied auxins, and certain *aux* genes from *Agrobacterium* have been shown to provide transformed cells with an additional source of auxin [25]. This is a clear advantage when considering the costs for large-scale cultivation. Hairy roots characteristically lack geotropism and have a high degree of lateral branching. In addition, hairy root cultures have demonstrated their ability to rapidly produce biomass as well as high contents of secondary metabolites, for example, tropane alkaloids [26, 27]. In Table 2, some pharmaceutical compounds produced by hairy root cultures are presented. Unlike crown gall tumors, hairy roots are capable of spontaneously regenerating into plants [57].

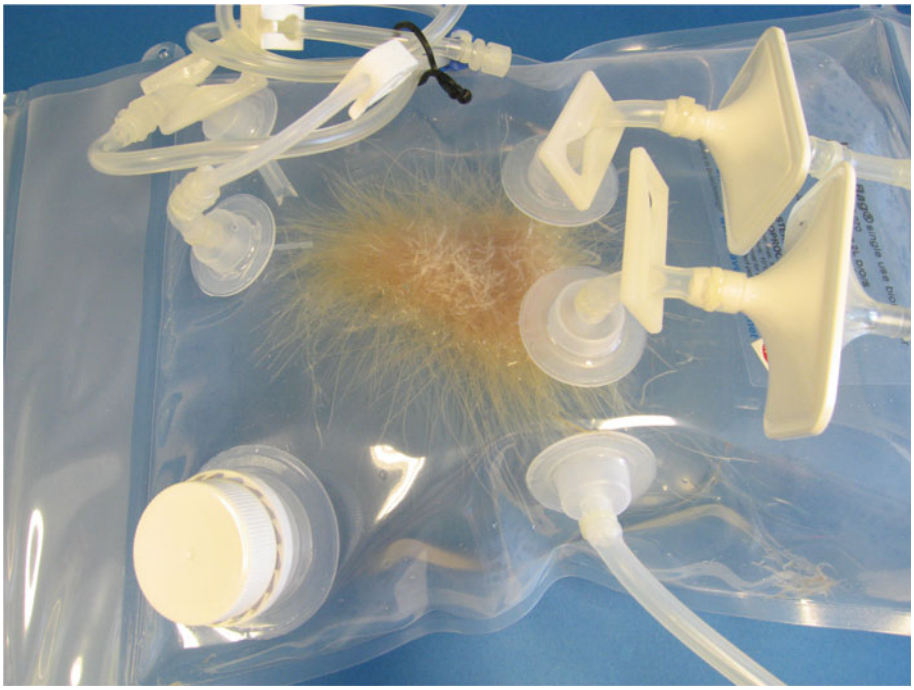
Bioreactors The selection of a suitable bioreactor type for the specific process depends on the desired product and the production material, for example, whether the production involves growing undifferentiated cells, hairy roots, or plantlets. Plants cells are larger in size than those of microbial cells, making them more sensitive to shear forces. For this reason, bioreactors have been designed where conventional mechanical impeller stirring have been replaced by bubble or wave-type agitation. Most widely used bioreactors are stirred tanks [58], but also airlift and bubble column reactors have been used in cultivation of plant cells. The classical production of shikonin is performed in airlift type of bioreactors. A balloon-type bubble bioreactor has been successfully used for the cultivation of, for example, ginseng roots [59].

One of the more recent developments in bioreactor design for plant cell applications has been the use of disposable bioreactors, usually plastic bags. Major advantages in these are that the capital costs are much lower than that of common stainless steel tanks. The production of glucocerobrosidase used for treating the enzyme deficiency cased in Gaucher's disease is performed in carrot cells grown in disposable bioreactors by Israeli company Protalix Biotherapeutics (www.protalix.com). The only secondary metabolite of pharmaceutical value, paclitaxel (Taxol®), is commercially produced in *Taxus* cells by German company Phyton Biotech (www.phytonbiotech.com). Moreover, lower expenses allow multiple parallel units to be employed, and high sterility requirements are met when there is no need for costly cleaning processes between runs. Disposable bioreactors may consist of a rigid cultivation container (tube, plate, flask, cylindrical vessel) or a flexible container (bag) [60]. Issues restricting the use of disposable bioreactors arise from a limited experience in their usage, insufficient strength of a plastic material, limited applicability of advanced automatization, and lack of suitable disposable sensors. Wave-mixed bioreactors [61], such as BioWave®, are well suited for small- to middle-scale processes for the production (Fig. 1) of, for example, plant-based secondary metabolites and therapeutic proteins, as well as cultivation of hairy roots [62, 63]. One of the highest productivities reported to date for paclitaxel production in *Taxus baccata* cell suspension cultures was achieved with immobilized cells cultivated in BioWave® system [64, 65].

Important factors when designing the cultivation of plant cell suspension cultures in bioreactors include

Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Table 2 Examples of metabolites produced by transformed hairy root cultures (adopted mainly from [28, 29])

Metabolite	Species	Activity	Reference
Ajmalicine, ajmaline	*Rauvolfia micrantha*	Antihypertensive	[30]
Artemisinin	*A. annua*	Antimalarial	[31]
Benzylisoquinoline alkaloids	*P. somniferum; E. californica*	Analgesic, antibiotic	[32]
Betalains	*Beta vulgaris*	Antioxidant, colorant	[33]
Camptothecin	*Ophiorrhiza pumila; Camptotheca acuminata*	Antitumor	[34, 35]
Iridoid glycosides	*Harpagophytum procumbens*	Anti-inflammatory, analgesic, and antidiabetic	[36]
3,4-Dihydroxy-L-phenylalanine	*Stizolobium hassjoo*	Therapeutic agent against Parkinson's disease	[37]
Rutin, hispidulin and syringin	*Saussurea involucrata*	Anti-inflammatory, antifungal	[38]
Scopolamine, hyoscyamine and atropine	*A. belladonna*	Anticholinergic	[24, 39]
Scopolamine and hyoscyamine	*Datura innoxia*	Anticholinergic	[40]
Scopolamine and hyoscyamine	*Datura quercifolia*	Anticholinergic	[41]
Scopolamine	*Duboisia leichhardtii*	Anticholinergic	[42]
Scopolamine and hyoscyamine	*Datura candida*	Anticholinergic	[43]
Scopolamine and hyoscyamine	*Datura innoxia*	Anticholinergic	[44]
Scopolamine and hyoscyamine	*H. niger*	Anticholinergic	[40]
Scopolamine and hyoscyamine	*H. muticus*	Anticholinergic	[26]
Scopolamine and hyoscyamine	*H. muticus, Nicotiana tabacum*	Anticholinergic	[45]
Scopolamine	*H. niger*	Anticholinergic	[46]
Solasodine	*Solanum khasianum*	Steroid hormone precursor	[47]
Paclitaxel	*Taxus brevifolia*	Anticancer	[48]
Terpenoid indole alkaloids	*C. roseus*	Antitumor	[49]
Thiarubrine A	*Ambrosia artemisiifolia*	Antifungal, antibacterail, antiviral	[50]
6-Methoxy-podophyllotoxin	*Linum album; Linum persicum*	Anticancer	[51]
Quinine, quinidine	*Cinchona ledgeriana*	Antimalarial	[52]
(+) catechin, (−) epicatechin-3-O-gallate, procyanidin B_2-3′-O-gallate	*Fagopyrun esculentum*	Antioxidant	[53]
Anthraquinone	*Rubia tinctoria*	Antimalarial, antineoplastic	[54]
Thiophene	*Tagetes patula*	Anti-inflammatory precursor	[55]
Valpotriates	*Valeriana officinalis*	Tranquilizing	[56]



Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Figure 1
Wave bioreactor is used to culture various types of plant cells. This is a 2-L disposable bag in a Wave® reactor containing tobacco hairy roots

guaranteed sterility through the whole process and low-shear mixing allowing still efficient nutrient transport without causing sedimentation or a loss in viability of the cells. In addition, the possibility for application of light induction for heterotrophic, photomixotrophic, and photoautotrophic cultures might be relevant [62]. Major physical process parameters regarding cultivation of plant cell and tissue cultures are temperature, viscosity, gas flow rates, and foaming.

Sometimes the lack of end-product formation may be due to the feedback inhibition, degradation of the product in the culture medium, or due to volatility of the substrates or end products. In such cases, adding of extra phase as a site for product accumulation might lead to increased production of the desired substance [66]. For example, addition of amberlite resin and charcoal resulted in increased accumulation of anthraquinones and vanilla, and coniferyl aldehyde, respectively [67–69]. On the other hand, bioconversion of water-insoluble substrates in cell culture systems can be aided by using cyclodextrins. They form inclusion bodies in their cyclodextrin cavity and by this way increase the water solubility of the substrates [70].

Enhancing the Production by Classical Optimization

Selection of High-Producing Lines

Selection of individual plants with desired traits has been a traditional approach in plant breeding. Similarly, high producers have been selected for further use, for example, for cloning and as a starting material for cell cultures. However, cell clones from the same origin may vary considerably in their metabolite production capacities. Selecting high producers is thus a very empirical approach, requiring a huge amount of screening work before good producing individuals are found [71, 72]. In order to obtain good producing cells, mutation strategies or application of various selective agents, such as *p*-fluorophenylalanine [73], 5-methyltryptophan [74], or biotin [75], have been used. Although undifferentiated plant cells can be maintained in an undifferentiated state using

phytohormones, they are not genetically or epigenetically stable. The concept of somaclonal variation was introduced by Larkin and Scowcroft in the beginning of 1980s, standing for the genetic variability in tissue culture–derived plants or cell culture clones [76]. These changes causing the variation can occur as large rearrangements in chromosomal level, for example, changes in chromosome number, karyotype modifications, or changes in gene level.

Somaclonal variation can be exploited when searching for high secondary metabolite producers or high producers of biomass, although a clear disadvantage is that these changes cannot be predicted or controlled and moreover, they are not always stable or heritable. The effect of culture age on growth rates were observed with *Nicotiana plumbaginifolia*, which showed higher growth rates with older cultures compared to newer cultures [77]. These differences were thought to appear as a cause of higher proportion of cells in older cultures exhibiting mutations which elevate cyclin-dependent kinases. Changes in ploidy levels are reported to affect regeneration capacity [78], gene silencing [79], and secondary metabolite production [80, 81]. After choosing good-producing cell lines, cultivation over time requires usually continuous selection in order to maintain high production levels. However, a gradual loss of secondary metabolite productivity over time is an obstacle in the development of commercial plant cell culture production systems [82, 83].

Optimization of Culture Medium

One of the major advantages in using plant cell cultures is the possibility of controlled and contained production systems. When attempting to reach high production levels, key roles are played by the composition of nutrient medium and other cultivation parameters, such as temperature, light, phytohormones, and gas exchange.

Because the plant cell is a production factory, the first requirement for obtaining high levels of products is the generation of high amounts of biomass or at least enough biomass for economic product yields. Plant cell cultures are usually grown heterotrophically using simple sugars as carbon source, sucrose being the most commonly used. Carbon source effects mainly on primary metabolism and by this way affects the overall productivity with either increased or decreased biomass production. Sucrose level may also have an indirect impact on secondary metabolite production, as inverse correlation between sucrose and hyoscyamine production was observed in *Hyoscyamus muticus* hairy root cultures [84]. This was probably due to the increased glycolysis and respiration rate with simultaneous overriding of secondary metabolite production. Sucrose is commonly applied in approximately 3% (w/v) concentration, but levels as high as 8% (w/v) have shown to increase the accumulation of indole and benzophenanthridine alkaloids in cell cultures of *Catharanthus roseus* and *Eschscholtzia californica*, respectively [85, 86].

Phosphate and nitrogen levels are perhaps the most important macronutrient factors effecting the secondary metabolite formation. Phosphate usually promotes cell growth, but often has been accompanied by lower secondary product formation. In fact, very often cell proliferation has been accompanied by decrease in secondary product formation and vice versa. For this reason, a two-stage cultivation system could be considered, where the cells are first cultivated in the medium optimized for cell multiplication and then transferred into medium limiting the biomass growth whereas enabling maximum product formation. As an example, shikonin produced by *Lithospermum erythrorhizon* in commercial scale by this type of two-phase system [87]. Low phosphate levels often have been correlated with high secondary metabolite formation, for example, in case of alkaloids in *Datura stramonium* [88], *Nicotiana tabacum* [89], and *C. roseus* [90]. Nitrogen is an important building block of amino acids, nucleic acids, proteins, and vitamins. Generally, nitrogen is added in the form of nitrate or ammonium, and the ratio of these salts plays an important role in secondary metabolite production of the plant cells. Reducing the levels of nitrogen generally leads to lower biomass production and thus leads to higher secondary metabolite production, as in the case of anthocyanin production by *Vitis vinifera* [91].

Phytohormones have an extensive effect not only on growth of plant cells, but also on differentiation and secondary metabolite production. Both the type and concentration of auxin and cytokinin as well as their ratio alter the growth and metabolite production dramatically in cultured plant cells. High auxin levels are known to inhibit the formation of secondary

metabolites in a large number of cases, for example, tobacco alkaloids [92] with the simultaneous activation of polyamine conjugate biosynthesis [93]. Sometimes, replacement of synthetic auxin 2,4-D (2,4-dichlorophenoxy acetic acid) by NAA (naphthalene acetic acid) or natural auxin IAA (indole acetic acid) has shown to enhance the production of anthraquinones, shikonin, or anthocyanins [94–96].

Commonly understanding of cell culture behavior has been relied on the measurements of culture average parameters, such as cell density and metabolite profiles. However, due to the nature of plant cell division, in which daughter cells often remain attached through cell wall, aggregates of various sizes in cell suspension culture are formed. Thus, each aggregate is exposed to different microenvironmental conditions with respect to nutrient and oxygen availability between inner and outer regions of the aggregate [97]. Understanding such subpopulation dynamics and cellular variability using tools such as flow cytometry is important in order to control the culture as a whole.

Effect of Elicitors

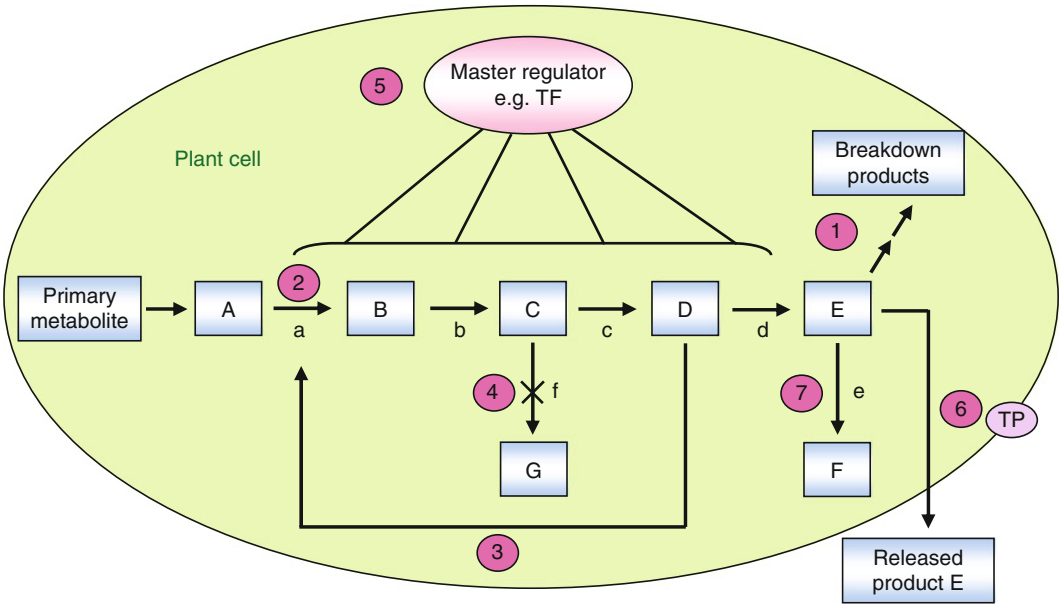
The enhanced production of secondary metabolites from plant cell and tissue cultures through elicitation has opened up a new area of research which could have beneficial influences for pharmaceutical industry. Elicitors are compounds, biotic or abiotic, or even physical factors, which can trigger various defense-related reactions, and thereby induce secondary metabolite formation in plant cells. The mechanisms of how elicitors activate the respective genes and the whole biosynthetic machinery in a plant cell are under active investigation. However, it is evident that the gene expression occurs very quickly after the elicitor contact and many hours before the secondary metabolites are accumulated in a plant cell [98].

In general, elicitors can be categorized based on their molecular structure and origin. Biotic elicitors include compounds such as chitosan, alginate, pectin, chitin or they may contain complex mixtures of compounds like those of fungal or yeast extracts [99]. Abiotic elicitors are chemical compounds of nonbiological origin, for example, heavy metals and vanadate derivatives, or physical factors such as thermal or osmotic stress, UV-irradiation, or wounding. In particular, widely used elicitors for plant cell culture systems are jasmonates and jasmonic acid derivatives, which are naturally occurring hormones involved in the regulation of defence-related genes and act as signaling compounds in these reactions [100]. Application of jasmonates can result in large alterations in desired metabolites in *Catharanthus* [101, 102], in *Taxus* [103], and in *Nicotiana* [98]. Even though plant cells accumulate secondary metabolites typical for species in question independent of the type of elicitor used, the accumulation kinetics may vary greatly with different elicitors. Moreover, elicitors can effect on the release of desired secondary metabolite from the cell to the cultivation medium [104]. This is beneficial when considering the biotechnological production facilitating thus the downstream processing.

Generally, both the elicitor concentration and the length of elicitor application have to be determined for each cell culture individually [104]. Commonly it is thought that the best growth phase for the start of the elicitation is during the exponential growth phase when the enzymatic machinery for elicitor response is most active [105]. In addition, the composition of the culture medium, especially phytohormones, has a major impact on elicitor response. For example, divergent regulation by auxins on the biosynthesis of different metabolites in terpenoid indole alkaloid pathway was observed by *C. roseus* cell cultures [102]. This regulation by auxins was shown to be partly dependent on the presence of methyl jasmonate. Production of various plant-derived medicinal compounds has been successfully induced by using elicitors [106]. Unfortunately, many elicitors also cause a loss of viability of the producing cells, thus a thorough optimization of the whole production process is required when using elicitation.

Metabolic Engineering

Functional genomics tools offer now huge potential to engineer plant metabolic pathways toward the targeted end product or alternatively to form entirely novel structures through combinatorial biochemistry. However, rational engineering of secondary metabolite pathways requires a thorough knowledge of the whole biosynthetic pathway and detailed understanding of the regulatory mechanisms controlling the flux of the pathway (Fig. 2) [7]. Such information is not



Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Figure 2
The hypothetic scheme how the secondary metabolite E could be formed from primary metabolites via different enzymatic steps and how the biosynthesis could be regulated in a plant cell [7]. Engineering of secondary metabolite pathways is a series of complex events. The following strategies could be used to modify the production of hypothetical plant metabolite E: (1) decrease the catabolism of the desired compound, (2) enhance the expression/activity of a rate limiting enzyme, (3) prevent feedback inhibition of a key enzyme, (4) decrease the flux through competitive pathways, (5) enhance expression/activity of all genes involved in the pathway, (6) compartmentalize the desired compound, and (7) convert an existing product into a new product. *TF* transcription factor, *TP* transporter gene

available for vast majority of secondary metabolites, explaining why only limited success has been obtained by metabolic engineering. New genome-wide transcript profiling techniques combined with up-to-date metabolomics allow us now to establish novel gene-to-gene and gene-to-metabolite networks which facilitate the gene discovery also in non-model plants that include most medicinal plants [102]. The ability to switch on entire pathways by ectopic expression of transcription factors suggests new possibilities for engineering secondary metabolite pathways (Fig. 2). Consequently, the utilization of plant cell cultures for biotechnological production of high-value alkaloids would thus become a true viable alternative.

Gene Discovery

Since the sequencing of *Arabidopsis* genome in 2,000 several other plants are being sequenced but still today very limited information exists for any medicinal plant. Therefore, also the biosynthetic pathways in these plants are largely unknown at the gene level. Several approaches have been developed to identify enzymes and the corresponding genes that are responsible for different biosynthetic pathway steps. One of the classical methods is the identification and isolation of intermediates and enzymes *via* precursor feeding [107]. The other very basic approach is to use cDNA libraries to identify genes by PCR amplification with primers designed to recognize conserved regions on the basis of enzyme homology from other plants with already known sequences [108]. More recently, methods based on differential display comparing mRNA transcripts of elicited and non-elicited cell culture samples have shown their potential in gene discovery. Goossens and coworkers [98] and Rischer and coworkers [102] utilized cDNA-AFLP technique for genome-wide gene hunt, whereas [109] supplemented their search with

homology-based analysis of a cDNA library of elicited cells. In addition, the use of random sequencing of elicited cDNA library can lead to identification of clones involved in the biosynthetic route in question as proven in case of *Taxus* biosynthesis [110].

The use of microarrays as widely used for model plants such as *Arabidopsis* is usually not applicable to medicinal plants simply because none has been sequenced with the very recent exception of tobacco http://www.pngg.org/tgi/index.html. The rapid advance of deep sequencing, however, will soon result in many important species being investigated at genome scale. The 454 pyrosequencing technique is currently perhaps the most widely used so-called next-generation sequencing technique for the *de novo* sequencing and analysis of transcriptomes in non-model organisms like medicinal plants are. For example, the GS FLX Titanium can generate one million reads with an average length of 400 bases at 99.5% accuracy. This technology was successfully used to discover putative genes involved in ginsenoside biosynthesis [111].

Once the candidate genes are discovered, they are functionally tested alone or in combination to find out their real mode of action, for example, improving or altering the production of desired metabolite. This is time consuming, and therefore new high-throughput systems have been developed, for example, miniaturized cell culture formats and multigene transformations.

Controlling the Expression of Transgenes

In order to be able to modify the metabolite profile of a respective medicinal plant or cell culture, the gene expression of target proteins and enzymes needs to be fine-tuned in an appropriate manner. For that purpose, the elements involved in transcriptional regulation of gene expression should be well characterized and evaluated to ensure correct spatial and temporal display. This also minimizes the potential adverse effects, and the outcome will be as wanted. Specific DNA sequences upstream of the encoding region of a gene that are recognized by proteins (transcription factors) involved in the initiation of transcription are determined as promoters. It is noteworthy that the promoter sequence itself is present in all tissues and cells, and thus the activity is controlled via transcription factors and their abundance. This opens the possibility to boost a cascade of enzymes and influence in the whole biosynthetic pathway in question by overexpressing transcription factors [112].

Promoters used for the metabolic engineering purposes can be divided into three classes:

1. Constitutive, that is, promoters that are continuously on in most or all of the tissues
2. Organ- or stage-specific, that is, promoters controlling spatiotemporal activity of the transgene
3. Inducible that are regulated by an external trigger of chemical or physical nature [113, 114].

As an example of the constitutive promoters and also the most used one in plant genetic engineering is the *Cauliflower mosaic virus 35S* promoter [115, 116]. The CaMV 35S promoter has been very thoroughly characterized and currently a typical CaMV 35S promoter in plant vectors consists of a bit more than one third of the full-length sequence [117]. It has also been observed that a partial duplication from −343 to −90 amplifies expression up to tenfold [118]. This promoter is also the most used one in metabolic engineering of plant cell cultures [119]. For the secondary metabolite production, the hairy root cultures have shown most potent, and little promise has been found with undifferentiated suspension cultures [120]. Actually there exist no studies for trying to find most suitable callus or suspension culture–specific promoters for efficient expression of target genes. This might be one factor why the success in using undifferentiated plant cell cultures for the production of valuable secondary metabolites has been so poor. However, the main blame for this is the current limited understanding of how the metabolic pathways and fluxes of secondary metabolites work in general.

Nowadays that the multigene transformations [121] are paving the way for more accurate and complex engineering of phenotypes, there is also more need to apply different promoter deployment strategies to reach the wanted goals. The delivery of 10–20 genes at the time is already very demanding, and thus there is no space for failure in running their expression. Roughly, two ways of proceeding can be drawn for promoter choice: utilization of the same promoter to run all the genes or combination of promoters to run different target genes in the generated multigene transformants.

The use of same promoter carries the risk of triggering gene silencing. It is very important to increase the promoter diversity via promoter discovery and generation of synthetic sequences to run the expression. Perhaps one of the most interesting ways is to apply bidirectional sequences which allow simultaneous expression of two genes, and thus halves the number of required promoters for multigene engineering [122].

Targeting the Metabolic Enzymes

From the genetic engineering perspective of medicinal plants, one of the key elements is to express the genes in question in right tissues, and even more importantly target the respective enzymes to correct, specific subcellular compartments. A good example of compartmentalization is the biosynthesis of terpenoids that are synthesized from universal five-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which in turn are formed via two alternate biosynthetic pathways localized in different subcellular compartments. The cytosolic mevalonic acid (MVA) pathway starts with condensation of two molecules of acetyl-CoA into acetoacetyl-CoA and finally gives rise to IPP. The methylerythritol phosphate (MEP) pathway takes place in plastids and leads to the formation of IPP and DMAPP from pyruvate and glyceraldehyde phosphate. The IPP and DMAPP precursors are then processed with prenyl diphosphate synthases in different compartments giving rise to intermediates that serve as substrates to a large group of terpene synthases resulting in construction of the final terpenoids [123, 124]. However, the picture is never black and white, and the subcellular localization studies as well as the genetic engineering experiments have shown that such a thing as a general rule does not apply to all tissues and species. From the rational genetic engineering point of view, this makes things even far more complex and we still need to reveal several aspects of biosynthetic pathways.

Targeting the biosynthetic enzymes to non-original compartment can also lead to interesting results. Precursors can be available in other compartments, and introduction of the respective enzyme can lead to increased accumulation of target compounds. For example, Wu and coworkers [125] showed that redirecting the sesquiterpene pathway from its natural cytosolic location to chloroplasts increased patchoulol accumulation even up to 10,000-fold when compared to native situation. Another example was given by introducing three different targeting modes: cytosolic, plastid, and ER of limonene synthase in transgenic tobacco plants [126]. Both the cytosolic and plastid targeting resulted in limonene formation, whereas ER targeting gave no response probably due to false folding or instability of the protein.

There has also been discussion on so-called metabolic channeling, which means that enzymes from the same pathway, especially the ones committing successive steps, form a protein complex resulting in efficient reactions and regulation of the pathway [127–129]. Aharoni and coworkers [130] interpreted that this might be a cause why some pathways do not seem to proceed even though substantial amount of substrate seem to be available. As a solution, an artificial channeling is suggested with the help of fusion constructs to be applied in the metabolic engineering. These studies also highlight the need for fluxomics and thorough understanding of metabolic pathways (see Sect. "Controlling the Expression of Transgenes").

Multigene Transformation

The first multigene-carrying transgenic plants were created either with several rounds of crossings between transgenic lines or by transforming transgenic plants with a new set of genes [131, 132]. The current multigene delivery systems are co-transformations with either linked or unlinked genes, that is, genes within a same vector or different vectors, respectively. The transfer itself is carried out either via *Agrobacterium*-mediated or direct transformation techniques. These systems have been developed mainly with crop plants, and the target pathways have been on nutritional composition like in engineering of the carotenoid pathway [133, 134]. These pioneer works have opened the possibility to engineer metabolic pathways of medicinal plants, and the potential in these can be seen almost as limitless. The future aim is the creation of a SMART locus (stable multiple arrays of transgenes), that is, a transgenic locus containing multiple genes, thus avoiding segregation in meiosis and possibly also minimizing rearrangements and silencing [121]. For medicinal plants, the possibility to modify entire

metabolic pathways, to introduce completely new pathways, and to study complex metabolic control circuits and regulations are perhaps the main future goals.

New Compounds by Engineered Enzymes/Proteins

In most common approaches, the intention of metabolic engineering is to either overexpress or repress genes leading to the accumulation of certain compounds (Fig. 2). The first successful genetic engineering approach to the medicinal plant was performed already almost 20 years ago. Yun and coworkers [135] introduced the gene-encoding hyoscyamine-6β-hydroxylase (H6H) from *Hyoscyamus niger* to the medicinal plant *A. belladonna*. As a result of the overexpression of *h6h*, the plants produced almost exclusively scopolamine, whereas in the control plants the production of hyoscyamine (precursor of scopolamine) was dominant. Later, the function of the same gene was demonstrated to be different in hairy roots of *Hyoscyamus muticus* [26]. The overexpression of *h6h* caused 100-fold increase in scopolamine production, whereas the hyoscyamine contents were not reduced.

There are also examples where genetic engineering can lead to formation of entirely new metabolites. Classically, this can, for example, be achieved by generating somatic hybrids, that is, by exposing enzymes and regulators derived from different genomes to new environments. A good example is the production of demissidine in somatic *Solanum* hybrids neither parent of which contained this specific metabolite but only a set of different precursors [136].

More recently, the combinatorial biochemistry concept which is based on the fact that enzymes often show relaxed substrate specificity, that is, that they can under certain conditions process substrates which differ from the preferred one is exploited in a stricter sense. Usually, native genes are modified with the aim of creating modified enzymes catalyzing new reactions. Initially, attempts to alter the substrate specificity of plant-derived terpenoid synthases by rather unspecific methods such as mutagenesis or truncation were quite unpredictable [137]. Meanwhile, however, it could be shown that preselection of a mutant strictosidine synthase with a specific point mutation according to substrate acceptance results in quite predictable events. *C. roseus* hairy roots expressing the gene formed unnatural terpenoid indole alkaloids when were fed with derivatized precursors in contrast to the wild type [138].

Future Directions

Different omics in techniques have opened totally new avenues to discover genes, to learn about their functions, for example, transcription, and to finally map the biosynthetic pathways leading to the formation of important secondary metabolites. Metabolomics, which deals with all cellular metabolites, was first defined in microbiology but has also been recognized as an important sector of post-genome plant science [139]. Even in the absence of any visible change in a cell or individual plant, metabolomics, which allows phenotyping by exhaustive metabolic profiling, can show how cells respond as a system. Plant metabolomics is of particular importance because of the huge chemical diversity in plants compared to microorganisms and animals [140]. The number of metabolites from the plant kingdom has been estimated at 200,000 or even more [6], and each plant has its own complex set of metabolites. By integrating transcriptome and metabolome data, one can build networks and get insight on how particular metabolites are formed in plants [102, 140]. This in turn helps us to identify the key genes that could be engineered for the production of improved medicinal plants.

Since cell physiology involves dynamic rather than static processes, the investigation of fluxes is needed to complement phenotyping by metabolomics which only allows inventory, although time-resolved snapshots. However, in contrast to mammalian and microbial cells, flux quantification in plants is much less advanced. This is mainly due to the high degree of subcellular compartmentation and the complexity which arises from intercompartmental transport. Labeling experiments have been very successfully used already in the past for the elucidation of biosynthetic pathways in plants [141], but flux determination has only recently gained pace due to the fast development of analytical and computational technology. Analytical techniques of choice are nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS) [142]. Generally, there are two fundamentally different methods

available facilitating flux measurement – steady-state and dynamic analysis – both of which have certain restrictions and benefits [143]. The latter, that is, kinetic approach is particularly interesting in the sense that it potentially could lead to predictive modeling in regard to secondary metabolism, while steady-state analysis is mainly used to measure carbon flux in well-defined pathways of primary metabolism [144].

In conclusion, modern genomic tools allow for mass gene discovery from plants although many biosynthetic pathways are incompletely resolved and medicinal plants have rarely been sequenced. Nevertheless, predictive metabolic engineering remains a goal of the future. This is because transgene integration in higher plants occurs through illegitimate rather than homologous recombination. DNA integration is random with a preference for gene-rich regions. Gene disruptions, sequence changes, and the production of new proteins constitute common consequences resulting in either predictable or unpredictable effects [145]. In this situation, the power of functional genomics tools allowing the comprehensive investigation of biological systems cannot be overemphasized. Genomics identifies all genes of a plant, while transcriptomics and proteomics provide information about their activities in cells or organs under certain conditions, and finally metabolomics and fluxomics account for the accumulation and kinetics of metabolites, that is, the phenotype. The individual techniques as such are thus invaluable to assign functions, but the real advantage lays in their combination, that is, the systems biology approach [140]. Interestingly at the same time, these tools allow not only the investigation of artificial situations generated by man but also for the first time broad assessment of natural variation.

Acknowledgments

This work has been financially supported by the SmartCell project (nr. 222176) from European Commission Framework 7 programme.

Bibliography

Primary Literature

1. Hostettmann K, Terreaux C (2000) Search for new lead compounds from higher plants. Chimia 54:652–657
2. Müller-Kuhrt L (2003) Putting nature back into drug discovery. Nat Biotechnol 21:602
3. Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. J Nat Prod 70: 461–477
4. Verpoorte R (1998) Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. Drug Discov Today 3:232–238
5. De Luca V, St Pierre B (2000) The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5: 168–173
6. Hartmann T, Kutchan TM, Strack D (2005) Evolution of metabolic diversity. Phytochemistry 66:1198–1199
7. Oksman-Caldentey K-M, Inzé D (2004) Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends Plant Sci 9:433–440
8. Verpoorte R (2000) Secondary metabolism. In: Verpoorte R, Alfermann AW (eds) Metabolic engineering of plant secondary metabolism. Kluwer, Dordrech, pp 1–29
9. Ziegler J, Facchini PJ (2008) Alkaloid biosynthesis – metabolism and trafficking. Annu Rev Plant Biol 59:735–769
10. Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187
11. Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffman NL, Woo SC (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci 80:4803–4807
12. Herrera-Estrella L, Depicker M, van Montagu M, Schell J (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303:209–213
13. Murai N, Sutton DW, Murray MG, Slightom JL, Merlo DJ, Reichert NA, Sengupta-Gopalan C, Stock CA, Barker RF, Kemp JD, Hall TC (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222:476–482
14. Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant J 23:11–28
15. Sheng J, Citovsky V (1996) *Agrobacterium* – plant cell DNA transport: have virulence proteins, will travel. Plant Cell 8:1699–1710
16. Chilton M-D, Tepfer DA, Petit A, David C, Casse-Delbart T, Tempé J (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295:432–434
17. Sahi SV, Chilton M-D, Chilton WS (1990) Corn metabolites affect growth and virulence of *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 87:3879–3883
18. Usami S, Morikawa S, Takebe I, Machida Y (1987) Absence in monocotyledonous plants at the diffusible plant factors inducing T-DNA circularization and *vir* gene expression in *Agrobacterium*. Mol Gen Genet 209:221–226
19. Narasimhulu SB, Deng X, Sarria R, Gelvin SB (1996) Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. Plant Cell 8:873–886

20. Hansen G (2000) Evidence for *Agrobacterium*-induced apoptosis in maize cells. Mol Plant Microbe Interact 13: 649–657
21. Nadolska-Orczyk A, Orczyk W, Przetakiewicz A (2000) *Agrobacterium* -mediated transformation of cereals – from technique development to its application. Acta Physiol Plant 22:77–88
22. Sevón N, Oksman-Caldentey K-M (2002) *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. Planta Med 68:859–868
23. Palazón J, Cusidó RM, Roig C, Piñol MT (1998) Expression of the *rol*C gene and nicotine production in transgenic roots and their regenerated plants. Plant Cell Rep 17:384–390
24. Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux M-A, Jacquin-Dubreuil A (2000) Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes* 15834 and *Agrobacterium tumefaciens* containing *rol A*, *B*, *C* genes only. J Biotechnol 81:151–158
25. Chriqui D, Guivarch A, Dewitte W, Prinsen E, van Onkelen H (1996) *Rol* genes and root initiation and development. Plant Soil 187:47–55
26. Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri T, Oksman-Caldentey K-M (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551
27. Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey K-M, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun K, Tang K (2004) Engineering tropane alkaloid pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci USA 101:6786–6791. doi:6786
28. Georgiev MI, Pavlov AI, Bley T (2007) Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol 74:1175–1185
29. Srivastava S, Srivastava AK (2007) Hairy root culture for mass-production of high-value secondary metabolites. Crit Rev Biotechnol 27:29–43
30. Sudha CG, Obul RB, Ravishankar GA, Seeni S (2003) Production of ajmalicine and ajmaline in hairy root cultures of *Rauvolfia micrantha* Hook F., a rare and endemic medicinal plant. Biotechnol Lett 25:631–636
31. Weathers P, Bunk G, McCoy MC (2005) The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. In Vitro Cell Dev B 41:47–53
32. Park S-U, Facchini P (2000) *Agrobacterium rhizogenes*-mediated transformation of opium poppy, *Papaver somniferum* L., and California poppy, *Eschscholzia californica* Cham., root cultures. J Exp Bot 347:1005–1006
33. Pavlov A, Bley T (2006) Betalains biosynthesis by *Beta vulgaris* L. hairy root culture in different bioreactor systems. Process Biochem 41:848–852
34. Saito K, Sudo H, Yamazaki M, Koseki-Nakamura M, Kitajima M, Takayama H, Aimi N (2001) Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila*. Plant Cell Rep 20:267–271
35. Lorence A, Medina-Bolivar F, Nessler CL (2004) Camptothecin and 10-hydroxycamptothecin from *Camptotheca acuminata* hairy roots. Plant Cell Rep 22:437–441
36. Georgiev M, Heinrich M, Kerns G, Pavlov A, Bley T (2006) Production of iridoids and phenolics by transformed *Harpagophytum procumbens* root cultures. Eng Life Sci 6:593–596
37. Sung H, Huang S-Y (2006) Medium optimization of transformed root cultures of *Stizolobium hassjoo* producing L-DOPA with response surface methodology. Biotechnol Bioeng 94:441–447
38. Fu C-X, Xu Y-J, Zhao D-X, Ma FS (2006) A comparison between hairy root cultures and wild plants of *Saussurea involucrata* in phenylpropanoids production. Plant Cell Rep 24:750–754
39. Jung G, Tepfer D (1987) Use of genetic transformation by the Ri T-DNA of *Agrobacterium rhizogenes* to stimulate biomass and tropane alkaloid production in *Atropa belladonna* and *Calystegia sepium* roots grown *in vitro*. J Ferment Bioeng 85:454–457
40. Shimomura K, Sauerwein M, Ishimaru K (1991) Tropane alkaloids in the adventitious and hairy root cultures of *Solanaceous* plants. Phytochemistry 30:2275–2278
41. Dupraz JM, Christen P, Kapetanidis I (1994) Tropane alkaloids in transformed roots of *Datura quercifolia*. Planta Med 60: 158–162
42. Mano Y, Ohkawa H, Yamada Y (1989) Production of tropane alkaloids by hairy root cultures of *Duboisia Leichhardtii* transformed by *Agrobacterium rhizogenes*. Plant Sci 59: 191–201
43. Christen P, Robert MF, Phillipson JD, Evans WC (1991) Alkaloids of hairy root cultures of a *Datura candida* hybrid. Plant Cell Rep 9:101–104
44. Dechaux C, Boitel-Conti M (2005) A Strategy for overaccumulation of scopolamine in *Datura innoxia* hairy root cultures. Acta Biol Cracov Bot 47:101–107
45. Häkkinen ST, Moyano E, Cusidó RM, Palazón J, Piñol MT, Oksman-Caldentey K-M (2005) Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous hyoscyamine-6beta-hydroxylase. J Exp Bot 420: 2611–2618
46. Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey K-M, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci 1117 USA 101:6786–6791
47. Jacob A, Malpathak N (2004) Green hairy root cultures of *Solanum khasianum* Clarke – a new route to *in vitro* solasodine production. Curr Sci 87:1442–1447
48. Huang Z, Mu Y, Zhou Y, Chen W, Xu K, Yu Z, Bian Y, Yang Q (1997) Transformation of *Taxus brevifolia* by *Agrobacterium rhizogenes* and taxol production in hairy root culture. Acta Bot Yunnanica 19:292–296
49. Palazón J, Cusidó RM, Gonzalo J, Bonfill M, Morales C, Piñol MT (1998) Relation between the amount of rolC gene product and indole alkaloid accumulation in *Catharanthus roseus* transformed root cultures. J Plant Physiol 153:712–718

50. Bhagwath SG, Hjortso MA (2000) Statistical analysis of elicitation strategies for thiarubrine. A production in hairy root cultures of *Ambrosia artemisiifolia*. J Biotechnol 80: 159–167
51. Wink M, Alfermann AW, Franke R, Wetterauer B, Distl M, Windhoevel J, Krohn O, Fuss E, Garden H, Mohagheghzadeh A, Wildi EJ, Ripplinger P (2005) Sustainable bioproduction of phytochemicals by plant *in vitro* cultures: anticancer agents. Plant Gene Res 3:90–100
52. Hamill JD, Robins RJ, Rhodes MJC (1989) Alkaloid production by transformed root cultures of *Cinchona ledgeriana*. Planta Med 55:354–357
53. Trotin F, Moumou Y, Vasseur J (1993) Flavanol production by *Fagopyrum esculentum* hairy and normal root cultures. Phytochemistry 32:929–931
54. Sato K, Yamazaki T, Okuyama E, Yoshihira K & Shimomura K (1991) Anthraquinones production by transformed root cultures of *Rubia tinctorum*: Influence of phytohormones and sucrose concentration. Phytochemistry 30:1507–1509
55. Croes AF, Vander Berg AJR, Bosveld M, Breteler H, Wullems GJ (1989) Thiophene accumulation in relation to morphology in roots of *Tagetes patula*. Effects of auxin and transformation by *Agrobacterium*. Planta Med 179:43–50
56. Granicher F, Christen P, Kapetandis I (1992) High-yield production of valepotriates by hairy root cultures of *Valeriana officnalis* L. var. *sambucifolia* Mikan. Plant Cell Rep 11:339–342
57. Oksman-Caldentey K-M, Kivelä O, Hiltunen R (1991) Spontaneous shoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. Plant Sci 78:129–136
58. Su WW (2006) Bioreactor engineering for recombinant protein production using plant cell suspension culture. In: Gupta SD, Ibaraki Y (eds) Plant tissue culture engineering. Springer, The Netherlands, pp 135–159
59. Choi YE, Kim YS, Paek KY (2006) Types and designs of bioreactors for hairy root culture. In: Gupta SD, Ibaraki Y (eds) Plant tissue culture engineering. Springer, The Netherlands, pp 161–172
60. Eibl R, Kaiser S, Lombriser R, Eibl D (2010) Disposable bioreactors: the current state-of-art and recommended applications in biotechnology. Appl Microbiol Biotechnol 86:41–49
61. Eibl R, Werner S, Eibl D (2009) Bag bioreactor based on wave-induced motion: characteristics and applications. Adv Biochem Eng Biotechnol 115:55–87
62. Eibl R, Eibl D (2002) Bioreactors for plant cell and tissue cultures. In: Oksman-Caldentey K-M, Barz WH (eds) Plant biotechnology and transgenic plants. Marcel Dekker, Basel, pp 163–199
63. Palazón J, Mallol A, Lettenbauer C, Cusidó RM, Piñol MT (2003) Growth and gingenoside production in hairy root cultures of *Panax ginseng* using a novel bioreactor. Planta Med 69:344–349
64. Bentebibel S, Moyano E, Palazón J, Cusidó RM, Bonfill M, Eibl R, Piñol MT (2005) Effects of immobilization by entrapment in alginate and scale-up on paclitaxel and baccatin III production in cell suspension cultures of *Taxus baccata*. Biotechnol Bioeng 89:647–655
65. Bonfill M, Bentebibel S, Moyano E, Palazón J, Cusidó RM, Piñol MT (2008) Paclitaxel and baccatin III production induced by methyl jasmonate in free and immobilized cells of *Taxus baccata*. Biol Plant 51:647–652
66. Ramachandra Rao S, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153
67. Robins RJ, Rhodes MJC (1986) The stimulation of anthraquinone production by *Cinchona ledgeriana* cultures with polymeric adsorbents. Appl Microbiol Biotechnol 24:35–41
68. Knuth ME, Sahai OP (1991) Flavour composition and method. US Patent 5,068,184, 26 Nov 1991
69. Beiderbeck R, Knoop B (1987) Two-phase culture. In: Constael F, Vasil I (eds) Cell culture and somatic cell genetics of plants, vol 5. Academic, San Diego, pp 255–266
70. Van Uden W, Woedenbag HJ, Pras N (1994) Cyclodextrins as a useful tool for bioconversion in plant cell biotechnology. Plant Cell Tiss Org 38:103–113
71. Oksman-Caldentey K-M, Vuorela H, Strauss A, Hiltunen R (1987) Variation in the tropane alkaloid content of *Hyoscyamus muticus* plants and cell culture clones. Planta Med 53:349–354
72. Mano Y, Ohkawa H, Yamada Y (1989) Production of tropane alkaloids by hairy root cultures of *Duboisia leichhardtii* transformed by *Agrobacterium rhizogenes*. Plant Sci 59:191–201
73. Berlin J (1980) Para-fluorophenylalanine resistant cell lines of tobacco. Z Pflanzenphysiol 97:317–324
74. Widholm JM (1974) Evidence for compartmentation of tryptophan in cultured plant tissues. Free tryptophan levels and inhibition of anthranilate synthetase. Physiol Plant 30:323–326
75. Wataneba K, Yano SI, Yamada Y (1982) Selection of cultured plant cell lines producing high levels of biotin. Phytochemicals 21:513–516
76. Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214
77. Zhang KR, John PCL (2005) Raised level of cyclin dependent kinase A after prolonged suspension culture of *Nicotiana plumbaginifolia* is associated with more rapid growth and division, diminished cytoskeleton and lost capacity for regeneration: implications for instability of cultures plant cells. Plant Cell Tissue Organ Cult 82:295–308
78. Shiba T, Mii M (2005) Visual selection and maintenance of the cell lines with high plant regeneration ability and low ploidy level in *Dianthus acicularis* by monitoring with flow cytometry analysis. Plant Cell Rep 24:572–580
79. Pikaard CS (2001) Genomic change and gene silencing in polyploids. Trends Genet 17:675–677
80. Hirasuna TJ, Pestchanker LJ, Srinivasan V, Shuler ML (1996) Taxol production in suspension cultures of *Taxus baccata*. Plant Cell Tiss Org 44:95–102
81. Wallaart TE, Pras N, Quax WJ (1999) Seasonal variations of artemisinin and its biosynthetic precursors in tetraploid

Artemisia annua plants compared with the diploid wild-type. Planta Med 65:723–728
82. Deus-Neumann B, Zenk MH (1984) Instability of indole alkaloid production in *Catharanthus roseus* cell suspension-cultures. Planta Med 50:427–431
83. Qu JG, Zhang W, Yu XJ, Jin MF (2005) Instability of anthocyanin accumulation in *Vitis vinifera* L. var. Gamay Freaux suspension cultures. Biotechnol Bioprocess Eng 10:155–161
84. Wilhelmson A, Häkkinen ST, Kallio P, Oksman-Caldentey K-M, Nuutila AM (2006) Heterologous expression of *Vitreoscilla* hemoglobin (VHb) and cultivation conditions affect the alkaloid profile of *Hyoscyamus muticus* hairy roots. Biotechnol Prog 22:350–358
85. Knobloch KH, Berlin J (1980) Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* L. G. Don. Z Naturforsch 35C:551–556
86. Berlin J, Forche E, Wray V, Hammer J, Hosel W (1983) Formation of benzophenanthridine alkaloids by suspension cultures of *Eschscholtzia californica*. Z Naturforsch 38:346–352
87. Fujita Y, Tabata M, Nishi A, Yamada Y (1982) New medium and production of secondary compounds with two-staged culture medium. In: Fujiwara A (ed) Plant tissue culture. Maruzen, Tokyo, pp 399–400
88. Payne J, Hamill JD, Robins RJ, Rhodes MJC (1987) Production of hyoscyamine by "hairy root" cultures of *Datura stramonium*. Planta Med 53:474–478
89. Mantell SH, Pearson DW, Hazell LP, Smith H (1983) The effect of initial phosphate and sucrose levels on nicotine accumulation in batch suspension cultures of *Nicotiana tabacum* L. Plant Cell Rep 1:73–77
90. Toivonen L, Ojala M, Kauppinen V (1991) Studies on the optimization of growth and indole alkalooid production by hairy root cultures of *Catharanthus roseus*. Biotechnol Bioeng 37:673–680
91. Do CB, Cormier F (1991) Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (*Vitis vinifera* L.) cell suspension. Plant Cell Rep 9:500–504
92. Ishikawa A, Yoshihara T, Nakamura K (1994) Jasmonate-inducible expression of a potato cathepsin D inhibitor-GUS gene fusion in tobacco cells. Plant Mol Biol 26:403–414
93. Tiburcio AF, Kaur-Sawhney R, Ingersoll R, Galston AW (1985) Correlation between polyamines and pyrrolidine in developing tobacco callus. Plant Physiol 78:323–326
94. Zenk MH, El-Shagi, E, Schulte U (1975) Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. Planta Med 28:79–101
95. Tabata M (1988) Naphtoquinones. In: Constael F, Vasil I (eds) Cell culture and somatic cell genetics of plants, vol 5. Academic, San Diego, pp 99–111
96. Rajendran L, Ravishankar GA, Venkataraman LV, Prathiba KR (1992) Anthocyanin production in callus cultures of *Daucus carota* L. as influenced by nutrient stress and osmoticum. Biotechnol Lett 14:707–714
97. Kolewe ME, Gaurav V, Roberst SC (2008) Pharmaceutically active natural product synthesis and supply via plant cell culture technology. Mol Pharm 5:243–256
98. Goossens A, Häkkinen ST, Laakso I, Seppänen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Söderlund H, Zabeau M, Inzé D, Oksman-Caldentey K-M (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci USA 100:8595–8600
99. Vasconsuelo AA, Boland R (2007) Molecular aspects of the early stages of elicitation of secondary metabolites in plants. Plant Sci 172:861–875
100. Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico JM, Bossche RV, Sewell J, Gil E, García-Casado G, Witters E, Inzé D, Long JA, De Jaeger G, Solano R, Goossens A (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464:788–791
101. Lee-Parsons CW, Royce AJ (2006) Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures. Plant Cell Rep 25:607–612
102. Rischer H, Orešič M, Seppänen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MCE, Inzé D, Oksman-Caldentey K-M, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci 103:5614–5619
103. Yukimune Y, Tabata H, Higashi Y, Hara Y (1996) Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. Nat Biotechnol 14:1129–1132
104. Sevón N, Hiltunen R, Oksman-Caldentey K-M (1992) Chitosan increases hyoscyamine content in hairy root cultures of *Hyoscyamus muticus*. Pharm Pharmacol Lett 2:96–99
105. Vasconsuelo AA, Giuletti AM, Picotto G, Rodriguez-Talou J, Boland R (2003) Involvement of the PLC/PKC pathway in chitosan-induced anthraquinone production by *Rubia tinctorium* L. cell cultures. Plant Sci 165:429–436
106. Namdeo AG (2010) Plant cell elicitation for production of secondary metabolites: a review. Pharmacogn Rev 1:69–79
107. Bringmann G, Wohlfarth M, Rischer H, Grüne M, Schlauer J (2000) A new biosynthetic pathway to alkaloids in plants: acetogenic isoquinolines. Angew Chem Int Ed 39:1464–1466
108. Wildung MR, Croteau R (1996) A cDNA clone for taxadiene synthase, the diterpene synthase, the diterpene cyclise that catalyzes the committed step of taxol biosynthesis. J Biol Chem 271:9201–9204
109. Kaspera R, Croteau R (2006) Cytochrome P450 oxygenases of taxol biosynthesis. Phytochem Rev 5:433–444
110. Jennewein S, Wildung MR, Chau M, Walker K, Croteau R (2004) Random sequencing of an induced *Taxus* cell cDNA library for identification of clones involved in taxol biosynthesis. Proc Natl Acad Sci USA 101:9149–9154
111. Sun C, Li Y, Wu Q, Luo H, Sun Y, Song J, Lui EMK, Chen S (2010) *De novo* sequencing and analysis of the American ginseng root transcriptome using a GS FLX titanium platform to

discover putative genes involved in ginsenoside biosynthesis. BMC Genomics 11:262–273
112. Memelink J, Verpoorte R, Kijne JW (2001) ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6:212–219
113. Potenza C, Aleman L, Sengupta-Gopalan C (2004) Targeting transgene expression in research, agricultural, and environmental applications:promoters used in plant transformation. In Vitro Cell Dev B 40:1–22
114. Yoshida K, Shinmyo A (2000) Transgene expression systems in plant, a natural bioreactor. J Biosci Bioeng 90:353–362
115. Guilley H, Dudley RK, Jonard G, Balázs E, Richards KE (1982) Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization of transcripts. Cell 30:763–773
116. Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812
117. Fang RX, Nagy F, Sivasubramanian S, Chua NH (1989) Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1:141–150
118. Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299–1302
119. Zárate R, Verpoorte R (2007) Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem Rev 6:475–491
120. Weathers PJ, Towler MJ, Xu J (2010) Bench to batch: advances in plant cell culture for producing useful products. Appl Microbiol Biotechnol 85:1339–1351
121. Naqvi S, Farré G, Sanahuja G, Capell T, Zhu C, Christou P (2010) When more is better: multigene engineering in plants. Trends Plant Sci 15:48–56
122. Peremarti A, Twyman RM, Gómes-Galera S, Naqvi S, Farré G, Sabalza M, Miralpeix B, Dashevskaya S, Yuan D, Ramessar K, Christou P, Zhu C, Bassie L, Capell T (2010) Promoter diversity in multigene transformation. Plant Mol Biol 73:363–378
123. Dudareva N, Negre F, Nagegowda DA, Orlova I (2006) Plant volatiles: recent advances and future prospects. Crit Rev Plant Sci 25:417–440
124. Nagegowda DA (2010) Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. FEBS Lett 584:2965–2973
125. Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nat Biotechnol 24:1441–1447
126. Ohara K, Ujihara T, Endo T, Sato F, Yazaki K (2003) Limonene production in tobacco with *Perilla* limonene synthase cDNA. J Exp Bot 54:2635–2642
127. Chappell J, Wolf F, Proulx J, Cuella R, Saunders C (1995) Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants. Plant Physiol 109:1337–1343
128. Winkel BSJ (2004) Metabolic channeling in plants. Annu Rev Plant Biol 55:85–107
129. Kristensen C, Morant M, Olsen CE, Ekstrøm CT, Galbraith DW, Lindberg Møller B, Bak S (2005) Metabolic engineering of dhurrin in transgenic *Arabidopsis* plants with marginal inadvertent effects on the metabolome and transcriptome. Proc Natl Acad Sci USA 102:1779–1784
130. Aharoni A, Jongsma MA, Bouwmeester HJ (2005) Volatile science? Metabolic engineering of terpenoids in plants. Trends Plant Sci 10:594–602
131. Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K, Lehner T (1995) Generation and assembly of secretory antibodies in plants. Science 268:716–719
132. Jobling SA, Westcott RJ, Tayal A, Jeffcoat R, Schwall GP (2002) Production of a freeze-thaw-stable potato starch by antisense inhibition of three starch synthase genes. Nat Biotechnol 20:295–299
133. Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc Natl Acad Sci USA 105:18232–18237
134. Fujisawa M, Takita E, Harada H, Sakurai N, Suzuki H, Ohyama K, Shibata D, Misawa N (2009) Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. J Exp Bot 60:1319–1332
135. Yun D-J, Hashimoto T, Yamada Y (1992) Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc Natl Acad Sci USA 89:11799–11803
136. Laurila J, Laakso I, Valkonen JPT, Hiltunen R, Pehu E (1996) Formation of parental-type and novel glycoalkaloids in somatic hybrids between *Solanum brevidens* and *S. tuberosum*. Plant Sci 118:145–155
137. Little DB, Croteau RB (2002) Alteration of product formation by directed mutagenesis and truncation of the multiple-product sesquiterpene synthases δ-selinene synthase and γ-humulene synthase. Arch Biochem Biophys 402:120–135
138. Runguphan W, O'Connor SE (2009) Metabolic reprogramming of periwinkle plant culture. Nat Chem Biol 5:151–153
139. Trethewey RN, Krozky AJ, Willmitzer L (1999) Metabolic profiling: a Rosetta stone for genomics? Curr Opin Plant Biol 2:83–85
140. Oksman-Caldentey K-M, Saito K (2005) Integrating genomics and metabolomics for engineering plant metabolic pathways. Curr Opin Biotechnol 16:174–179
141. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365–369
142. Ratcliffe RG, Shachar-Hill Y (2005) Revealing metabolic phenotypes in plants: inputs from NMR analysis. Biol Rev 80:27–43
143. Kruger NJ, Ratcliffe RG (2007) Dynamic metabolic networks: going with the flow. Phytochemistry 68:2136–2138

144. Kruger NJ, Huddleston JE, Le Lay P, Brown ND, Ratcliffe RG (2007) Network flux analysis: impact of 13C-substrates on metabolism in *Arabidopsis thaliana* cell suspension cultures. Phytochemistry 68:2176–2188
145. Rischer H, Oksman-Caldentey K-M (2006) Unintended effects in genetically modified crops: revealed by metabolomics? Trends Biotechnol 24:102–104

Books and Reviews

Allen DK, Libourel IG, Shachar-Hill Y (2009) Metabolic flux analysis in plants: coping with complexity. Plant Cell Environ 32:1241–1257
Bhagwath SG, Hjortso MA (2000) J Biotechnol 80:159–167
Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux M, Jacquin-Dubreuil A (2000) J Biotechnol 81:151–158
Buchanan BB, Gruissem W, Russell LJ (eds) (2000) Biochemistry & molecular biology of plants. American Society of Plant Physiologists, Rockville, p 1367
Christen P, Robert MF, Phillipson JD, Evans WC (1991) Plant Cell Rep 9:101–104
Croes AF, Vander Berg AJR, Bosveld M, Breteler H, Wullems GJ (1989) Planta Med 179:43–50
Dechaux C, Boitel-Conti M (2005) Acta Biol Cracov Bot 47:101–107
Du H, Huang Y, Tang Y (2010) Genetic and metabolic engineering of isoflavonoid biosynthesis. Appl Microbiol Biotechnol 86:1293–1312
Dudareva N, Pichersky E (2008) Metabolic engineering of plant volatiles. Curr Opin Biotechnol 19:181–189
Dupraz JM, Christen P, Kapetanidis I (1994) Planta Med 60:158–162
Fu C-X, Xu Y-J, Zhao D-X, Ma FS (2006) Plant Cell Rep 24:750–754
Georgiev M, Heinrich M, Kerns G, Pavlov A, Bley T (2006) Eng Life Sci 6:593–596
Georgiev MI, Pavlov AI, Bley T (2007) Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol 74:1175–1185
Granicher F, Christen P, Kapetandis I (1992) Plant Cell Rep 11:339–342
Häkkinen ST, Moyano E, Cusidó RM, Palazón J, Piñol MT, Oksman-Caldentey K-M (2005) J Exp Bot 420:2611–2618
Häkkinen ST, Oksman-Caldentey K-M (2004) Regulation of secondary metabolism in tobacco cell cultures. In: Nagata T, Hasezawa S, Inzé D (eds) Biotechnology in agriculture and forestry, vol 53, Tobacco BY-2 cells. Springer, Berlin/Heidelberg, pp 231–249
Hamill JD, Robins RJ, Rhodes MJC (1989) Planta Med 55:354–357
Huang Z, Mu Y, Zhou Y, Chen W, Xu K, Yu Z, Bian Y, Yang Q (1997) Acta Bot Yunnanica 19:292–296
Jacob A, Malpathak N (2004) Curr Sci 87:1442–1447
Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Oksman-Caldentey K-M (1999) Enhancement of scopolamine production in *Hyscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551
Jung G, Tepfer D (1987) J Ferment Bioeng 85:454–457
Lorence A, Medina-Bolivar F, Nessler CL (2004) Plant Cell Rep 22:437–441
Mano Y, Ohkawa H, Yamada Y (1989) Plant Sci 59:191–201
Nascimiento NC, Fett-Neto AG (2010) Plant secondary metabolism and challenges in modifying its operation: an overview. Meth Mol Biol 643:1–13
Oksman-Caldentey K-M, Barz W (eds) (2002) Plant biotechnology and transgenic plants. Marcel and Dekker, New York, p 719
Oksman-Caldentey K-M, Inzé D (2004) Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends Plant Sci 9:433–440
Oksman-Caldentey K-M, Inzé D, Orešič M (2004) Connecting genes to metabolites by a systems biology approach. Proc Natl Acad Sci USA 101:9949–9950
Palazón J, Cusidó RM, Gonzalo J, Bonfill M, Morales C, Piñol MT (1998) J Plant Physiol 153:712–718
Park S-U, Facchini P (2000) J Exp Bot 347:1005–1006
Pavlov A, Bley T (2006) Process Biochem 41:848–852
Rischer H, Oksman-Caldentey K-M (2005) Biotechnological utilization of plant genetic resources for the production of phytopharmaceuticals. Plant Gen Resour 3:83–89
Saito K, Dixon RD, Willmitzer L (2006) Plant metabolomics. In: Nagata T, Lörz H, Widholm JM (eds) Biotechnology in agriculture and forestry, vol 57. Springer, Berlin/Heidelberg, p 347
Saito K, Sudo H, Yamazaki M, Koseki-Nakamura M, Kitajima M, Takayama H, Aimi N (2001) Plant Cell Rep 20:267–271
Saito K, Yamazaki T, Okuyama E, Yoshihira K, Shimomura K (1991) Phytochemistry 30:2977–2980
Samuelsson G (2004) Drugs of natural origin. A textbook of pharmacognocy, 5th edn. Swedish Pharmaceutical Press, Stockholm, pp 473–575
Schäfer H, Wink M (2009) Medicinally important secondary metabolites in recombinant microorganisms or plants: progress in alkaloid biosynthesis. Biotechnol J 4:1684–1703
Sevón N, Oksman-Caldentey K-M (2002) *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. Planta Med 68:859–868
Shimomura K, Sauerwein M, Ishimaru K (1991) Phytochemistry 30:2275–2278
Srivastava S, Srivastava AK (2007) Hairy root culture for mass-production of high-value secondary metabolites. Crit Rev Biotechnol 27:29–43
Sudha CG, Obul RB, Ravishankar GA, Seeni S (2003) Biotechnol Lett 25:631–636
Sung H, Huang S-Y (2006) Biotechnol Bioeng 94:441–447
Trotin F, Moumou Y, Vasseur J (1993) Phytochemistry 32:929–931
Verpoorte R, Alfermann AW (eds) (2000) Metabolic engineering of plant secondary metabolism. Academic, Dordrecht, p 286
Verpoorte R, Alfermann AW, Johnson TS (eds) (2007) Applications of plant metabolic engineering. Springer, Dordrecht, p 332
Weathers P, Bunk G, McCoy MC (2005) In Vitro Cell Dev B 41:47–53
Wink M, Alfermann AW, Franke R, Wetterauer B, Distl M, Windhoevel J, Krohn O, Fuss E, Garden H, Mohagheghzadeh A, Wildi EJ, Ripplinger P (2005) Plant Gene Res 3:90–100
Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey K-M, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Proc Natl Acad Sci USA 101:6786–6791

Molecular Breeding Platforms in World Agriculture

Jean-Marcel Ribaut, Xavier Delannay, Graham McLaren, Frederick Okono
Integrated Breeding Platform, Consultative Group on International Agricultural Research, Generation Challenge Programme, c/o CIMMYT, Texcoco, Edo. Mexico

Article Outline

Glossary

Analytical pipeline A sequence of data management and statistical analysis algorithms which can be applied to one or more data sets to produce a result which can be interpreted and applied in decision making.

Capacity building Assistance that is provided to entities, usually institutions in developing countries, which have a need to develop a certain skill or competence, or for general upgrading of capability.

Cyberinfrastructure (CI) Computer-based research environments that support advanced data acquisition, data storage, data management, data integration, data mining, data visualization, and other computing and information processing services over the Internet. In scientific usage, CI is a technological solution to the problem of efficiently connecting data, computers, and people with the goal of enabling derivation of novel scientific theories and knowledge.

Gene Segment of DNA specifying a unit of genetic information; an ordered sequence of nucleotide base pairs that produce a certain product that has a specific function.

Information system (IS) An integrated set of computing components and human activities for collecting, storing, processing, and communicating information.

Integrated breeding platform (IBP) Term to describe a Molecular Breeding Platform (see below) in a broader sense including the availability of tools and services suitable for conventional breeding based on phenotypic selection only.

Molecular breeding (MB) Identification, evaluation, and stacking of useful alleles for agronomic traits of importance using molecular markers (MMs) in breeding programs. MB encompasses several modern breeding strategies, such as marker-assisted selection (MAS), marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), and genome-wide selection (GWS).

Molecular breeding platform (MBP) A term that has come to indicate a virtual platform driven by modern information and communication technologies through which MB programs can access genomic resources, advanced laboratory services, and analytical and data management tools to accelerate variety development using marker technologies.

Plant breeding The science of improving the genetic makeup of plants in order to increase their value. Increased crop yield is the primary aim of most plant breeding programs; benefits of the hybrids and new varieties developed include adaptation to new agricultural areas, greater resistance to disease and insects, greater yield of useful parts, better nutritional content of edible parts, and greater physiological efficiency especially under abiotic stress conditions.

Quantitative trait locus (QTL) A region of the genome that contains genes affecting a quantitative trait. Though not necessarily genes themselves, QTLs are stretches of DNA that are closely linked to the genes that underlie the corresponding trait.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Definition of the Subject

In the last decade, private seed companies have benefitted immensely from molecular breeding (MB) [1]. A private sector-led "gene revolution" has boosted crop adaptation and productivity in developed countries, by applying and combining the latest advances in molecular biology with cutting-edge information and communication technologies combined with accurate plant phenotyping.

MB allows the stacking of favorable alleles, or genomic regions, for target traits in a desired genetic background thanks to the use of polymorphic molecular markers (MMs) that monitor differences in genomic composition among cultivars, or genotypes, at specific genomic regions, or genes, involved in the expression of those target traits. The use of MMs generally increases the genetic gain per crop cycle compared to selection based on plant phenotyping only, and therefore reduces the number of needed selection cycles, hastening the delivery of improved crop varieties to the farmers.

In contrast to the private sector, MB adoption is still limited in the public sector, and is hardly used at all in developing countries. This is the result of several factors, among which are the following: (1) scientists from the academic world are more interested in discovering new genes or QTLs to be published than in applied biology; (2) until recently access to genomic resources was limited in the public sector, especially for less-studied crops; (3) public access to large-scale genotyping facilities was not easily available; and (4) although a broad set of stand-alone tools are available to conduct the multiple types of analyses necessitated by MB, no single analytical pipeline is available today in the public sector allowing integrated analysis in a user-friendly mode.

The situation is even more critical in developing countries as additional limitations include shortage of well-trained personnel, inadequate laboratory and field infrastructure, lack of ISs with applicable and flexible analysis tools, as well as inappropriate funding – simply put, resource-limited breeding programs. As a result, the developing world has yet to benefit from the MB revolution, and most of the countries indeed lack the fundamental prerequisites for a move to informatics powered breeding.

Under those circumstances, developing and deploying a sustainable web-based Molecular Breeding Platform (MBP) as a one-stop shop for information, analytical tools, and related services to help design and conduct marker-assisted breeding experiments in the most efficient way will alleviate many of the bottlenecks mentioned earlier. Such a platform will enable breeding programs in the public and private sectors in developing countries to accelerate variety development using marker technologies for different breeding purposes: major genes or transgene introgression via marker-assisted backcrossing (MABC), gene pyramiding via marker-assisted selection (MAS), marker-assisted recurrent selection (MARS) and, in a not too distant future, genome-wide selection (GWS).

Introduction

Since the dawn of agriculture, mankind has sought to improve crops by selecting individual plants with the most desirable characteristics or traits. Agricultural productivity has been progressively enhanced by constant innovation, including improved crop varieties to increase production in specific environments [2]. The major objective of crop improvement is to identify within heterogeneous materials those individuals for which favorable alleles are present at the highest proportion of loci involved in the expression of key traits [3]. The classical plant breeding method is based on increasing the probability of selecting such individuals from populations generated from sexual matings. Selection has traditionally been carried out at the whole-plant level (i.e., phenotype), which represents the net result of genotype and environment (and their interactions). Phenotypic selection has delivered tremendous genetic gains in most cultivated crop species, but is severely limited when faced with traits that are heavily modulated by the environment [4]. In addition, the nature of some traits can make the phenotypic testing procedure itself complex, unreliable, or expensive (or a combination of these).

The recent remarkable development of molecular genetics and associated technologies represents a quantum leap in our understanding of the underlying genetics of important traits for crop improvement. The ongoing revolutions in molecular biology and information technology offer tremendous and

unprecedented opportunities for enhancing the effectiveness and efficiency of MB programs. Indirect selection, based on genetic markers, presents an efficient complementary breeding tool to phenotypic selection. Individual genes or QTLs having an impact upon target traits can be identified and linked with one or more markers, and then the marker loci can be used as a surrogate for the trait, resulting in greatly enhanced breeding efficiency [5–8].

Molecular techniques can have an impact upon every stage of the breeding process from parental selection and cross prediction [9], to introgression of known genes [10] and population enhancement. Selection of beneficial alleles of known genes can be done through marker-assisted selection (MAS) – the selection of specific alleles for traits conditioned by a few loci [10] – or through marker-assisted backcrossing (MABC) – transferring specific alleles of a limited number of loci from one genetic background to another, including transgenes [11, 12]. For marker-assisted population improvement, individuals selected from a segregating population based on their marker genotype are intermated at random to produce the following generation, at which point the same process can be repeated a number of times [13]. A second approach aims at direct recombination between selected individuals as part of a breeding scheme, seeking to generate an ideal genotype or ideotype [14]. The ideotype is predefined on the basis of QTL mapping within the segregating population, combined with the use of multi-trait selection indices that can also consider historical QTL data. This variety development approach is commonly referred to as marker-assisted recurrent selection (MARS) [15–17], or genotype construction. An alternative is to infer a predictive function using all available markers jointly, without significant testing and without identifying a priori a subset of markers associated with the traits of interest. This more recent approach coming from genomic medicine [18, 19], and then applied successfully in animal breeding [20] named genome-wide selection (GWS), also appears to be quite promising in crop improvement [7].

Concomitantly with the evolution of marker technologies becoming increasingly "data rich," the amount of data produced by plant breeding programs has increased dramatically in recent years. Increasingly, the critical factor determining the rate of progress in plant breeding programs is their capacity to manage large amounts of data efficiently and subsequently maximize the timely extraction of meaningful information from that data for use in selection decisions. If genotyping has become less of an issue, the efficient management of genotyping data in a broad sense, including sequence information, is increasingly becoming a major challenge in modern plant breeding. This was recognized early on in the private sector where the establishment of platforms or pipelines integrating field and laboratory processes with powerful data management systems (DMS) that merged and analyzed the data collected at every step and guided the process of crop improvement toward the release of improved cultivars has been the key to successful adoption of MB.

A few initiatives have taken place in the public sector to establish efficient data management or ISs [21, 22]. One of these has been led by several centers of the Consultative Group on International Agricultural Research (CGIAR) which have worked over the past decade, along with advanced research institutes (ARIs) and national agricultural research systems (NARS) in developing countries, to develop an open-source generic IS, the International Crop Information System (ICIS), to handle pedigree information, genetic resource, and crop improvement information [23]. Based on some elements of ICIS, the CGIAR Generation Challenge Programme (GCP, http://www.generationcp.org) has invested in integrating crop information with genomic and genetic information and in using existing or developing new public decision-support tools to access and analyze information resources in an integrated and user-friendly way [24]. Another initiative has been led by Primary Industries and Fisheries (PI&F) of the Queensland Government Department of Employment, Economic Development and Innovation in Australia, which recognized that effective data management is an essential element in obtaining maximum benefit from their investment in plant breeding. In conjunction with the New South Wales Department of Primary Industries (NSW DPI) and more recently Dart Pty Ltd (http://www.diversityarrays.com/) they are in the process of developing a linked IS for plant breeding (Katmandoo) that includes applications for capturing field data using hand-held computers, barcode-based seed management systems, and databases to store and link field

trial data, laboratory data, genealogical data, and marker data [25].

Although an IS involves far more than a database, the development and implementation of a suitable database system alone remains a real challenge because of the fast turnover in technologies, the need to manage and integrate increasingly diverse and complex data types, and the exponential increase in data volume. Previous solutions, such as central databases, journal-based publication, and manually intensive data curation, are now being enhanced with new systems for federated databases, database publication, and more automated management of data flows and quality control. Along with emerging technologies that enhance connectivity and data retrieval, these advances should help create a powerful knowledge environment for genotype–phenotype information [26].

In addition to efficient data management, advances in statistical methodology [27–29], graphical visualization tools, and simulation modeling [9, 30–32] have greatly enhanced these ISs. The availability of molecular data linked to computable pedigrees [33] and phenotypic evaluation now makes genotype–phenotype analysis a practical reality [34].

In order to realize the full potential of marker technologies and bioinformatics in plant breeding, tools for molecular characterization, accurate phenotyping, efficient ISs, and effective data analysis must be integrated with breeding workflows managing pedigree, phenotypic, genotypic, and adaptation data. The goals of this integration of technologies are to (1) create genotype–phenotype trait knowledge for breeding objectives, and (2) use that knowledge in product development and deployment [4].

This entry generally explores the pace of innovation in world agriculture and the rise of MB. It particularly illustrates the accelerating application of information and communication technologies to the information management challenges of MB and, as a result, the emergence of virtual molecular breeding platforms (MBPs) as a vital tool for accelerating genetic gains and rapidly developing more resilient and more productive cultivars.

This entry reviews the rationale for access to MB technology and services and the status of existing public analytical pipelines and ISs for MB, and offers a detailed case study for the CGIAR GCP Integrated Breeding Platform (IBP) – the pioneer public sector MBP specifically targeting developing country breeding programs. It explores the gaps between countries and between crops in the application of informatics-powered MB approaches, and the potential for adopting MBPs to close these gaps; and it reviews institutional, governmental, and public support for these approaches. The entry discusses the challenges and opportunities inherent in MBPs, and the potential economic impact of MB. Finally, the entry explores the future directions and perspectives of MBPs.

Marker Technologies and Service Laboratories

Markers are "characters" whose pattern of inheritance can be followed at the morphological (e.g., flower color), biochemical (e.g., proteins and/or isozymes), or molecular (DNA) levels. They are so called because they can be used to elicit, albeit indirectly, information concerning the inheritance of "real" traits. The major advantages of molecular over other classes of markers are that their number is potentially unlimited, their dispersion across the genome is complete, their expression is unaffected by the environment and their assessment is independent of the stage of plant development [35]. During the past two decades, DNA technology has been exploited to advance the identification, mapping, and isolation of genes in a wide range of crop species. The first generation of DNA markers, restriction fragment length polymorphisms (RFLPs), was used to construct the earliest genome-wide linkage maps [36] and identify the first QTLs [37, 38]. During the 1990s, emphasis switched to assays based on the polymerase chain reaction (PCR), which are much easier to use and potentially automatable [39]. The development of simple sequence repeats (SSRs) [40], amplified fragment length polymorphisms (AFLPs) [41], and single nucleotide polymorphism (SNP) [42] opened the door for large-scale deployment of marker technology in genomics and progeny screening.

SNPs are amenable to very high throughput and a wide range of detection techniques has been developed for them, from singleplex systems to high-density arrays. They can be used in fully integrated robotic systems going from automated DNA extraction to automated scoring in high-throughput detection platforms. The combination of increase in throughput and lowering in costs makes SNPs highly suitable to

intensive marker applications in plant breeding such as MARS and the emerging approach of GWS. Based on SNP technology, production of molecular marker (MM) data expanded more than 40-fold between 2000 and 2006 at Monsanto, while cost per data point decreased to one sixth of the original cost [43].

With the transition from SSRs to SNPs and the concomitant large increase in the demand for genotyping as markers get more and more widely used in a broad range of applications from medicine to plant breeding, marker genotyping laboratories have evolved from relatively low-tech operations to highly automated, high-throughput laboratories using an array of sophisticated equipment (pipetting robots, high-density PCR, high-throughput SNP detection machines, high-level informatics). Although large private seed companies have had the need and the resources to put in place large-scale genotyping laboratories for their own uses, smaller programs, especially in the public sector, have typically not had the resources or the justification to establish such large operations to respond to their increasing need for SNP genotyping data. In response to this need, a few private marker service laboratories have sprung up over the past few years, which can provide complete genotyping services for their customers, from DNA extraction to generation of large numbers of SNP or other datapoints. Due to their broad customer base (from medical research laboratories to animal and plant breeding operations, both public and private), these laboratories can have a large volume of datapoint production which may lead to low costs for the customer and high throughput. They are able to invest in the most advanced equipment to keep up with the constant evolution of genotyping technologies and are able to pass on the resulting benefits to their customers. Processes have now been put in place for rapid shipment of leaf samples from any location (field or laboratory) around the world without any restrictions. Examples of such companies that can service breeding programs from around the world are DNA LandMarks, Inc. of Saint-Jean-sur-Richelieu, Quebec, Canada (http://www.dnalandmarks.ca/english/) and KBioscience Ltd. of Hoddesdon Herts, UK (http://www.kbioscience.co.uk/). For many public breeding programs and small companies, especially in developing countries, it is now more efficient to use those types of contract genotyping services than to try to support their growing MB needs through the establishment of an in-house laboratory. Functional and reliable SNP laboratories are especially difficult to establish in many developing countries due to the unreliability of the power supply, difficulties in shipping and storing and a low level of resources for the purchase and maintenance of sophisticated equipment. The GCP is facilitating the linkage between users and service laboratories through its marker services, a component of the breeding services offered through the GCP's IBP.

Analytical Tools, Software, and Pipelines

One of the achievements of the plant biotechnology revolution of the last two decades has been the development of molecular genetics and associated technologies, which have led to the development of an improved understanding of the basis of inheritance of agronomic traits. The genomic segments or QTLs involved in the determination of phenotype can be identified from the analysis of phenotypic data in conjunction with allelic segregation at loci distributed throughout the genome. Because of this, the mode of inheritance, as well as the gene action underlying the QTL, can be deduced [44]. As with the improvement in marker technologies, the statistical tools needed for QTL mapping have evolved from a rudimentary to a very sophisticated level [45]. Previous approaches based on multiple regression methods, using least squares or generalized least squares estimation methods [46, 47], have evolved to composite interval mapping [9], mixed model approaches using maximum likelihood or restricted maximum likelihood (REML) [48], and Markov Chain Monte Carlo (MCMC) algorithms [49, 50], which use Bayesian statistics to estimate posterior probabilities by sampling from the data. In parallel, with progress in the characterization of genetic effects at QTLs and refinement of QTL peak position through meta-analysis [51], advances have also been made in understanding the impact of the environment on plant phenotype. The mapping of QTLs for multiple traits has allowed the quantification of QTL by environment interaction (QEI) [52] and, more recently, approaches using factorial regression mixed models have been applied to model both genotype by environment interaction [53] and QEI [48, 54, 55]. Recent approaches are now

implemented to evaluate gene networking [56] and epistasis, based on Bayesian approaches [57, 58] or through stepwise regression by considering all marker information simultaneously [59, 60]. Epistasis and balanced polymorphism influence complex trait variation [61, 62], and classical generation means analyses, estimates of variance components, and QTL mapping indicated an important role of digenic and/or higher-order epistatic effects for all biomass-related traits in model plants [63] and in crops [64–66]. It will be critical to implement the most efficient MB strategies in order to evaluate and include these genetic effects in breeding schemes [60].

All tools necessary to run MB projects, from the simplest to the most complicated approaches, are available today in the public domain. They are based on different algorithms and statistical approaches, from the very simple to the more complex. One challenge is the diversity of tools available for a given analytical function or along the different steps of an analytical pathway, making the choice of the "right" tool difficult and the move from one analytical step to the next very tedious due to the complete lack of common standards and formatting across tools. The number of applications available for QTL analysis illustrates well the multiplicity and diversity of tools that are available for a given analysis. The following software packages have been developed over the past 20 years:

- Mapmaker/QTL [67]
- MapQTL [68, 69]
- QTL Cartographer [9, 70]
- PLABQTL [71]
- QGene [72, 73]
- Map Manager QT [74]
- iCIM [59, 60]

For most of these applications, the first versions were already available 15 years ago and the multiplicity and possible duplication generated by the independent development of these tools were already identified at the Gordon Research Conference on Quantitative Genetics and Biotechnology held in February 1997 in Ventura, California. A main objective of that workshop was to survey participants on the attributes of several software packages for QTL mapping and to define their analytical needs which were not presently met by the existing software packages. The workshop covered software for QTL mapping in inbred and outcrossed populations and the conclusions are available at: http://www.stat.wisc.edu/~yandell/statgen/software/biosci/qtl.html. In those conclusions one can read that "[a] consensus was reached that there is considerable overlap in the kinds of matings handled and statistics produced by the various QTL mapping software packages," clearly identifying the need for better coordinated efforts. Such coordination never took place, as is often the case in public research. As a result, most of those QTL packages are still available today, although in more sophisticated versions. They are all suitable for QTL mapping but use different statistical algorithms, present a different user interface, and necessitate different input and output file formats.

Some specialists in the field realized that the public software packages are usually too specialized and too technical in statistics to permit a thorough understanding by the many experimental geneticists and molecular biologists who would want to use them. In addition, the fast methodological advances, coupled with a range of stand-alone software, make it difficult for expert as well as non-expert users to decide on the best tools when designing and analyzing their genetic studies. Based on this rationale, a few commercial analytical pipelines emerged about a decade ago that include some of the QTL packages mentioned above. Two of them are Kyazma and GenStat®. These applications assist plant scientists by providing easy access to statistical packages for phenotypic and genotypic data. Kyazma was founded in the spring of 2003 (http://www.kyazma.nl/), and offers powerful methods for genetic linkage mapping and QTL analysis. Since 2003 Kyazma has taken over the development of the software packages JoinMap® and MapQTL® from Biometris of Plant Research International. Kyazma handles the distribution and support of JoinMap and MapQTL and, in collaboration with the statistical geneticists of Biometris, Kyazma provides introductory courses on genetic linkage mapping and QTL analysis in order to make the use of the software even more accessible. GenStat encompasses statistical data analysis software for biological and life science markets worldwide. GenStat includes the ASReml algorithm (average information algorithm for REML) to undertake very efficient meta-analyses of data with linear mixed models. The development of GenStat at Rothamsted

began in 1968, when John Nelder took over from Frank Yates as Head of Statistics. Roger Payne took over leadership of the GenStat activity when John Nelder retired in 1985 (http://www.vsni.co.uk/). An important feature of GenStat is that it has been developed in (and now in collaboration with) a Statistics Department whose members have been responsible for many of the most widely used methods in applied statistics. Examples include analysis of variance, design of experiments, maximum likelihood, generalized linear models, canonical variates analysis, and recent developments in the analysis of mixed models by REML.

These commercial analytical pipelines offer a set of quality tools to researchers in plant science. However, they cover only a part of the configurable workflow system that is required for integrated breeding activities. In addition, there is a need to have tools and analytical pipelines that are freely available and, if possible, based on open source code to avoid dependence on private companies that might discontinue support and ensure access to the tools even with limited financial resources, which is a critical constraint in the arena of research for development, of which breeding programs of developing countries are key partners. It is important to underline that a version of GenStat that does not include the most advanced version of the different tools but allows users to run most basic analyses is available for breeding programs in developing countries. The web site for the GenStat Discovery Edition is http://www.vsni.co.uk/software/genstat-discovery/, but this version of the pipeline does not include QTL selection based on the mixed model approach, which is available in the commercial version.

The issue of open source code is an important one as, even for freely-available tools, the lack of availability of the source code limits the further expansion and customization of the tools. It also reduces the opportunity of researchers in developing countries to participate in methodology development. Over the last decade, a programming language and software environment for statistical computing and graphics, R, is becoming the reference in open source code for a broad range of biological applications, including genetic analysis (http://www.r-project.org/). Its source code is freely available under the *GNU General Public License* (http://en.wikipedia.org/wiki/GNU_General_Public_License). The R language has become a de facto standard among statisticians for the development of statistical software. It compiles and runs on a wide variety of UNIX, Windows, and MacOS platforms. R is similar to other programming languages, such as C, Java, and Perl, in that it helps people perform a wide variety of computing tasks by giving them access to various commands. For statisticians, however, R is particularly useful because it contains a number of built-in modules for organizing data, running calculations on the information, and creating graphical representations of the data sets. R provides a wide variety of statistical (linear and nonlinear modeling, classical statistical tests, time-series analysis, classification, clustering, etc.) [29] and graphical techniques, and is highly extensible. Close to 1,600 different packages reside on just one of the many web sites devoted to R, and the number of packages has grown exponentially. However, R is difficult to use directly and procedures based on R must be wrapped in user-friendly menu systems if field biologists are to use them.

Information Systems

A functional IS involves far more than an analytical pipeline; it is a complete system that should include:

- A project planning module
- A germplasm management module
- A robust relational database
- Analytical standards
- Data collection and cleaning tools
- Analytical and decision support tools
- Query tools
- A cyber infrastructure (CI) that links the different tools in a cohesive and user-friendly way

Key elements of an IS are obviously the CI and the DMS as described in the following section. The value of an IS does not only reside in the quality of the individual tools or modules that are part of it, but rather in the CI or middleware that ensures cohesion across tools and efficient communication with databases.

There are not many examples of breeding ISs in the public domain. One example is the ICIS (http://www.icis.cgiar.org, [23]). ICIS is an open source IS for managing genetic resource and breeding information for any crop species. It has been developed over the last 10 years through collaboration between centers of the

CGIAR, some NARS, and private companies. The ICIS system is Windows-based, and distributable on CD-ROM or via the Internet. It contains a genealogy management system (GMS, [33]) to capture and process historical genealogies as well as to maintain evolving pedigrees and to provide the basis for unique identification using internationally accepted nomenclature conventions for each crop; a seed inventory management system (IMS); a DMS [75] for genetic, phenotypic, and environmental data generated through evaluation and testing, as well as for providing links to genomic maps; links to geographic ISs that can manipulate all data associated with latitude and longitude (e.g., international, regional, and national testing programs); applications for maintaining, updating, and correcting genealogy records and tracking changes and updates; applications for producing field books and managing sets of breeding material, and for diagnostics such as coefficients of parentage and genetic profiles for planning crosses; tools to add new breeding methods, new data fields, and new traits; and tools for submitting data to crop curators and for distributing data updates via CD-ROM and electronic networks. The community of ICIS collaborators communicates via the ICIS Wiki (http://www.icis.cgiar.org), where all design and development decisions are documented. Feature requests and bug reports are made through the ICIS Communications project and the source code is published through various other ICIS projects on CropForge (http://cropforge.org). A commercial company, Phenome-Networks, has implemented a Web-based IS based on ICIS (http://phnserver.phenome-networks.com/).

Another system available is the Katmandoo Biosciences Data Management System (http://www.katmandoo.org/, [25]), which is a freely available, open source DMS for plant breeders developed by PI&F, NSW DPI, and DArT Pty. Ltd. It comprises linked ISs for plant breeding including applications for capturing field data using hand-held computers, barcode-based seed management systems, and databases to store and link field trial data, laboratory data, genealogical data, and marker data. A particular focus is on the use of whole-genome MM information to create graphical genotypes, track the ancestral origin of chromosomal regions, validate pedigrees, and infer missing data. It includes the applications of the Pedigree-Based Marker-Assisted Selection System (PBMASS) developed by PI&F as well as a seed management system, a digital field book for hand-held computers, and a system for directly recording weights of barcoded samples.

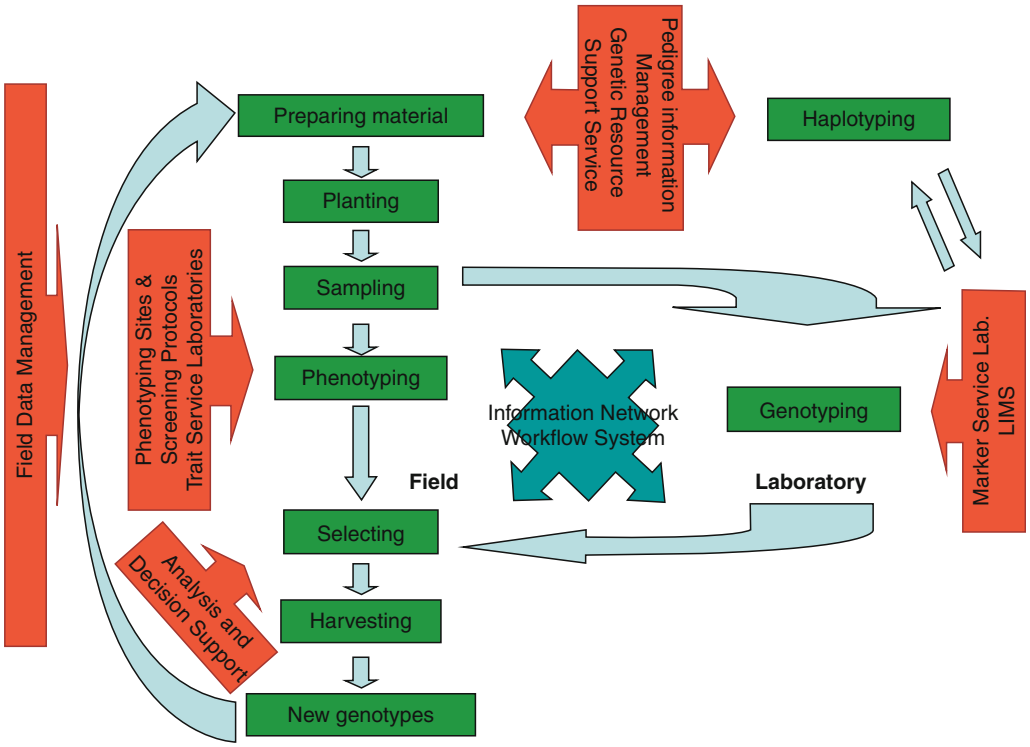
Both ISs struggle with the problem of integrating the different components into a single configurable system which matches the workflows of different breeding projects. Such a workflow should provide the user all tools and analytical means required to run a crop cycle: from germplasm preparation and planting, through the collection of phenotypic and the production of the genotypic data and their analysis, to the identification of genotypes to be crossed or the selection of suitable genotypes to be planted in the next cycle (Fig. 1).

In order to do this effectively, a CI is required which allows syntactic linkage between different data resources and applications.

Cyberinfrastructure and Data Management

We have referred to the revolution in Information and Communication Technology and the opportunities it presents for improving the efficiency of plant breeding. However, plant breeding is not the only area of biology being affected by this revolution and, in fact, the successful deployment of MB depends on other fields of information-intensive biology delivering knowledge (markers and methodology) to plant breeding. Even more is expected of the information and communications technology (ICT) revolution in the developing world, as it offers an opportunity for scientists there to overcome some of the constraints of isolation, the "brain drain," and the lack of infrastructure which have prevented them from fully participating in science for development in the past [76].

It is generally recognized that upstream biology is increasingly reliant on networks of integrated information and on applications for analyzing and visualizing that information. Discipline-specific (sequence and protein databases) and model organism ISs such as Graingenes (http://wheat.pw.usda.gov/GG2/index.shtml), Gramene (http://www.gramene.org/), MaizeGDB (http://www.maizegdb.org/), and Soybase (http://www.soybase.org/) have been developed to facilitate exchanges in molecular biology and functional genomics. As noted above, plant breeding



Molecular Breeding Platforms in World Agriculture. Figure 1
Different activities conducted during the crop cycle of an MB experiment presented in a generic way

depends on these upstream sciences of molecular biology, functional genomics, and comparative biology to deliver the knowledge needed to deploy MB. The bottleneck in the overall network has been the technology needed to integrate diverse and distributed information resources, and many information scientists have been working on this problem [24, 26, 77].

One constraint to integration of scientific information is the necessity to have a standard terminology for biological concepts across species and disciplines. A successful example of such standardization is the Gene Ontology (GO) initiative (http://www.geneontology.org, [78]). Another more specialized ontology initiative, especially pertinent to agriculture, is the Plant Ontology Consortium (POC: http://www.plantontology.org, [79–81]). However, these formal descriptions remain somewhat limited to biology of model plants and controlled environments. A key challenge will be to extend such standards to describe characteristics of plants growing in the unique, stress-prone environments found within the developing world to ensure a wider impact of such standards on international agriculture. The GCP has been working with POC to expand these ontologies to economic traits and farming environments so that they can be used in the field of plant breeding [82].

Another constraint to the efficient utilization of genomic information is the sheer volume of sequence data that can now be generated very cheaply across numerous genotypes. ISs to handle this volume of information are struggling to keep up. In plant biology, some examples of systems aiming to handle these torrents of data are the Germinate database ([83], http://bioinf.scri.ac.uk/public/?page_id=159) and the Genomic Diversity and Phenotype Connection (GDPC, http://www.maizegenetics.net/gdpc/). The primary goal of Germinate is to develop a robust database which may be used for the storage and retrieval of a wide variety of data types for a broad range of plant species. Germinate focuses on genotypic, phenotypic, and passport data, but has been designed to potentially handle a much wider range of data including, but not

limited to, ecogeographic, genetic diversity, pedigree, and trait data, and will permit users to query across these different types of data. The developers have aimed to provide a versatile database structure, which can be simple, requires little maintenance, may be run on a desktop computer, and yet has the potential to be scaled to a large, well-curated database running on a server. The design of Germinate provides a generic database framework from which interfaces ranging from simple to complex may be used as a gateway to the data. The data tables are structured in a way that they are able to hold information ranging from simple data associated with a single accession or plant, to complex data sets, images, and detailed text information. Features of the Germinate database structure include its ability to access any information associated with a group of accessions and to relate different types of information through their association with an accession. The GDPC database was designed as a research database to support association genetics applications such as Tassel (http://www.maizegenetics.net/index.php?option=com_content&task=view&id=89&Itemid=119) and is being extended to handle higher and higher densities of genotyping and sequence data. The second version of Germinate seems quite similar to GDPC and if new databases are developed to handle the large data files to be generated soon through high-throughput sequencing, some conversion tools should be easily developed to migrate data from one system to another.

Finally, the problem of integrating all these diverse and widely-distributed information resources is a major informatics challenge, which is being tackled on several fronts at several levels of complexity. The BioMOBY project ([84], http://www.biomoby.org, [85]) and the Semantic Web seek to define standards that will allow computer programs to interpret requests for information or services, find informatics resources capable of fulfilling those requests, and return the results without the authors of the interacting software having specifically collaborated. In the private sector, solutions have been more pragmatic and Enterprise Software solutions have been developed to link data resources and applications with specific services. The iPlant Collaborative (http://www.iplantcollaborative.org/) is a National Science Foundation (NSF)-funded initiative designed to bring these Enterprise Software solutions to the biological sciences in the form of CI which can support any biological data resource and analytical application. iPlant and the GCP are collaborating on integrating plant breeding information resources and applications into the infrastructure. This will automatically link these resources to upstream biological applications using the same infrastructure such as that used by the Systems Biology Knowledgebase initiative (http://genomicscience.energy.gov/compbio/#page=news) of the US Department of Energy which will be producing knowledge needed for crop improvement.

With all the progress achieved in marker technology, software development, analytical pipelines, and DMS, it is time to provide an IS, available through a public platform, that will offer breeding programs in developed and developing countries access to modern breeding technologies, in an integrated and configurable way, to boost crop quality and productivity.

Case Study: GCP's Integrated Breeding Platform

To fill this gap in the public sector and in particular in the arena of research for development, the GCP has been coordinating the development of the IBP (www.generationcp.org/ibp) in collaboration with scientists from ARIs, CGIAR centers, and national research programs since mid-2009. In a first phase the IBP aims at serving the needs of a set of 14 pioneer "user cases" – MB projects for eight crops in 16 developing countries in Africa and Asia. Leading scientists of those user cases help in testing the prototypes developed for the different tools of the analytical pipeline and contribute to the monitoring and evaluation of the platform development. This ensures that IBP development is driven by real breeding needs and its interface is user-friendly.

Objective of the IBP

The overall objective of the IBP project is to provide access to modern breeding technologies, breeding material, and related information and services in a centralized and functional manner to improve plant breeding efficiency in developing countries and hence

facilitate the adoption of MB approaches. The short-term objective of the project (the initial phase) is to establish – through a client-centered approach – a minimum set of tools, data management infrastructure, and services to meet the needs and enhance the efficiency of the 14 user cases.

To achieve the overall objective, GCP is developing and deploying a sustainable IBP as a one-stop shop for information, analytical tools, and related services to design, implement, and analyze MB experiments. This platform should enable breeding programs in the public and private sectors to accelerate variety development for developing countries using marker technologies – from simple gene or transgene introgression to gene pyramiding and complex MARS and GWS projects. Hence IBP aims at bringing cutting-edge breeding technologies to breeding programs that are too resource-restricted to invest in the requisite genotyping and data management infrastructure and capacity on their own.

The IBP Partnerships

The primary stakeholders of the platform are plant scientists – at this time specifically breeders leading the selected MB projects of the 14 pioneer user cases. These pioneer user cases are all recently initiated marker-assisted breeding projects with specific budgets, objectives, and work plans. The needs of the projects are defining the user requirements, and hence the design and development prioritization of the different elements of the platform. In selecting the user cases, crop diversity was a primary consideration, since the platform is supposed to address the needs of a broad variety of crops. The platform's reciprocal contribution to these breeding projects is in helping them overcome bottlenecks that would compromise final product delivery and in enhancing their overall efficiency and chances of success by providing appropriate tools and support.

The developmental phase of the IBP brings together highly regarded public research teams – institutes and individuals who have been working on the challenges of crop information management and analysis, biometrics, and quantitative genetics. This team of bioinformaticians, statisticians, and developers aims to design and develop the different elements of the platform, based on needs and priorities defined by the user cases.

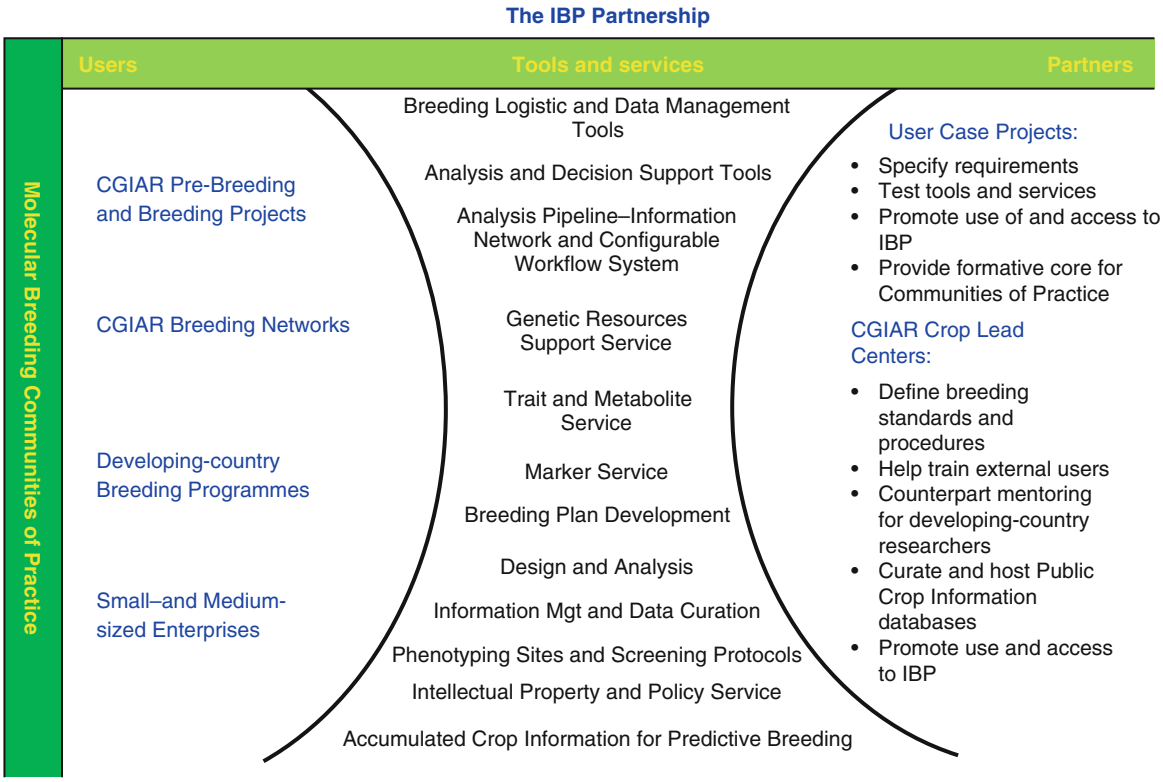
A continuous dialogue between users, developers, and service providers ensures a healthy balance between having a user-driven platform on the one hand, with a reasonable degree of "technology push" on the other hand, to ensure that users are kept abreast of technological solutions they may not be aware of but that would facilitate and accelerate breeding work.

The private sector has led the application of MB approaches and utilization of MBPs. The IBP is the first public sector effort of this magnitude aimed at developing and deploying an MBP. Given that MB for complex polygenic traits, and more so MARS, is still in its infancy in the public sector, it is recognized that efficient partnerships with the major private sector transnational seed companies is a strong prerequisite for the success of the IBP project. Consultations are ongoing with leaders in MB at Limagrain, Monsanto, Pioneer-DuPont, and Syngenta. Partnership with the private sector includes mainly some technology transfer, especially for stand-alone tools, and access to human resources to advise on the development of the platform and contribute to developing new tools or implement data management. The users, tools and services, and partnership of the platform are presented in Fig. 2.

The Platform

The IBP has three broad components (see Fig. 3): a Web-based portal and helpdesk, an open-source IS incorporating an adaptable breeding workflow system, and breeding and support services.

The stepwise development of the breeding workflow includes: (1) access to existing tools, (2) development of stand-alone new tools or adapted versions of existing tools to address the needs of the user cases, and (3) the integration of those tools into a CI (collaboration with the iPlant initiative) or through a thin middleware linking with local database to form a user-friendly configurable workflow system (CWS). A first version of the CWS, including an adequate set of tools, should be available by mid-2012, with full unfettered access scheduled for 2014.



Molecular Breeding Platforms in World Agriculture. Figure 2
The IBP partnership

Component 1: The Integrated Breeding Portal and Helpdesk

Inaugurated by mid-2011, the portal is the online gateway through which users access all the tools and services of the IBP. Through the portal, users will select and download tools and instructions, order materials, and procure laboratory services.

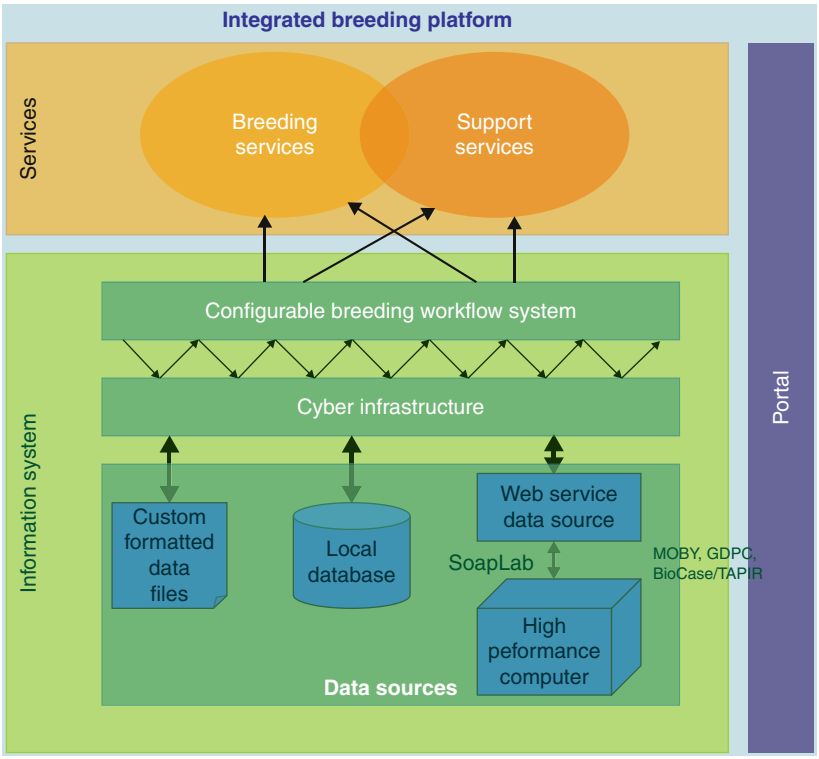
The portal's helpdesk facilitates its use and ensures access for users who cannot efficiently use the Web interface by providing the elements they need via email, compact disc, and other offline media.

Through their user-friendly networking components, the Portal and Helpdesk will stimulate the development of collaborative crop-based and discipline-based communities of practice (CoPs). The CoPs are expected to promote the application of MB techniques and the utilization of facilitative information management technologies, enhance data and germplasm sharing, and generally advance modern breeding capacity by linking CGIAR Centers and ARIs with developing-country breeding programs and research organizations. There is a strong hope that CoPs will facilitate and accelerate a paradigm shift to a more collaborative, outward-looking, technology-enhanced approach to breeding.

Component 2: The Information System

The IBP IS is structured as a CWS, with access to both local databases and distributed resources, such as central crop databases, molecular databases from GCP partner sites and from public initiatives such as Gramene and GrainGenes.

The Configurable Workflow System This CWS is the operational representation of the IS and will be implemented by assembling informatics tools into applications configured to match specific breeding workflows (e.g., for MAS, MABC, or MARS; Fig. 4). The tools are organized in a series of functional modules comprising



Molecular Breeding Platforms in World Agriculture. Figure 3
The IBP and its three main components

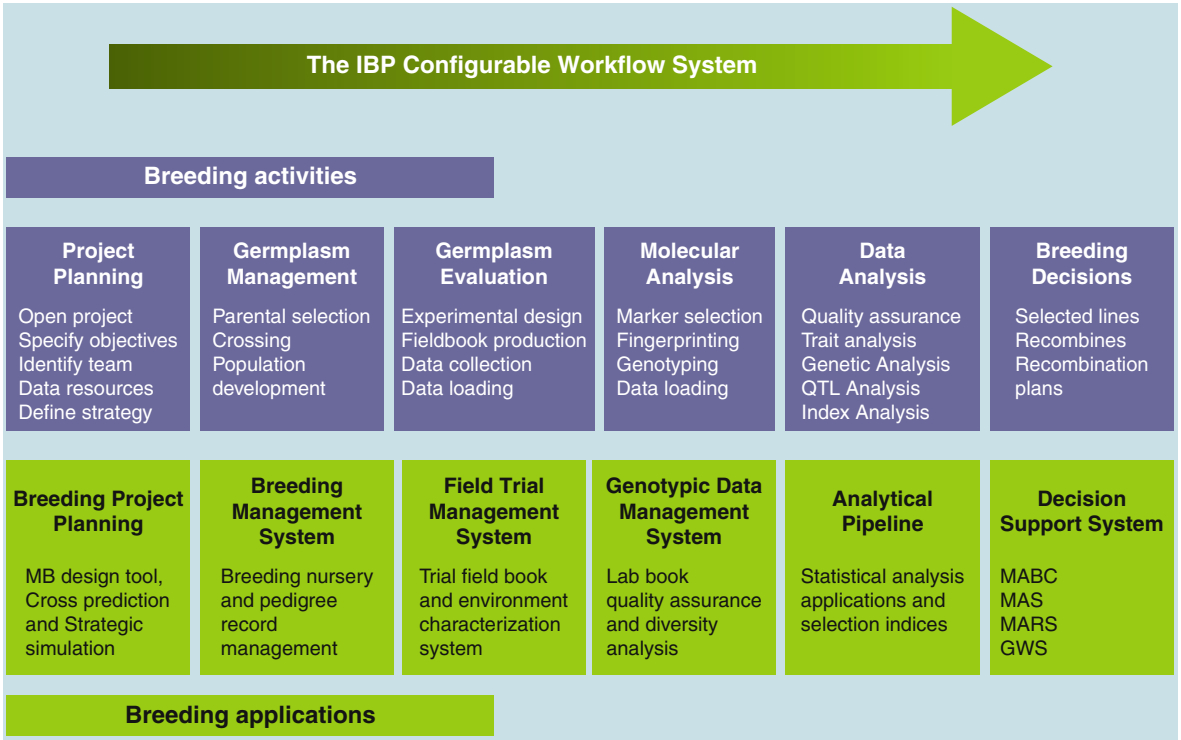
the Integrated Breeding Workbench, which is really the background structure that implements the CWS.

The IBP CWS drives the users through the different practical steps or activities of an MB project. The setup of the experiment and the germplasm management are the first steps of any project, to be followed by a set of activities that can be repeated during subsequent crop cycles, depending on the breeding objective of the experiment:

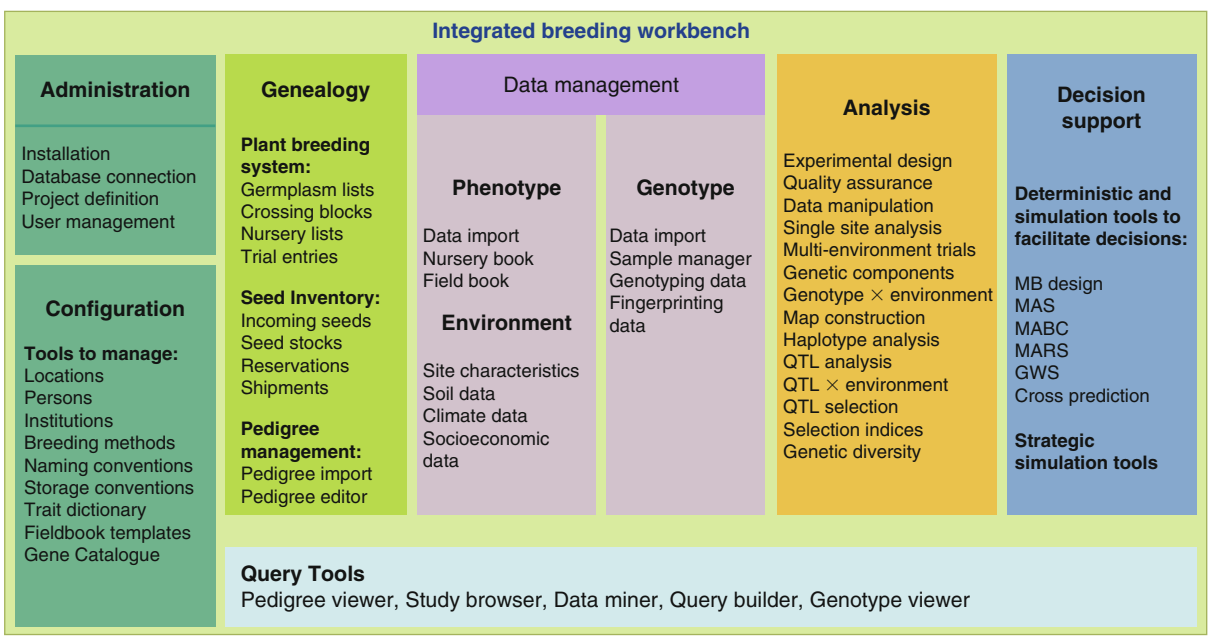
- Germplasm evaluation
- Genetic analysis
- Data management
- Data analysis, and
- Breeding decisions

The Integrated Breeding Workbench The workbench starts as a blank slate and the first task for the user is to open or create a project. A project manages a breeding workflow for a particular crop and a specified user. The initial sets of tools which should be available are grouped in seven modules: Administration Tools, Configuration Tools, Query Tools, and Workflow Initialization Tools (genealogy, data management, analysis, and decision support; Fig. 5).

The administration module of the workbench specifies the crop, which identifies the central (public) data resources that will be accessible to the project. This includes a central genealogy database, a central phenotype database, a public gene management database, and a central genotype database. Each installation provides access to local (private) data resources. These data resources include a private or local database for the above data types as well as a seed inventory management system. Each installation has at least one user with administrative privileges. Users are identified by authentication codes (username and password) for access to specific private data resources. ("Private" simply means "requiring authentication for access" and several users may have access to the same private data.)



Molecular Breeding Platforms in World Agriculture. Figure 4
The IBP configurable workflow system



Molecular Breeding Platforms in World Agriculture. Figure 5
The integrated breeding workbench

The first functionality of the workbench asks the user to open a project by selecting from a list of available project configuration "files." Once the configuration is selected, the availability of the public data resources should be checked, the user authentication codes verified, and the local data resources checked. Next, the list of modules should be reviewed and checked for availability and, depending on the state of the workflow, icons or menus should be made available for modules and tools.

The configuration tools allow users to:

- Select or specify naming conventions for germplasm, germplasm lists, studies, etc.
- Use and update ontologies such as germplasm methods and the trait dictionary
- Update breeding, testing, or collection locations
- Create and modify study templates

The query tools will depend on the data resources specified in the project configuration, and examples are:

- A germplasm and pedigree viewer
- A study browser to view phenotype or genotype data
- A data miner for identifying data patterns
- A cross-study query builder for linking different data sets
- A gene catalog viewer for viewing genetic diversity
- A genotype and trait viewer for visualizing graphical genotypes and trait markers

The workflow initialization tools comprise a set of modules (genealogy, data management, analysis, and decision support tools) that provide the user with a choice of different tools to achieve precise breeding objectives. Users might construct different breeding workflows to match their project activities. The user will only see the workbench tools and settings for those tools required to execute the steps in a particular breeding workflow, and at the appropriate step in that workflow.

The development of each tool is overseen by a team of IBP researchers, developers, and users who design, mock up, and prototype the tools of the breeding application and pass the specifications to a software engineering team. They will then monitor the development and test and support the application. For each application, the team develops a description of the application, functional specifications of all the tools, workflow specifications for the application, and an interface mockup. A workflow for a MARS project is shown in Fig. 6.

Component 3: IBP Services

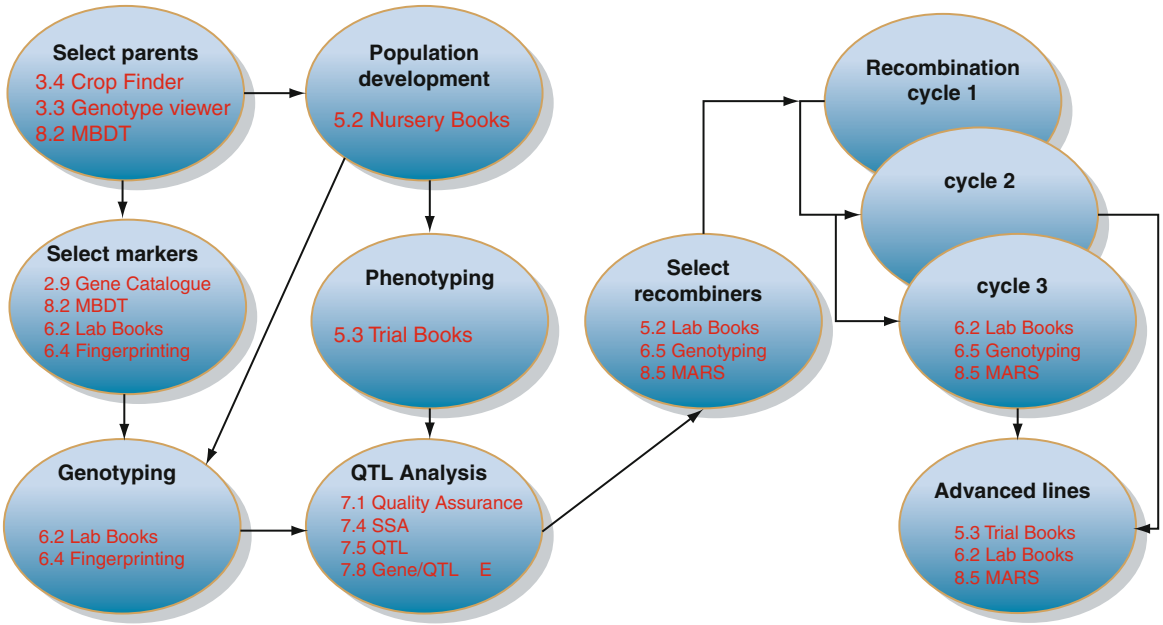
The Services component comprises two modules. The first module, Breeding Services, provides services to conduct MB projects. The second module, Support Services, deals with training and capacity-building, aiming to provide support and improve capacity of NARS breeders to deliver improved germplasm through marker approaches – essential for the adoption of MB approaches and the MBP.

Breeding Services These services provide access to specific germplasm, and assist with contracting a service laboratory to conduct the marker work or to quantify specific traits, such as metabolite profiles or grain quality parameters. The module has three elements (Fig. 7):

Genetic Resource Support Service: Access to suitable germplasm and related information from the different partners is a critical element of the portal. To address this, a Genetic Resource Support Service (GRSS) plans to tap into the CGIAR System-wide Genetic Resources Program (SGRP), a collaborative effort between GCP and existing gene banks in the CGIAR and NARS. The GRSS should ensure quality control, maintenance, and distribution of genetic resources, including reference sets and segregating populations acquired or generated through projects supported by GCP, and material generated from other sources and deposited with the GRSS (e.g., maize introgression lines from Syngenta).

Marker Service: The portal provides a set of online options for users to access different high-throughput marker service laboratories in the public and private sectors with clear contractual conditions. Service Laboratories have been selected on the basis of competitive cost, compliance with quality control requirements, and expeditious delivery, but are currently accessible by offline processes pending deployment of the IBP portal.

Trait and Metabolite Service: The portal provides a set of options for users to access laboratories



Molecular Breeding Platforms in World Agriculture. Figure 6
Breeding workflow for an MARS experiment

specialized in the evaluation and analysis of specific traits, such as quality traits, pathology screening, or metabolite quantification. Analyses of certain secondary traits and metabolites that are indicative of plant stress tolerance can potentially provide valuable information to be used in breeding. Such analyses are generally prohibitively expensive if done locally, as it is difficult to maintain assay quality and devote the necessary resources for expertise, quality control, and specialized facilities.

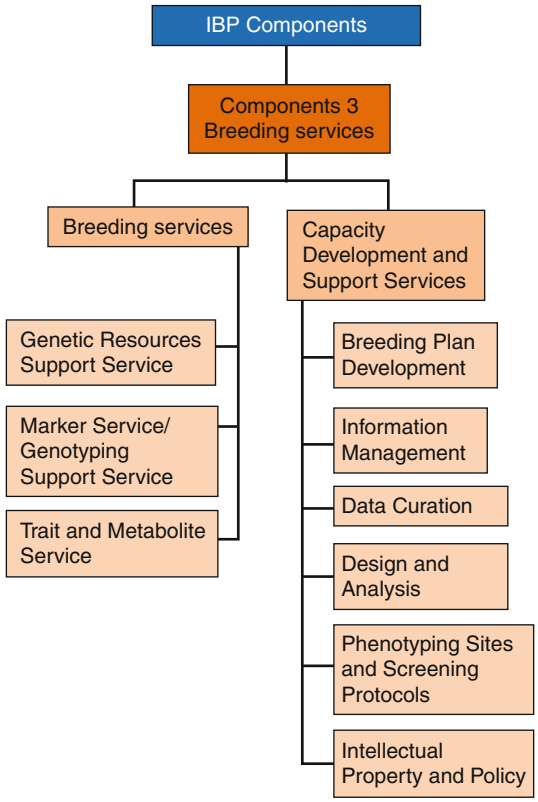
Capacity Development and Support Services
Capacity development is an integral part of the project, encompassing training and support in using MB techniques and markers, designing breeding strategies, quality data management, information analysis and decision modeling, phenotyping protocols, and protection of intellectual property (IP).

The main objective of this set of services is therefore to provide backstopping and training in a broad set of disciplines, to complement the elements of the breeding services and address specific technical and logistical bottlenecks. Such expert assistance is essential for the adoption and proper use of new technologies. Services that will be available include:

Breeding plan development: It is essential to develop a breeding plan with a cost–benefit analysis before conducting a multi-cycle MB project. Depending on the nature of the experiment, such a plan may be quite simple or very elaborate, from the transfer of a single region (e.g., transgene) to complex selection that can consider the simultaneous transfer of dozens of regions. The critical factor is that the plan must detail all the activities over time, and the costs and benefits of the project to determine if it is worthwhile conducting the experiment. The platform provides templates and associated cost calculation sheets for different breeding schemes.

Information management: Under this service, assistance is provided in installing and parameterizing the platform IS for use by specific breeding projects.

Data curation: This service assists with capturing and curating current data for particular breeding projects, and in entering them into the integrated IS. This step is absolutely critical for quality control and further sharing of the information, and a contact person for each of the pioneer user cases has been identified to ensure good communication between the platform and the users.



Molecular Breeding Platforms in World Agriculture.
Figure 7
Organogram of the services provided by the IBP

Design and analysis: This service provides support on statistics, bioinformatics, quantitative genetics, and molecular biology. It includes training in data generation, handling, processing, and interpretation, as well as experimental design from field planting to MAS and MABC schemes. It provides assistance with the "translation" of the molecular context to the breeding context, and it will ensure that the methodology developed for the design and analysis of breeding trials is rapidly available to the users.

Phenotyping sites and screening protocols: Through this service, users can access information on phenotyping sites, protocols, and potential collaborators to ensure that selection is carried out under appropriate biotic and abiotic stresses and that the adaptation of germplasm is well characterized. Characterization of phenotypic sites includes geographical information, meteorological historical data, soil composition, and field infrastructure.

Genotyping Support Service (GSS): The GSS aims to facilitate access by developing country national agricultural research institutes to genotyping technologies, and bridge the gap between lab and field research. This service provides financial and technical support for NARS breeders to access cost-efficient genotyping services worldwide and supports training activities in experimental design and data analysis for MB projects.

Intellectual property (IP) and policy: This service provides support on IP rights and freedom to operate in the arena of biotechnology and germplasm use. The service is currently being provided on an experimental basis through a virtual IP Helpdesk hosted by the GCP web site at http://www.generationcp.org/iphelpdesk.php.

Integrated Breeding Hubs

If today few question the usefulness of local basic laboratories, it is also generally accepted that large-scale genotyping activities are best outsourced to cost-effective, high-throughput service laboratories, irrespective of location. Following that rationale, the IBP provides access to marker service laboratories as the main avenue to generate the large amount of genotyping data that will be necessary to support the extensive MABC programs of the future, starting with the user cases, but the GCP also recognizes the need to provide breeders in developing countries with access to some regional hubs. At the beginning of the project four regional hubs are envisioned, covering the needs of the Americas – Centro Internacional de Agricultura Tropical (CIAT, www.ciat.cigiar.org); Africa – BioSciences eastern and central Africa (BecA, http://hub.africabiosciences.org); South Asia – International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, www.icrisat.org); and South East Asia – International Rice Research Institute (IRRI, www.irri.org).

These regional hubs are expected to provide the following services:

- In-house hands-on training (different formats are possible from short- to medium-length periods), with the objective of exposing scientists to new technologies and their applications to breeding.
- Training courses for selected groups of researchers, targeting basic knowledge of marker technologies and their applications, as well as data analysis.

These courses can be used for the testing and validation of learning materials, which will then be continuously upgraded.

- Facilitation of small genomic and genotyping projects led by national programs, academia, and small and medium enterprises (SMEs).
- Marker services for "small" and "orphan" crops that do not have mass demand from breeding programs and would therefore not benefit from large service providers, due to the lack of availability of SNP markers and the need to use lower-throughput SSR or other markers that can more easily be handled in lower-tech laboratories.

The Genomics and Molecular Breeding Hubs should help raise the visibility of the IBP and thus help promote the adoption of MB. Collaboration between the IBP and the regional hubs is anticipated to occur through sharing information, guiding users to apply for the appropriate service, organizing training events, and planning other developments of common interest.

Scope and Potential for Molecular Breeding Platforms

Gaps Across Countries and Crops

The application of MB approaches is now routine in developed countries, as is the integration of facilitative information and communication technologies, which are critical given the immense volumes of data necessary for, and generated by, these breeding processes. However, the situation is very different in developing countries, where MB is still far from routine in its application in breeding programs, particularly in Africa. This is especially critical due to the monumental and urgent imperative to rapidly achieve food security and improve livelihoods for a rapidly growing population through breeding for biotic stresses (including weeds, pests, and diseases) and abiotic stresses (including physical soil degradation, nitrogen deficiency, drought, heat, cold, and salinity) – conditions that make accurate phenotyping challenging. Fortunately, the history of modern breeding in developing countries is comparatively short, allowing a larger potential for crop improvement relative to the genetic gains that can be obtained at this time in developed countries, in which extensive breeding has been applied to crops for a longer time.

To address these issues, the capacity of national research institutions in terms of funds, infrastructure and expertise is directly related to the strength of their national economies [86]. This is reflected in the sharp differences in the capacity to conduct and apply biotechnology research as observed across developing countries (FAOBioDeC, http://www.fao.org/biotech/inventory_admin/dep/default.asp), and by the same token in their capacity to establish and/or utilize MBPs. The result is a three-tier typology of developing countries, directly attributable to the level of each country's investment in agricultural R&D [87].

Tier-1 countries, comprising newly industrialized countries (NICs) such as Brazil, China, India, Mexico, South Africa, and Thailand, substantially invest in technology and R&D and are self-reliant in most aspects of marker technologies [88, 89]. These countries have the simultaneous potential to effectively adopt, adapt, and apply information and communication technologies to enhance research efficiency and outputs. They are therefore naturally at the vanguard in adopting MBPs.

Mid-level developing world economies (tier-2) such as Colombia, Indonesia, Kenya, Morocco, Uruguay, and Vietnam are well aware of MB's importance, and some effectively apply marker technologies for germplasm characterization [90–93] and selection of major genes [94–99]. These countries have a matching potential for a limited utilization of MBPs, a potential that can be enhanced fairly rapidly in the medium to long term.

Low-level developing world economies (tier-3 countries) are struggling to sustain even basic conventional breeding. They have very limited or no application of MB approaches and are unlikely to adopt MBPs except in the long term.

Especially for tier-3 countries, resource-limited breeding programs in many developing countries are severely hampered by a shortage of well-trained personnel, low level of research funding, inadequate access to high-throughput genotyping capacity, poor and inadequate phenotyping infrastructure, lack of ISs and appropriate analysis tools, and by the logistical difficulty of integrating new approaches with traditional breeding methodologies – including problems of scale when scaling up from small to large breeding programs.

Until recently, the scarcity of available genomic resources for clonally propagated crops, for some neglected cereals such as millet, and for less-studied crops such as most tropical legumes, which are all very important crops in developing countries, represented a further constraint to agricultural research for development [100], thereby limiting the application of molecular approaches and hence the potential for MBPs. However, the recent emergence of affordable large-scale marker technologies (e.g., DArT [101]), the sharp decline of sequencing costs boosting marker development based on sequence information [102], and the explicit efforts of national agricultural research programs (e.g., India [103]) and international initiatives such as GCP [104]) have all resulted in a significant increase in the number of genomic resources available for less-studied crops. As a result, most key crops in developing countries now have adequate genomic resources for meaningful genetic studies and most MB applications.

Similarly, international efforts such as GCP's IBP are designed to help overcome the challenges of developing-country breeders – exploiting economies of scale by making available convenient and cost-effective collective access to cutting-edge breeding technologies and informatics hitherto unavailable to them, including genomic resources, advanced laboratory services, and robust analytical and data management tools. Together, this increasing availability of genomic resources and tools for previously neglected but important crops and the access to initiatives targeting the resource-challenged NARS of the developing world will hasten the adoption of MBPs for these countries.

Institutional, Governmental, and Public Support

While corporate and other proprietary MBPs need only meet the specific requirements of a particular corporation or of specific paying clients, the development of platforms targeted at breeding programs in the developing world require a broad consensus among the parties that would use them and support them from multiple overseeing organizations. This is because these platforms are built on the premise of minimizing costs and maximizing benefits through economies of scale generated through collective access by multiple partners.

The public-access MBPs would therefore be critically dependent on well-structured MB programs, which may not be a reality in many developing countries. A good structure would entail compliance with common or compatible:

- Good field infrastructure, including meteo station
- Good agronomical practices at experimental stations
- Crop ontology information system
- Data collection, management, and analysis protocols
- Breeding plan design
- Information and communication technology infrastructure
- Informatics tools for analysis, decision support purposes, and eventually modeling and simulation

Traditionally, developing world breeding programs have largely been poorly funded and poorly supported, and have been primarily driven by donor organizations [105, 106]. The lack of in-country support has often limited the dependent breeding activities to no more than a basic level. Under such circumstances, it was unrealistic to anticipate the adoption of new biotechnologies – including the utilization of MBPs. Fortunately, this scenario is changing. In 2003, through the Comprehensive Africa Agriculture Development Programme (CAADP, http://www.caadp.net/implementingcaadp-agenda.php), African governments committed to invest more in food security and in agriculture-led growth. Since then, many countries in Africa and elsewhere have developed comprehensive agricultural development strategies.

There is also a growing participation by foundations and nongovernmental organizations, and more recently the emergence of public–private sector partnerships (e.g., US Global Food Security Plan, http://www.state.gov/s/globalfoodsecurity/129952.htm). This governmental and institutional commitment is critical for the adoption of biotechnologies in general [8, 107] and for MB adoption in tier-2 countries in particular, with the attendant establishment and utilization of MBPs.

Challenges, Risks, and Opportunities

Challenges hampering the potential of MBPs in developing countries include both factors applicable generally to MB and those specific to MBPs. These

factors encompass infrastructure capacity, human resource, and operational and policy issues. But amidst the challenges there are also actual and potential opportunities.

Human Capacity Human capacity for MB technologies in developing countries is a challenge, and limitations include substandard agriculture programs at universities; difficulties in keeping up to date with relevant developments, including failures by others; poor technical skills in core disciplines; isolation as a result of insufficient peer critical mass in the workplace; and poor incentives to attract and retain scientists, resulting in brain drain and staff turnover [108].

To partially offset the undesirable trend of losing the "champions" and to "generate" more "champions," novel international initiatives like Alliance for a Green Revolution in Africa (AGRA) support high-quality education in the South. Examples include the African Centre for Crop Improvement (ACCI, http://www.acci.org.za/) based at the University of KwaZulu–Natal in South Africa and the University of Ghana-based West African Centre for Crop Improvement (WACCI, http://www.wacci.edu.gh/). Both institutes offer doctorate degrees in modern breeding to African students, with the fieldwork component being carried out in the students' home countries.

While obtaining their Ph.D. in plant breeding, these scientists study the principles of marker technologies, equipping them to undertake MB activities. To retain this much-needed expertise in Africa, the WACCI and ACCI programs also provide post-Ph.D. funds for these scientists to conduct research in their home countries and, in some cases, provide matching funds for their career advancement.

Precise Phenotyping There can be no successful MB program without precise phenotyping of the target traits. Reliable phenotypic data is a must for good genetic studies [109] and most developing countries lack suitable field infrastructure for good trials and collection of accurate phenotypic data. As part of the services of a good MBP, guidelines on best practice must be provided on how to design and run a trial and conduct precise phenotyping for genetic studies under different target environments. Improving access to homogeneous field areas, and paying attention to good soil preparation and homogeneous sowing are critical. The development of new geographic IS tools [102, 110], experimental designs, phenotyping methodologies [111, 112], and advanced statistical methods [113] will facilitate the understanding of the genetic basis of complex traits [114] and of genotype-by-environment (G×E) interactions [48, 115]. Improving phenotyping infrastructure in developing countries must thus be a top priority to promote modern breeding and utilization of MBPs [106].

Laboratories for Markers Services Genotyping can be expensive when it is performed in small laboratories using labor-intensive and low-throughput markers such as SSRs. This has traditionally limited the use of MMs in developing countries beyond the fingerprinting of germplasm with a small number of markers or the use of MAS for a few key traits. Operational efficiency is also vital, because fundamental timelines must be respected to ensure that no crop cycle is lost. Indeed, at every selection cycle, a service laboratory may have only a few weeks (time between DNA being extracted from leaves harvested on plantlets and the flowering time) to conduct the analysis and return the data to the breeders to enable them to conduct appropriate crosses among selected genotypes.

There is general agreement today that basic local laboratories at national and regional levels can be useful at least to service small local needs such as fingerprinting of limited number of accessions, GMO detection or MAS for specific traits, or for teaching and training purposes. It is also generally accepted that large-scale genotyping activities are best outsourced to advanced, modern, cost-effective high-throughput service laboratories, irrespective of the original location of the needs. This outsourcing is driven by the evolution in marker technologies. The advent of SNP genotyping led the shift from the low-throughput, primarily manual world of SSRs to high-throughput platforms powered by robotics and automated scoring, better handled by dedicated service laboratories [102, 116, 117]. As a result, genotyping costs have decreased by up to tenfold while data throughput has increased by the same magnitude. An example for MARS is provided in Fig. 6. SNP markers are increasingly available for most mainstream crops

and for several less-studied crops [118, 119], which are important in developing countries.

A particular effort will be needed to ensure an easy and reliable way to track samples from the field to the laboratory, and back to the field – it will hence be vital to carefully identify DNA samples from material collected in the field. Such documentation should optimally be through bar-coding, and all information pertaining to management of field trials or experiments should be recorded in electronic field books. Marker work would of necessity be subcontracted to a service lab with a good and preferably platform-compatible laboratory information management system (LIMS).

Data Management For breeders to efficiently access relevant information generated by themselves and by other researchers, reliable data management (including sample tracking, data collection and storage, and modern analytical methodologies and tools for accurate decision making, among others) is critical both within a given MB program and across programs. In view of this, it is essential that breeders manage pedigree, phenotypic, and genotypic information through common or mutually compatible crop databases, in keeping with the collective access principle of a public MBP. The format of databases would need to be user-friendly and compatible with field data collection devices and applications to encourage both adoption and compliance. Ultimately, data collection and management processes would need to seamlessly link with a platform-resident analysis, modeling, simulation, and a decision support workbench for full utility of the breeding platform.

Paradigm Shift: Collaborative Work and Data Sharing Access to information and products generated by fellow users is a potentially critical incentive for breeders to use the platform and share their own data with other users. However, this would require a fundamental paradigm shift from the present data-hoarding, inward-looking approach to research common to breeders. This may, however, only be achievable if it is a clear requirement in the terms of engagement for membership of a "platform community," or if distinct financial and other incentives are offered for such sharing.

Technology-Push Versus Demand-Driven An MBP is by nature a high-level technological solution. It carries with it the inherent risk of failing to address fundamental practical problems of developing-world breeding programs, which will often by nature be technology-deficient. Such platforms therefore face the challenge of ensuring that they meet targeted user objectives and address practical constraints.

However, with this challenge comes an opportunity to introduce advanced MB methodologies to developing world breeders, by encouraging change that will enable them to take advantage of the efficiencies and economies of scale offered by the MBP. This opportunity would be particularly reachable with bottom-up platform design and development that actively engages and involves the breeders – including elements of human resource capacity development and support in usage.

Adoption and Use by Breeders An MBP would only make a difference if it is adopted and widely used by the breeders. The most important element influencing this would be credibility – a function of the quality of the technology, the awareness of potential users, the ease of access, and initial incentives. There is a need for successful public sector developing-country examples to demonstrate that the platform can effectively enhance the efficiency of breeders through the use of modern approaches – a clear demonstration of the added value of using the platform.

Sustainability of the Platform Sustainability would be a challenge for MBPs targeting developing world breeding programs, given their resource limitations. These programs may not be able to meet the full cost of platform usage, and the cost of maintaining and updating the different elements of the platform on a regular basis – particularly tools and facilities that must keep abreast with evolving information and communication technologies.

Of course, platform sustainability is directly linked to its adoption by breeders, and sustainability strategies must be adapted to the diversity and financial resources of the potential clients, from developing-world national agricultural research institutes with limited resources to SMEs. Service costs might also be adjusted

if clients are willing to share data and release germplasm through the platform.

Platform managers may also have to consider other innovative options like on-platform advertising by agriculture-related commercial enterprises. However, ongoing donor support would most likely still be required in the medium to long term.

Communities of Practice The development of platform-based MB communities of practice, to connect groups of crop researchers, mainly breeders, willing to share experiences and information on modern breeding methods, best field practices, and development of improved varieties, and to practice peer-to-peer mentoring, are an additional potential avenue for platform adoption and sustainability, besides providing means to quickly and efficiently resolve recurring breeding problems. Partnerships between developed and developing-country institutions, and between the private and public sectors, are also an opportunity for realizing the full potential of MB [87, 108].

Many other hurdles limit successful public sector utilization of MB opportunities [120, 121]. However, the potential of virtual MBPs made possible by the revolution in information and communication technologies provides opportunities to counter and overcome many of those shortcomings.

Potential Economic Impact of Molecular Breeding Platforms

By its nature, MB improves the efficiency of crop breeding – progressively increasing genetic gains by selecting and stacking favorable alleles at target loci. The utilization of MBPs accelerates and amplifies the advantages of MB by introducing significant efficiencies in resource and time usage. Predictive or designer breeding, which would be the ultimate result of information-rich MB, attainable through the use of MBPs by numerous different breeding programs that freely share data and germplasm, would particularly bring about these savings in resources and time.

However, a direct comparison of the cost-effectiveness of MB with phenotypic selection is not straightforward. Firstly, factors other than cost – such as trade-offs between time and money – play an important role in determining the selection method. Secondly, this choice is further complicated by the fact that the two methods are rarely mutually exclusive or direct substitutes for each other [122]. On the contrary, under most breeding schemes, they are in fact complementary. Where operating capital is not a limitation, MB maximizes the net present value, especially when strengthened through MBPs [123]. With the increasing ease of accessing marker service laboratories and the declining cost per marker data point, MB costs are shrinking, making it extremely attractive from a purely economic perspective.

However, once the technological hurdles are overcome, the ultimate impact of new technologies (such as MBPs) is often limited by the lack of, or ineffective, seed distribution systems or by distant markets. SMEs are critical in promoting access to, and distribution of, improved seeds, thus helping alleviate a major bottleneck to the impact of improved breeding on smallholder farmers [124, 125].

Few economic analyses have been conducted to objectively assess the potential impacts of MB in the public sector, and none for MBPs that are just now emerging as a tool for breeding in the public sector.

Of the few analyses done to date, one evaluates the economic benefits of MABC using preexisting MMs in developing rice varieties tolerant to salinity and P-deficiency [126] in Bangladesh, India, Indonesia, and the Philippines. Encompassing a broad set of economic parameters, the study concluded that MABC saves an estimated minimum of 2–3 years, resulting in significant incremental benefits in the range of USD 300–800 million depending on the country, the extent of abiotic stress encountered, and the lag for conventional breeding [127].

Future studies are likely to confirm the positive economic benefits of MB and, given that MBPs amplify the benefits of MB, it can be reasonably inferred that the emerging platforms would indeed further enhance those economic benefits.

Future Directions

MBPs will inevitably have a significant impact on crop breeding in developing countries in the medium to long term because of:

- The needs-driven demand for improved crop varieties to counter the global food crisis

- The exponential development of genomic resources
- The ever-declining cost of marker technologies
- The increasing occurrence of public–private partnerships, where the public sector can learn from private companies about best practices for integrating MB into their breeding programs
- The need for innovative solutions to the challenges of resource and operational limitations

The first challenge of MBPs will be to meet the immediate needs of the breeders in developing-country public and private programs. The first step will be to provide them with the tools for enhancement of their current breeding programs, through the implementation of field books, pedigree management, and basic statistical analytical tools necessary to optimally conduct their current breeding efforts. In close succession with these first applications, tools will need to be made available to facilitate the integration of MB into their breeding programs. Databases will need to be developed for storing genotypic and phenotypic data, integrated analytical tools will need to be made available to breeders for analysis of this accumulated data and for the identification of important simple trait loci or QTLs to monitor and recombine in their breeding programs, and decision support tools will need to be developed to help breeders decide on the next steps to engage in based on the data they generated from their MB activities.

In the near future, more complex tools will need to be developed for the storage and analysis of the large amounts of genotypic data that will be generated by new next-generation sequencing technologies and for their application in GWS. A tight linkage will also have to be established with the wealth of information that is being generated and will continue to be generated even faster in the genomics area, leading to the dissection of the genome and to the discovery of the location and function of major genes having an impact upon the performance of crops in environments relevant to developing-country programs.

Eventually, the accumulation of large amounts of genetic information linked to specific haplotypes will lead to the increasing use of predictive breeding in combination with traditional MB usage and appropriate tools will also need to be developed to support those efforts.

Although it is critical for a platform to anticipate all the new possible features of MB, ensuring that new technologies and ISs will find their way in a flexible infrastructure, it is also quite probable that most of the breeding programs in developing countries will work at the short- and mid-term mainly with simple MB approaches as they will never reach the critical size of crosses and germplasm evaluation requested to maximize complex approaches.

Conclusion and Prospective Scenarios

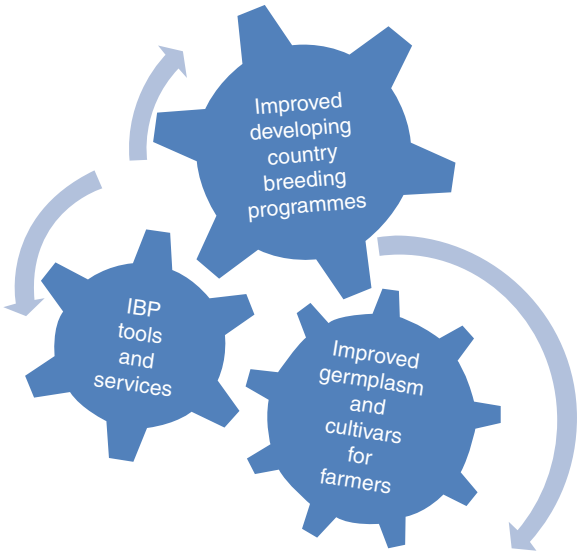
Through international initiatives like the ones coordinated by the CGIAR centers and programs, several notable developing-world MB successes have already been reported.

A well-known example is the development of submergence-tolerant rice cultivars through MABC led by IRRI [128]. The introgression of the Sub1 gene from FR13A (the world's most flood-tolerant variety) into widely grown varieties like Swarna improved yields in more than 15 million hectares of rain-fed lowland rice in South and Southeast Asia.

MB in general and the use of MBPs in particular have definitely been shown to be an efficient approach for reducing the number of required selection cycles and for increasing the genetic gain per crop cycle to a point where the required human and operational resources can be kept to a minimum.

However, for sustainable adoption, the use of modern breeding strategies requires a breeder-led bottom-up approach. As a start, simple MB approaches adapted to local environments should be tested first by individual breeders to evaluate their success and impact under those breeders' conditions. Once proven, these approaches can then be implemented more widely or integrated to an MBP for enhanced efficiency. In case of individual success the adoption of MB by those breeders should be quite straightforward.

It is clear that the extent, speed, and scope of adoption of MB approaches and of utilization of MBPs will vary somewhat across tier-1, tier-2, and tier-3 countries, depending on the local priorities and on the resources available in given breeding programs. It is unrealistic to expect that large-scale MB breeding activities, including utilization of MBPs, will be widely implemented across the board in developing



Molecular Breeding Platforms in World Agriculture. Figure 8
IBP as a key component to boost NARS breeding capacities and therefore crop productivity in developing countries

countries in the near term. However, the prospects are bright for individual breeders in these countries (particularly in tiers 1 and 2) to access germplasm, data, tools, and methodology that will allow them to conduct efficient MB projects by taking advantage of large international initiatives specifically targeting developing-country breeding programs. This will, however, happen in different ways and on different timelines for each tier.

For tier-1 countries, the impact would be evident in the shorter term – say in 3–6 years. These countries will benefit from new tools and platforms by increasing the rate of MB adoption. The biggest change is likely to occur in tier-2 countries, as these countries would be starting MB from scratch, but the impact would realistically be measurable only in the medium term, meaning in about a decade from now. For countries currently in tier-3 to advance to tier-2, basic breeding programs must first be established, which is highly dependent on governmental priorities and on subsequent resource allocation.

All in all, implementing MB (and catalyzing and accelerating its impact through MBPs) will boost crop production, which will translate into higher farm productivity per unit of land, better nutrition, higher incomes, poverty alleviation, and ultimately improved livelihoods in developing countries (Fig. 8). These gains will be amplified by sustained use, by continuously improving expertise, and by growth and development of homegrown capacity for the application of advanced breeding approaches.

Bibliography

1. Crosbie TM, Eathington SR, Johnson GR, Edwards M, Reiter R, Stark S, Mohanty RG, Oyervides M, Buehler RE, Walker AK, Dobert R, Delannay X, Pershing JC, Hall MA, Lamkey KR (2006) Plant breeding: past, present, and future. In: Lamkey KR, Lee M (eds) Plant breeding: the Arnel R. Hallauer international symposium. Blackwell, Ames, pp 3–50
2. Falck-Zepeda J, Zambrano P, Cohen JI, Borges O, Guimarães EP, Hautea D, Kengue J, Songa J (2008) Plant genetic resources for agriculture, plant breeding, and biotechnology. EPTD Discussion Paper 00762. International Food Policy Research Institute, Washington, DC
3. Goodman RM, Hauptli H, Crossway A, Knauf VC (1987) Gene transfer in crop improvement. Science 236:48–54
4. Cooper M, Smith OS, Merrill RE, Arthur L, Polich DW, Loffler CM (2006) Integrating breeding tools to generate information for efficient breeding: past, present, and future. In: Lamkey KR, Lee MA (eds) Plant breeding: the Arnel R. Hallauer international symposium. Blackwell, Ames, pp 141–154
5. Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7:257–264
6. Ribaut J-M, Hoisington DA (1998) Marker-assisted selection: new tools and strategies. Trends Plant Sci 3:236–239
7. Bernardo R (2008) Molecular markers and selection for complex traits in plants: Learning from the last 20 years. Crop Sci 48:1649–1664
8. Moose SP, Mumm RH (2008) Molecular plant breeding as the foundation for 21st century crop improvement. Plant Phys 147:969–977
9. Wang S, Basten CJ, Zeng Z-B (2005) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh
10. Collard BCY, Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philos Trans R Soc B 363:557–572
11. Ribaut J-M, Jiang C, Hoisington D (2002) Efficiency of a gene introgression experiment by backcrossing. Crop Sci 42:557–565
12. Mumm RH (2007) Backcross versus forward breeding in the development of transgenic maize hybrids: theory and practice. Crop Sci 47(S3):S164–S171
13. Hospital F, Charcosset A (1997) Marker-assisted introgression of quantitative trait loci. Genetics 147:1469–1485
14. Stam P (1995) Marker-assisted breeding. In: Van Ooijen JW, Jansen J (eds) Biometrics in plant breeding: applications of

molecular markers. Proceedings of the ninth meeting of the EUCARPIA section biometrics in plant breeding, CPRO-DLO, Wageningen, pp 32–44
15. Peleman JD, Van Der Voort JR (2003) Breeding by design. Trends Plant Sci 7:330–334
16. Johnson R (2004) Marker-assisted selection. Plant Breed Rev 24:293–309
17. Bernardo R, Charcosset A (2006) Usefulness of gene information in marker-assisted recurrent selection: a simulation appraisal. Crop Sci 46:614–662
18. Guttmacher AE, Collins FS (2002) Genomic medicine – a primer. N Engl J Med 347:1512–1520
19. de los Campos G, Gianola D, Allison DB (2010) Predicting genetic predisposition in humans: the promise of whole-genome markers. Nat Rev Genet 11:880–886. doi:10.1038/nrg2898
20. Goddard ME, Hayes BJ (2007) Genomic selection. J Anim Breed Genet 124:323–330
21. Tinker NA, Yan W (2006) Information systems for crop performance data. Can J Plant Sci 86:647–662
22. Yan W, Tinker NA (2007) DUDE: a user-friendly crop information system. Agron J 99:1029–1033
23. McLaren CG, Bruskiewich RM, Portugal AM, Cosico B (2005) The international rice information system. A platform for meta-analysis of rice crop data. Plant Physiol 139:637–642
24. Bruskiewich R, Senger M, Davenport G, Ruiz M, Rouard M, Hazekamp T, Takeya M, Doi K, Satoh K, Costa M, Simon R, Balaji J, Akintunde A, Mauleon R, Wanchana S, Shah T, Anacleto M, Portugal A, Ulat VJ, Thongjuea S, Braak K, Ritter S, Dereeper A, Skofic M, Rojas E, Martins N, Pappas G, Alamban R, Almodiel R, Barboza LH, Detras J, Manansala K, Mendoza MJ, Morales J, Peralta B, Valerio R, Zhang Y, Gregorio S, Hermocilla J, Echavez M, Yap JM, Farmer SA, Gary, Lee J, Casstevens T, Jaiswal P, Meintjes A, Wilkinson M, Good B, Wagner J, Morris J, Marshall D, Collins A, Kikuchi S, Metz T, McLaren G, van Hintum T (2008) The Generation Challenge Programme platform: semantic standards and workbench for crop science. J Plant Genom 2008, Article ID 369601, 6 p. doi: 10.1155/2008/369601
25. Rodgers D, Jordan D (2009) Information management systems for plant breeders. Primary Industries and Fisheries (PI&F) of the Queensland Government, Department of Employment, Economic Development and Innovation in Australia, Queensland, Australia
26. Gudmundur A, Thorisson JM, Brookes AJ (2009) Genotype–phenotype databases: challenges and solutions for the post-genomic era. Nat Rev 10:9–18
27. Smith A, Cullis B, Thompson R (2001) Analyzing variety by environment data using multiplicative mixed models and adjustments for spatial field trend. Biometrics 57: 1138–1147
28. Burgueño J, Crossa J, Cornelius PL, Trethowan R, McLaren G, Krishnamachari A (2007) Modeling additive × environment and additive × additive × environment using genetic covariances of relatives of wheat genotypes. Crop Sci 47:311–320
29. Butler D, Cullis BR, Gilmour AR, Gogel BJ (2007) ASReml reference manual, release 2.00. VSN, Hemel Hempstead
30. Hammer G, Cooper M, Tardieu F, Welch S, Walsh B, van Eeuwijk F, Chapman S, Podlich D (2006) Models for navigating biological complexity in breeding improved crop plants. Trends Plant Sci 11:587–593
31. Wang J, Chapman SC, Bonnett DG, Rebetzke GJ, Crouch J (2007) Application of population genetic theory and simulation models to efficiently pyramid multiple genes via marker-assisted selection. Crop Sci 47:580–588
32. Chapman S (2008) Use of crop models to understand genotype by environment interactions for drought in real-world and simulated plant breeding trials. Euphytica 161: 195–208
33. DeLacy IH, Fox PN, McLaren G, Trethowan R, White JW (2009) A conceptual model for describing processes of crop improvement in database structures. Crop Sci 49: 2100–2112
34. Crossa J, Burgueño J, Dreisigacker S, Vargas M, Herrera S, Lillemo M, Singh RP, Trethowan R, Franco J, Warburton M, Reynolds M, Crouch JH, Ortiz R (2007) Association analysis of historical bread wheat germplasm using additive genetic covariance of relatives and population structure. Genetics 177:1889–1913
35. Lee M (1995) DNA markers and plant breeding programs. Adv Agron 55:265–344
36. Helentjaris T, Slocum M, Wright S, Schaefer A, Nienhuis J (1986) Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor Appl Genet 72:761–769
37. Edwards MD, Stuber CW, Wendel JF (1987) Molecular-marker-facilitated investigations of quantitative-trait loci in maize. I. Numbers, genomic distribution and types of gene action. Genetics 116:113–125
38. Paterson AH, Lander ES, Hewitt JD, Peterson S, Lincoln SE, Tanksley SD (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721–726
39. Mullis K (1990) The unusual origin of the polymerase chain reaction. Sci Am 262:56–65
40. Senior ML, Heun M (1993) Mapping maize microsatellites and polymerase chain reaction confirmation of the target repeats using a CT primer. Genome 36:884–889
41. Vos P, Hogers R, Bleeker M, Reijans M, Tho L, van der Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414
42. Gilles PN, Wu DJ, Foster CB, Dillon PJ, Chanock SJ (1999) Single nucleotide polymorphic discrimination by an electronic dot blot assay on semiconductor microchips. Nat Biotechnol 17:365–370
43. Eathington SR, Crosbie TM, Edwards MD, Reiter RS, Bull JK (2007) Molecular markers in commercial breeding. Crop Sci 47:154–163

44. Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199
45. Borevitz J (2004) Genomic approaches to identifying quantitative trait loci: lessons from *Arabidopsis thaliana*. In: Cronk QCB, Whitton J, Ree RH, Taylor IEP (eds) Molecular genetics and ecology of plant adaptation. Proceedings of an international workshop, December 2002, Vancouver, NCR Research Press, Ottawa, pp 53–60
46. Haley CS, Knott SA (1992) A simple regression method for mapping quantitative loci in line crosses using flanking markers. Heredity 69:315–324
47. Martinez O, Curnow RN (1992) Estimating the locations and the sizes of the effects of quantitative trait loci using flanking markers. Theor Appl Genet 85:480–488
48. Malosetti M, Ribaut J-M, Vargas M, Crossa J, van Eeuwijk FA (2008) A multi-trait, multi-environment QTL mixed model with an application to drought and nitrogen trials in maize (*Zea mays* L.). Euphytica 161:241–257
49. Bink MCAM, Janss LLG, Quaas RL (2000) Markov chain Monte Carlo for mapping a quantitative trait locus in outbred populations. Genet Res 75:231–241
50. Bink MCAM, Boer MP, ter Braak CJF, Jansen J, Voorrips RE, van de Weg WE (2007) Bayesian analysis of complex traits in pedigreed plant populations. Euphytica 161:85–96. doi:10.1007/s10681-007-9516-1
51. Chardon F, Virlon B, Moreau L, Falque M, Joets J, Decousset L, Murigneux A, Charcosset A (2004) Genetic architecture of flowering time in maize as inferred from quantitative trait loci meta-analysis and synteny conservation with the rice genome. Genetics 168:2169–2185
52. Jiang C, Zeng ZB (1995) Multiple trait analysis of genetic mapping for quantitative trait loci. Genetics 140:1111–1127
53. van Eeuwijk FA, Malosetti M, Boer MP (2007) Modelling the genetic basis of response curves underlying genotype x environment interaction. In: Spiertz JHJ, Struik PC, van Laar HH (eds) Scale and complexity in plant systems research. gene-plant-crop relations. Springer, Dordrecht, pp 115–126
54. Boer MP, Wright D, Feng L, Podlich DW, Luo L, Cooper M, van Eeuwijk FA (2007) A mixed-model quantitative trait loci (QTL) analysis for multiple-environment trial data using environmental covariables for QTL-by-environment interactions, with an example in maize. Genetics 177: 1801–1813
55. Malosetti M, Ribaut J-M, van Eeuwijk FA (2011) The statistical analysis of multienvironment data: modelling genotype-by-environment interaction and its genetic basis. In: Drought phenotyping in crops: from theory to practice (Monneveux Philippe and Ribaut Jean-Marcel, eds). CGIAR Generation Challenge Programme, Texcoco, Mexico. In press
56. Zhang F, Zhai H-Q, Paterson AH, Xu J-L, Gao Y-M et al (2011) Dissecting genetic networks underlying complex phenotypes: the theoretical framework. PLoS ONE 6(1):e14541. doi:10.1371/journal.pone.0014541
57. Yi N, Yandell BS, Churchill GA, Allison DB, Eisen EJ, Pomp D (2005) Bayesian model selection for genome-wide epistatic quantitative trait loci analysis. Genetics 170:1333–1344
58. Xu S, Jia Z (2007) Genome wide analysis of epistatic effects for quantitative traits in barley. Genetics 176:611–623
59. Li H, Ye G, Wang J (2007) A modified algorithm for the improvement of composite interval mapping. Genetics 175:361–374
60. Li H, Ribaut J-M, Li Z, Wang J (2008) Inclusive composite interval mapping (ICIM) for digenic epistasis of quantitative traits in biparental populations. Theor Appl Genet 116: 243–260
61. Kroymann J, Mitchell-Olds T (2005) Epistasis and balanced polymorphism influencing complex trait variation. Nature 435:95–98
62. Zeng Z-B (2005) Modeling quantitative trait loci and interpretation of models. Genetics 169:1711–1725
63. Kusterer B, Muminovic J, Utz HF, Piepho H-P, Barth S, Heckenberger M, Meyer RC, Altmann T, Melchinger AE (2007) Analysis of a triple testcross design with recombinant inbred lines reveals a significant role of epistasis in heterosis for biomass-related traits in Arabidopsis. Genetics 175:2009–2017
64. Frascaroli CEMA, Landi P, Pea G, Gianfranceschi L, Villa M, Morgante M, Pè ME (2007) Classical genetic and quantitative trait loci analyses of heterosis in a maize hybrid between two elite inbred lines. Genetics 176:625–644
65. Gu X-Y, Foley ME (2007) Epistatic interactions of three loci regulate flowering time under short and long daylengths in a backcross population of rice. Theor Appl Genet 114: 745–754
66. Melchinger AE, Piepho H-P, Utz HF, Muminović J, Wegenast T, Törjék O, Altmann T, Kusterer B (2007) Genetic basis of heterosis for growth-related traits in Arabidopsis investigated by Testcross progenies of near-isogenic lines reveals a significant role of epistasis. Genetics 177: 1827–1837
67. Landers ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181
68. Jansen RC (1993) Interval mapping of multiple quantitative trait loci. Genetics 135:205–211
69. Ooijen V (2004) MapQTL® 5, Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen
70. Zeng Z-B (1994) Precision mapping of quantitative trait loci. Genetics 136:1457–1468
71. Utz HF, Melchinger AE (1996) PLABQTL: a program for composite interval mapping of QTL. J Agric Genom 2:1–5. http://probe.nalusda.gov:8000/otherdocs/jqtl/jqtl1996-01/utz.html (verified 10 September 1999)
72. Nelson JC (1997) QGene: software for marker-based genomic analysis and breeding. Mol Breed 3:229–235
73. Joehanes R, Nelson JC (2008) QGene 4.0, extensible Java QTL-analysis platform. Bioinformatics 24:2788–2789

74. Manly KF, Olson JM (1999) Overview of QTL mapping software and introduction to Map Manager QT. Mamm Genome 10:327–334
75. Portugal A, Balachandra R, Metz T, Bruskiewich R, McLaren G (2007) International crop information system for germplasm data management. In: Plant bioinformatics: methods and protocols. Humana, Totowa, pp 459–471, Chapter 22
76. McLaren CG, Metz T, van den Berg M, Bruskiewich R, Magor NP, Shires D (2009) Informatics in agricultural research for development. Adv Agron 102:135–157
77. Parkhill J, Birney E, Kersey P (2010) Genomic information infrastructure after the deluge. Genome Biol 11:402
78. Gene Ontology Consortium (2008) The Gene Ontology project in 2008. Nucleic Acids Res 36(Database issue): D440–D444
79. Avraham S, Tung CW, Ilic K, Jaiswal P, Kellogg EA, McCouch S, Pujar A, Reiser L, Rhee SY, Sachs MM, Schaeffer M, Stein L, et al (2008) The plant ontology database: A community resource for plant structure and developmental stages controlled vocabulary and annotations. Nucleic Acids Res 36(Database issue): D449–D454
80. Ilic K, Kellogg EA, Jaiswal P, Zapata F, Stevens PF, Vincent LP, Avraham S, Reiser L, Pujar A, Sachs MM, Whitman NT, McCouch SR et al (2007) The plant structure ontology, a unified vocabulary of anatomy and morphology of a flowering plant. Plant Physiol 143(2):587–599
81. Plant Ontology Consortium (2002) The Plant Ontology Consortium and plant ontologies. Comp Funct Genomics 3:137–142
82. Bruskiewich R, Davenport G, Hazenkamp T, Metz T, Ruiz M, Simon R, Takeya M, Lee J, Senger M, McLaren G, van Hintum T (2006) The Generation Challenge Programme (GCP)—Standards for crop data. OMICS 10:215–219
83. Lee JM, Davenport GF, Marshall D, Ellis TH, Ambrose MJ, Dicks J, van Hintum TJ, Flavell AJ (2005) GERMINATE. A generic database for integrating genotypic and phenotypic information for plant genetic resource collections. Plant Physiol 139(2):619–631
84. BioMoby Consortium (2008) Interoperability with Moby 1.0—It's better than sharing your toothbrush! Brief Bioinform 9(3):220–231. doi:10.1093/bib/bbn003
85. Wilkinson M, Schoof H, Ernst R, Haase D (2005) BioMOBY successfully integrates distributed heterogeneous bioinformatics web services. The PlaNet exemplar case. Plant Physiol 138:1–13
86. Ribaut J-M, Monneveux P, Glaszmann JC, Leung H, Van Hintum T, de Vicente C (2008) International programs and the use of modern biotechnologies for crop improvement. In: Moore P, Ming R (eds) Genomics of tropical crop plants. Springer, New York, pp 21–63
87. Sonnino A, Carena MJ, Guimarães EP, Baumung R, Pilling D, Rischkowsky B (2007) An assessment of the use of molecular markers in developing countries. In: Guimarães EP, Ruane J, Scherf BD, Sonnino A, Dargie JD (eds) Marker-assisted selection: Current status and future perspectives in crops, livestock, forestry and fish. FAO, Rome, pp 15–26
88. Huang J, Rozelle S, Pray C, Wang Q (2002) Plant biotechnology in China. Science 295:674–677
89. Suresh P, Devi SV, Choudhary UN (2008) Resources and priorities for plant biotechnology research in India. Curr Sci 95:1400–1402
90. Ghneim Herrera T, Posso Duque D, Pérez Almeida I, Torrealba Nuñez G, Pieters AJ, Martínez CP, Tohme JM (2008) Assessment of genetic diversity in Venezuelan rice cultivars using simple sequence repeats markers. Electron J Biotechnol. doi:10.2225/vol11-issue5-fulltext-6
91. Khadari B, Oukabli A, Ater M, Mamouni A, Roger JP, Kjellberg F (2004) Molecular characterization of Moroccan fig germplasm using intersimple sequence repeat and simple sequence repeat markers to establish a reference collection. Hortic Sci 40:29–32
92. Onguso JM, Kahangi EM, Ndiritu DW, Mizutani F (2004) Genetic characterization of cultivated bananas and plantains in Kenya by RAPD markers. Sci Hortic 99:9–20
93. Paredes M, Becerra V, González MI (2008) Low genetic diversity among garlic (*Allium sativum* L.) accessions detected using random amplified polymorphic DNA (RAPD). Chil J Agric Res 68:3–12
94. Abalo G, Tongoonaa P, Derera J, Edema R (2009) A comparative analysis of conventional and marker-assisted selection methods in breeding maize streak virus resistance in maize. Crop Sci 49:509–520
95. Danson JW, Mbogori M, Kimani M, Lagat M, Kuria A, Diallo A (2006) Marker-assisted introgression of opaque2 gene into herbicide-resistant elite maize inbred lines. Afr J Biotechnol 5:2417–2422
96. Okogbenin E, Porto MCM, Egesi C, Mba C, Espinosa E, Santos LG, Ospina C, Marin J, Barrera E, Gutierrez J et al (2007) Marker-assisted introgression of resistance to cassava mosaic disease into Latin American germplasm for the genetic improvement of cassava in Africa. Crop Sci 47:1895–1904
97. Leung H, Wu J, Liu B, Bustaman M, Sridhar R, Singh K, Redona E, Quang VD, Zheng K, Bernardo M et al (2004) Sustainable disease resistance in rice: current and future strategies. In: New directions for a diverse planet. Proceedings of the 4th international crop science congress, 26 September–1 October, Brisbane
98. Sagredo B, Mathias M, Barrientos C, Acuña I, Kalazich J, Santosrojas J (2009) Evaluation of a SCAR RYSC3 marker of the RYadg gene to select resistant genotypes to potato virus Y (PVY) in the INIA potato breeding program. Chil J Agric Res 69:305–315
99. Stevens R (2008) Prospects for using marker-assisted breeding to improve maize production in Africa. J Sci Food Agric. doi:10.1002/jsfa.3154
100. Hartwich F, Tola J, Engler A, González C, Ghezan G, Vázquez-Alvarado JMP, Silva JA, Espinoza JJ, Gottret MV (2007) Building public–private partnerships for agricultural innovation, Food security in practice technical guide series. International Food Policy Research Institute, Washington, DC

101. Jaccoud D, Peng K, Feinstein D, Kilian A (2001) Diversity arrays: a solid state technology for sequence information independent genotyping. Nucleic Acids Res 29:e25
102. Ganal MW, Altmann T, Roder M (2009) SNP identification in crop plants. Curr Opin Plant Biol 12:211–217
103. Varshney RK, Penmetsa RV, Dutta S, Kulwal PL, Saxena RK, Datta S, Sharma TR, Rosen B, Carrasquilla-Garcia N, Farmer A et al (2009) Pigeonpea genomics initiative (PGI): an international effort to improve crop productivity of pigeonpea (*Cajanus cajan* L.). Mol Breed 26:393–408. doi:10.1007/s11032-009-9327-2
104. Varshney RK, Close TJ, Singh NK, Hoisington DA, Cook DR (2009) Orphan legume crops enter the genomics era! Curr Opin Plant Biol 12:1–9
105. Ajani EN, Madukwe MC, Agwu AE, Onwubuya EA (2009) Assessment of technology generating institutions in biotechnology innovation system of South-Eastern Nigeria. Afr J Biotechnol 8:2258–2264
106. O'Toole JC, Toenniessen GH, Murashige T, Harris RR, Herdt RW (2001) The Rockefeller Foundation's international program on rice biotechnology. In: Khush GS, Brar DS, Hardy B (eds) Rice genetics IV. Proceedings of the 4th international rice genetics symposium, Los Baños. International Rice Research Institute, pp 39–59
107. Kelemu S, Mahuku G, Fregene M, Pachico D, Johnson N, Calvert L, Rao I, Buruchara R, Amede T, Kimani P et al (2003) Harmonizing the agricultural biotechnology debate for the benefit of African farmers. Afr J Biotechnol 2:394–416
108. Morris M, Edmeades G, Peju E (2006) The global need for plant breeding capacity: what roles for the public and private sectors? Hortic Sci 41:30–39
109. Salekdeh GH, Reynolds M, Bennett J, Boyer J (2009) Conceptual framework for drought phenotyping during molecular breeding. Trends Plant Sci 14:488–496
110. Hyman G, Fujisaka S, Jones P, Wood S, de Vicente C, Dixon J (2008) Strategic approaches to targeting technology generation: assessing the coincidence of poverty and drought-prone crop production. Agric Syst 98:50–61
111. Hamer G, Cooper M, Tardieu F, Welch S, Walsh B, van Euuwijk F, Chapman S, Polish D (2006) Models for navigating biological complexity in breeding improved crop plants. Trends Plant Sci 11:587–593
112. Ribaut J-M, Betran J, Monneveux P, Setter T (2008) Drought tolerance in maize. In: Bennetzen J, Hake S (eds) Maize handbook, vol 1. Springer, New York, pp 311–344
113. Cooper M, van Eeuwijk F, Hammer GL, Podlich DW, Messina C (2009) Modeling QTL for complex traits: detection and context for plant breeding. Curr Opin Plant Biol 12: 231–240
114. Mackay TFC, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. Nat Rev Genet. doi:10.1038/nrg2612
115. Cooper M, van Eeuwijk FA, Chapman SC, Podlich DW, Löffler C (2006) Genotype-by-environment interactions under water-limited conditions. In: Ribaut JM (ed) Drought adaptation in cereals. Haworth, Binghampton, pp 51–95
116. Chagné D, Batley J, Edwards D, Forster JW (2007) Single nucleotide polymorphism genotyping in plants. In: Oraguzie NC, Rikkerink EHA, Gardiner SE, de Silva HN (eds) Association mapping in plants. Springer, New York, pp 77–94
117. Angaji SA (2009) Single nucleotide polymorphism genotyping and its application on mapping and marker-assisted plant breeding. Afr J Biotechnol 8:908–914
118. Muchero M, Diop NN, Bhat PR, Fenton RD, Wanamaker S, Pottorff M, Hearne S, Cisse N, Fatokun C, Ehlers JD et al (2009) A consensus genetic map of cowpea [*Vigna unguiculata* (L) Walp.] and synteny based on EST-derived SNPs. Proc Natl Acad Sci USA 106:18159–18164
119. Kawuki RS, Ferguson M, Labuschagne M, Herselman L, Kim DJ (2009) Identification, characterisation and application of single nucleotide polymorphisms for diversity assessment in cassava (*Manihot esculenta* Crantz). Mol Breed 23:669–684
120. Dwivedi SL, Crouch JH, Mackill DJ, Xu Y, Blair MW, Ragot M, Upadhyaya HD, Ortiz R (2007) The molecularization of public sector crop breeding: progress, problems, and prospects. Adv Agron 95:163–318. doi:10.1016/S0065-2113(07)95003-8
121. Xu Y, Crouch JH (2008) Marker-assisted selection in plant breeding: from publications to practice. Crop Sci 48:391–407
122. Dreher K, Khairallah M, Ribaut J-M, Morris M (2003) Money matters (I): costs of field and laboratory procedures associated with conventional and marker-assisted maize breeding at CIMMYT. Mol Breed 11:221–234
123. Morris M, Dreher K, Ribaut J-M, Khairallah M (2003) Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Mol Breed 11:235–247
124. Delmer DP (2005) Agriculture in the developing world: connecting innovations in plant research to downstream applications. Proc Natl Acad Sci USA 102:15739–15746
125. Guimarães EP, Kueneman E, Carena MJ (2006) Assessment of national plant breeding and biotechnology capacity in Africa and recommendations for future capacity building. Hortic Sci 41:50–52
126. Ismail AM, Heuer S, Thomson MJ, Wissuwa M (2007) Genetic and genomic approaches to develop rice germplasm for problem soils. Plant Mol Biol 4:547–570
127. Alpuerto VE, Norton GW, Alwang J, Ismail AM (2009) Economic impact analysis of marker-assisted breeding for tolerance to salinity and phosphorous deficiency in rice. Rev Agr Econ 31:779–792
128. Septiningsih EM, Pamplona AM, Sanchez DL, Neeraja CN, Vergara GV, Heuer S, Ismail AM, Mackill DJ (2009) Development of submergence-tolerant rice cultivars: the Sub1 locus and beyond. Ann Bot 103:151–160

Mussel Culture, Open Ocean Innovations

Richard Langan
Atlantic Mariner Aquaculture Center, University of New Hampshire, Durham, NH, USA

Article Outline

Glossary

Suspension culture A production method for mussels and other shellfish that employs ropes, cages, or nets suspended in the water column from either rafts or longlines.

Surface longline An anchored structure consisting of surface floatation supporting one or more horizontal lines from which ropes, cages, or nets can be suspended in the water column.

Open ocean farming Refers to aquaculture production of marine organisms in open ocean or offshore waters that are removed from any significant influence of land masses.

Submerged longline Subsurface structure consisting of anchors and submerged floatation from which ropes, cages, or nets can be suspended.

Site selection The process for selecting farming sites based on specified parameters such as depth, current and wave climate, temperature, and primary productivity.

Environmental effects The effects of farming activities on the physical, biological, and chemical properties of the marine environment *and* the effects of the environment on cultured organisms and consumers of cultured food products.

Seston Particulate material suspended in the water column of water bodies consisting of both living and dead organic material and inorganic particles.

Pseudofeces Suspended particles that have been rejected as food by filter feeding bivalve mollusks. The rejected particles are wrapped in mucus and expelled without being passed through the digestive tract.

Definition of the Subject

Aquaculture production of several species of mussels in sheltered marine waters is well established and occurs in many countries worldwide. The primary method of production of high quality mussels is suspension of ropes with attached mussels from floating rafts or surface longlines that are anchored to the seafloor. While demand for fresh, frozen, and canned mussel products continues to increase, growth in production is hampered by a lack of suitable space for expansion in sheltered waters. For more than a decade, there has been interest in developing production methods suitable for open ocean environments where wind and wave conditions preclude the use of either rafts or surface longlines. Recent advances in the use of longlines that can be submerged below the sea surface and therefore avoid the upper portion of the water column that is most affected by wave energy indicate that open ocean production is feasible. However, additional development in technology and methods to improve production efficiency and insure worker safety, as well as changes to political and regulatory frameworks are needed in order to achieve large-scale production.

Introduction

Population growth and consumer preference have resulted in a growing demand for seafood, a trend that is projected to continue into the future [1]. Production from capture fisheries has leveled off, and by most projections will remain stagnant or decline, depending on management and regulatory measures implemented by fishing nations [2, 3]. In contrast,

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

aquaculture production has increased by nearly 10% each year since 1980, and has played an important role in filling the gap between seafood supply and demand. Only a few decades ago, wild-caught fish and shellfish supplied nearly all edible seafood, though with essentially flat growth since 1980 and the rise of aquaculture over the same time period, capture fishing now accounts for only about half of the total [1]. In the most optimistic scenarios, wild-caught fisheries production will remain stagnant [2]; therefore, growth in the global seafood supply will continue to rely on aquaculture production.

There are signs, however, that the rate of growth for global aquaculture may have peaked for land-based and nearshore marine culture due to political, environmental, economic, and resource constraints [1]. Expansion of land-based culture is limited primarily by economics, particularly in developed countries where costs associated with land, capital equipment, and energy required to pump and treat water are prohibitive. In addition, very few marine species are appropriate for land-based culture. For example, the space and volume of phytoplankton required to produce large quantities of filter feeding mollusks in land-based systems would be enormous, and therefore not economically viable.

For nearshore marine farming, available and suitable space is the primary limiting factor as sheltered coastal waters are for most countries quite constrained to begin with and are already used for a multitude of commercial and recreational activities with which aquaculture must compete for space [4]. Expansion of large-scale finfish farming in coastal waters is also limited by environmental concerns. While there are also concerns about potential environmental effects of bivalve mollusk culture, they are minor in comparison to net pen culture of finfish and are balanced by recognition of the ecosystem services such as enhanced habitat complexity and filtration capacity provided by mollusks [5]. It is rather the effect of environmental conditions on mollusk culture, and specifically the effects of pollution on product safety that is limiting expansion in nearshore waters. Rapid coastal development and population growth and the resulting increase in human sources of pollution have affected the sanitary quality of nearshore waters, rendering shellfish grown there unsafe for consumption. As a consequence, many otherwise suitable sheltered sites for mollusk culture are off limits due to public health restrictions.

In developed countries, conflict with coastal residents and tourist-related businesses over aesthetic values, primarily over water views from shorefront property, have also affected the establishment of new farming sites. As the demographic of coastal communities continues to change and new residents place more value on views and recreation than food production, these conflicts are likely to increase. Given the constraints on expansion of current methods of production, it is clear that alternative approaches are needed in order for the marine aquaculture sector to make a meaningful contribution to the world's seafood supply.

Farming in open ocean waters has been identified as one potential option for increasing production and has been a focus of international attention for more than a decade. Despite the global interest in open ocean farming, development to date has been measured, primarily due to the significant technical and operational challenges posed by wind and wave conditions in most of the world's oceans [4]. Farming in fully exposed open ocean waters requires a different engineering approach since equipment and methods currently used in sheltered nearshore sites are largely unsuitable for the open ocean. In addition, the scale of investment required to develop and demonstrate new technologies and methods for offshore farming is yet to be determined, though most engaged in this endeavor would agree that it will likely be substantial.

Despite these challenges, there is sufficient rationale for pursuing the development of open ocean farming. Favorable features of open ocean waters include ample space for expansion, tremendous carrying capacity, less conflict with many user groups, reduced exposure to human sources of pollution, the potential to moderate some of the negative environmental and aesthetic impacts of high density coastal farming [6–8], and optimal environmental conditions for some bivalve mollusk species [9, 10]. For many countries, where cost, environmental concerns, limited space, and competing uses have restricted growth of land-based and nearshore marine farming, few other options for significant expansion exist.

Of the many species of finfish and shellfish that have been considered for open ocean farming, several

species of mussels have emerged as attractive candidates. There are several reasons for this. Like all filter-feeding mollusks, mussels derive all their nutritional needs from naturally occurring phytoplankton and organic particulates. Therefore, daily visits to deliver formulated feed by service vessels and farm personnel, which may be prohibited for extended periods by sea conditions, are not needed, nor is on-site infrastructure for automated feeding, which is both costly and vulnerable to damage from storms. Unlike many cultured species that have gradually transitioned from wild capture to aquaculture, farming has been the primary means of production for mussels for many decades; therefore, methods used in sheltered waters are well developed, highly automated, and very efficient [11]. Mussels are also relatively fast growers, with production cycles ranging from 12 to 18 months [9, 12].

Production methods in sheltered nearshore waters include bottom culture, which is practiced in some locations such as the Netherlands, Scandinavia, and the USA (Maine), and pole or "bouchot" culture, which is practiced in France; however, suspension culture, because of superior product quality, accelerated growth, and opportunities for mechanization, has emerged as the leading method of production [11]. Techniques and materials used for suspension culture may vary somewhat from place to place; however, in general, culture methodology consists of suspending mussel ropes or "droppers" from either rafts or longlines [13]. Raft culture was pioneered in Spain and from there became established in Scotland and more recently in Maine USA and in the Pacific Northwest coast of North America [11]. While rafts can be highly productive, they are suitable for use only in very sheltered embayments. Longline technology, which was developed in Japan, consists of either surface or submerged longlines, held in place with anchors and supported by buoys or floats. As with raft culture, surface longlines are only suitable for use in sheltered waters [13]; therefore, in locations where adverse sea conditions or drift ice occur, submerged longlines are the only option. Submerged longlines have been used primarily in locations (e.g., Atlantic Canada) where winter ice would impact buoys and lines [14]. It is only in recent years that the technology has been used in fully exposed open ocean locations [9].

Characterization and Selection of Open Ocean Farming Sites

Before discussing approaches to the development of open ocean mussel culture, it is important to first define what is meant by the term "open ocean." For most engaged in this sector, it is used synonymously with "offshore" and is generally accepted to mean farming in locations that are subjected to ocean waves and currents and removed from any significant influence of land masses rather than a set distance from shore. Clearly, a wide range of sea conditions falls under this broad definition. Ryan [4] reported on a site classification system for marine waters developed in Norway that is based on significant wave height exposure (Table 1).

While this classification method is instructive, knowledge of the full range of conditions at a particular site is needed to develop appropriate technologies and safe and efficient operating procedures.

There are a number of criteria that determine the suitability of open ocean sites for farming, many of which are also considerations for sheltered waters. These include proximity to infrastructure such as ports, processing and distribution centers, as well as physical and biological criteria such as bathymetry, seabed characteristics and contour, current velocities, temperature profiles, dissolved oxygen, turbidity, the quantity of quality of phytoplankton, and the frequency of occurrence of harmful algal blooms. The most important additional feature of offshore sites is wave climate. Significant wave heights, wave periods, the frequency and duration of high energy storm

Mussel Culture, Open Ocean Innovations. Table 1 Norwegian classification of offshore waters based on significant wave heights (From Ryan [4])

Site Class	Significant wave height (m)	Degree of exposure
1	<0.5	Small
2	0.5–1.0	Moderate
3	1.0–2.0	Medium
4	2.0–3.0	High
5	>3.0	Extreme

conditions, and the combined forcing of waves and currents must be known in order to determine whether a site is suitable, accessible by service vessels and personnel with reasonable frequency, and if so, what type of technology is required for farming.

It is imperative that a thorough evaluation of the parameters described above be conducted before proceeding with development of a site for farming. The requirements for data and subsequent analysis can be substantial; however, the use of advanced oceanographic technologies can greatly facilitate this task [8]. Multibeam sonar and three-dimensional visualization can generate a wealth of data on seafloor contours and texture to inform mooring system design and placement. Collection of time intensive data on temperature, salinity, dissolved oxygen, turbidity, and fluorescence can be greatly facilitated by strategic deployment of in situ instrumentation at appropriate depth intervals in the water column. Additional instrumentation should include Acoustic Doppler Current Profilers (ADCP) that can measure and record current velocity and direction throughout the water column, wave sensors that can give precise data on wave height, direction, steepness, and period, and meteorological sensors to measure air temperature and wind speed and direction. Many countries have buoy arrays in coastal waters that can provide long-term data on regional climatology to aid site evaluation; however, collection of site-specific data is critical. Assessment of the potential for the effects of global climate change on critical parameters such as water temperature should also be considered.

The data collection period required for site evaluation will vary, depending on local and regional environmental and meteorological conditions. Good baselines for some parameters can be established in a relatively short time frame (1 year), others such as the frequency, duration, and severity of storms or blooms of toxic algae are less predictable and it may take longer to determine the suitability of a particular site.

While most of the focus on open ocean development has been on cage culture of finfish, there has also been growing interest in offshore culture of bivalve mollusks. Some of the same drivers such as ample space and the opportunity to avoid user conflicts are identical to those for finfish culture, though perhaps more importantly, reduced risk of exposure to human sewage and industrial pollution presents a major advantage of open ocean waters over coastal locations.

There are, however, possible limitations as well as advantages. Open ocean waters in many areas of the world are nutrient deficient, so careful attention must be paid during site selection to the quantity, quality, and seasonality of phytoplankton available to dense arrays of filter feeding mollusks. Macroscale information on primary productivity can be obtained from ocean color satellite data generated by instruments such as Sea-viewing Wide Field-of-view Sensor (SeaWiFS) and Moderate Resolution Imaging Spectroradiometer (MODIS). Site-specific data on concentration and composition can be generated by in situ fluorometry and microscopic analysis of the plankton community. Phytoplankton concentration at different depths is also an important factor, as farmers will wish to maximize the use of vertical space for production in deep ocean waters. The frequency and duration of harmful algal blooms (HABs) is also a critical consideration for offshore mollusk farming. In some locations, blooms of toxic algae originate and persist in offshore waters (e.g., *Alexandrium sp.* In the Gulf of Maine, USA) and can result in extended public health closures with severe economic impact on producers.

In addition to physical, chemical, and biological characteristics of a site, other human uses in the vicinity such as shipping, fishing, and mining must be identified in order to avoid conflicts. Involvement of the appropriate permitting authorities in the early stages of development of an open ocean farming site is also critical [15]. Other factors such as use of the area by marine mammals, proximity to foraging areas of predators (e.g., diving ducks), location of sensitive biological communities, presence of parasitic organisms (e.g., pea crabs, trematodes, and copepods), and sediments contaminated by toxic substances must also be considered [16].

Technologies for and Methods Open Ocean Mussel Farming

Technologies for open ocean mussel farming are essentially adaptations of suspension culture methods employed in sheltered marine waters. Designs and prototypes for submersible rafts have

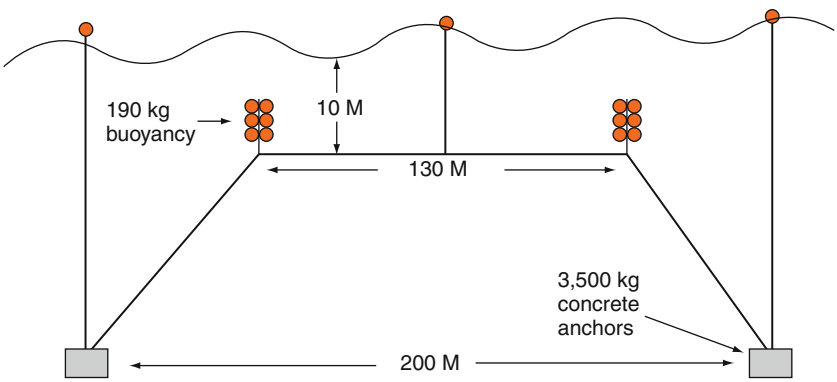
been developed [17, 18]; however, submerged longlines are the most commonly used method. This technology was developed in Japan and has been in use there for several decades for deep water suspended scallop culture, though not in fully exposed open ocean conditions. The technology has been successfully adapted for sheltered water mussel culture in Atlantic Canada where winter and spring drift ice can damage surface longlines [14]. More recently, the technology has been shown to be effective for mussel production in very high-energy open ocean conditions (e.g., significant wave heights >10 m) in the northeast USA [9] and at a test site in the German Bight with significant wave heights >8 m, and current velocity up to 1 ms^{-1} [19]. The technology is quite simple and it consists of relatively inexpensive materials. A design currently in use in North America is presented in Fig. 1.

The structural stability of a submerged longline is maintained by the opposing forces of submerged flotation at the ends of a single horizontal backbone, connected by lines set at a 45° angle to seafloor anchors. The most commonly used anchors are large (3–6 tons) deadweight concrete anchors, though both plow type and screw anchors have been used in some locations. Submergence depth of the backbone is dictated by site-specific wave climate and can range from 3 to 15 m. Surface floatation is minimized to prevent the transfer of wave-induced motion the backbone, and consists of nonstructural marker buoys for the anchor lines and a mid-backbone pick-up line that provides access to the crop from a service vessel. Anchors are generally spaced from 100 to 200 m apart, and depending upon the depth of the water and desired depth of submergence, the backbone length can range from 70 to 130 m. Ropes or "droppers" of mussels are suspended from the backbone, and additional submerged floatation is added as the crop gains mass during growout (Fig. 2).

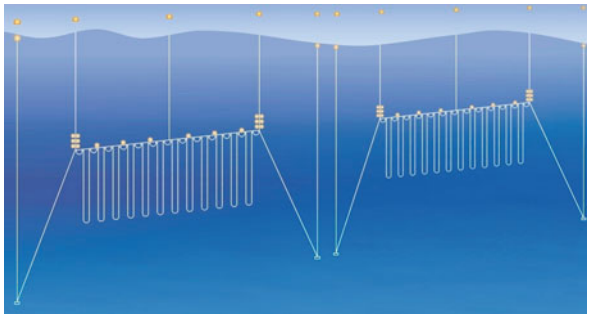
At some of the open ocean farms that have been established, converted fishing vessels are currently used to tend offshore longlines. The deck equipment required for tending lines to seed growout ropes and to inspect and harvest crops is similar to that in use for sheltered sites and includes rail mounted starwheels (Fig. 3) and an articulating crane (Fig. 4).

In addition, equipment common to many fishing vessels such as a lobster or crab trap hauler or a rotating boom is needed for lifting the submerged line to the surface. If there is sufficient deck space, bulk processing equipment such as declumping and debyssing machines can be used during harvest operations to reduce the need for extensive processing at shore-based facilities. Though converted fishing vessels may be used as this sector develops, it is likely that large, seaworthy, specialized vessels that can carry the harvesting and primary processing gear, provide a stable platform for lifting operations and a large load capacity for the harvest will be required to support large-scale operations. Vessels of this nature are in use in France and New Zealand [20].

In addition to submerged longlines, some experimental efforts have employed a submersible ring-like structure attached to a wind turbine tower, which has



Mussel Culture, Open Ocean Innovations. Figure 1
A schematic of a submerged longline used for suspension culture of mollusks in open ocean environments



Mussel Culture, Open Ocean Innovations. Figure 2
A diagram of a submerged longlines showing the attachment of mussel growing ropes to the backbone and the placement of floatation added to the backbone as the crop increases in mass during growout (From Langan and Horton [9])

Mussel Culture, Open Ocean Innovations. Figure 3
A forward looking view of the starboard side of a service vessel showing the backbone of a submerged longline set into aft (foreground) and forward starwheels. Growing ropes with seed mussels are attached to the backbone for the growout cycle

Mussel Culture, Open Ocean Innovations. Figure 4
A hydraulic articulating crane on a service vessel, shown here being used to unload equipment, is used extensively in mussel farming operations

been used for offshore macroalgae growout [21]. This device could potentially be used for mussel cultivation; however, there may be scaling issues in reaching the desired biomass.

Mussel Species in Open Ocean Cultivation

There are several species of mussels that are cultivated in open ocean waters; however, regardless of species or location, production is currently minor by comparison with well-established nearshore production sites. In North America, small quantities of blue mussels (*Mytilus edulis)* are produced in offshore farms in New England (USA) and Atlantic Canada and Mediterranean mussels (*M. galloprovincialis*) are being grown at an offshore farm off the southern California (USA) coast [22]. In Europe, *M. galloprovincialis* are grown on submerged longlines at exposed locations in the Mediterranean coast of France [23] and in the Turkish Black Sea. Culture trials have been initiated for *M. edulis* in the North Sea off the coast of Germany, [19] and in the Belgian North Sea [24]. Other European

countries, including Portugal, Spain, Italy, and Ireland are developing strategies for offshore mussel production.

In New Zealand, where the nearshore greenshell mussel (*Perna canaliculus*) industry is well developed and highly mechanized, there is a great deal of interest in developing large-scale ocean farms, as lease sites in sheltered nearshore waters have become difficult to obtain [25]. Initial efforts at open ocean mussel farming involved moving the double longline surface technology into more exposed sites and some success was achieved in wave conditions up to 2.5 m [26]. However, failure of surface longline systems in higher energy sites has led to the development of submerged technologies and a small number of open ocean mussel farms are operating in New Zealand offshore waters, with many new farms proposed [27]. This scale of expansion is projected to provide a threefold increase in production and export earnings by 2020 [28].

While data is limited to a few locations in North America and France, there are indications that production cycles and product quality for mussels grown in open ocean waters are highly favorable. Open ocean farms off the New Hampshire coast in the northeast USA have consistently produced market-sized (55 mm) blue mussels in 12–14 months from spat settlement with meat yields ranging from 42% to 58% [9]. Similar data has been reported for blue mussels at sites off the coast of Martha's Vineyard [29]. By comparison, rope-grown blue mussels from nearby estuaries and bays can take up to 18 months to reach market size [30]. Mediterranean mussels produced at an open ocean site in California have also demonstrated excellent growth and quality, reaching market size in 6–8 months and nearly 50% meat yield [22]. Trials in the North Sea have shown that the growth conditions in the German Bight are very favorable for mussel cultivation. Market-size (50–55 mm) can be reached by 12–15 months and infestation by parasites is much lower than in nearshore sites [10]. Faster growth at offshore sites may to be due to a more stable temperature and salinity conditions and therefore lower stress, reduced turbidity, and better water exchange [20].

Open Ocean Mussel Farming in Multiuse Facilities

Open ocean mussel farming can be practiced in isolation of other activities; however, there may be economic or environmental advantages to combining mussel culture with offshore fish farming or energy production. At a nearshore marine farming site in New Brunswick, Canada, Lander et al. [31] demonstrated better growth rates for raft-cultured mussels 100 m down current of a salmon farm than at reference sites, and was able to document that organic wastes, primarily fine particulates from feed emanating from the salmon farm contributed to the diet of the mussels. In open ocean sites, creating mussel culture "zones" in proximity to finfish farms may offset the effects of organic loading to the environment [32].

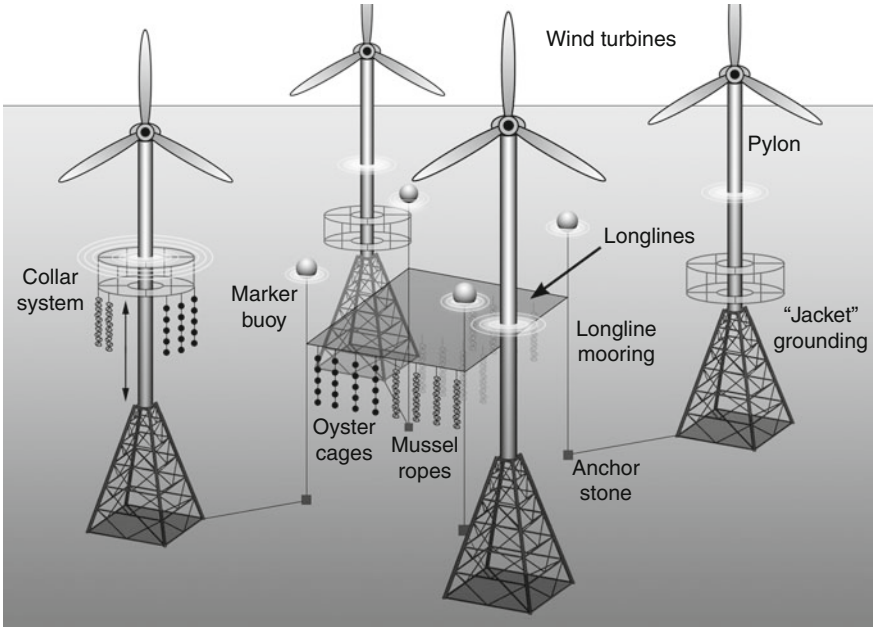
Energy installations may also provide structure for deployment of mussel culture systems. Mussels (*M. galloprovincialis*) have been harvested from oil platforms in California, USA for many years [33], and there is interest in using decommissioned offshore oil platforms as attachment points for mussel culture infrastructure.

Buck et al. [34] investigated the possibility of integrating suspension culture of oysters and mussels at existing offshore wind energy platforms in the North Sea (Fig. 5).

There are a number of advantages for conducting mussel cultivation activities within the footprint offshore wind farms. The placement of aquaculture production facilities in defined corridors between wind farm turbines eliminates the need for a separately permitted facility and reduces the space required if the two facilities were located separately [34]. Also, infrastructure for regular servicing may be shared. As both industries need a multifunctional service vessel, preferably with lifting capacities to install and change plant components and execute farming operations, and sufficient deck space to carry equipment and stock, the opportunity to share high-priced infrastructure exists [35]. Further, a combined environmental impact assessment for both users may reduce costs.

Environmental Considerations for Open Ocean Mussel Farming

Like all forms of food production, the culture of marine species, whether practiced in land-based, nearshore, or open ocean locations will have some effect on the environment. The effect can be both negative and positive and can vary depending upon the species,



Mussel Culture, Open Ocean Innovations. Figure 5
A schematic of shellfish growing systems associated with wind turbine towers (from Buck et al. [34])

location, and farming practices. In the past 3 decades of marine farming in sheltered marine waters, adverse impacts from aquaculture of both molluskan shellfish and finfish have been documented, though most of the concerns and controversy are centered on finfish. Mollusk culture is generally perceived as environmentally benign or even beneficial [5]; however, there have been documented environmental impacts from nearshore mussel farming that merit consideration for development of the offshore sector.

Though mussels feed on naturally occurring seston and no external feed is provided to the organisms, deposition of feces and pseudofeces can enrich bottom sediments beneath culture systems and impact benthic communities [36, 37]. Occurrences of sediment impacts have been associated with very dense culture in shallow embayments; therefore, if offshore farms are sited in locations with sufficient depth and adequate water circulation to disperse wastes, enrichment of bottom sediments should not be an issue [7]. High-density mussel culture can also deplete the water column of planktonic food, affecting both the growth and fitness of the cultured organisms as well as naturally occurring filter feeders in the system [38]. This too, is an impact that has been observed in sheltered embayments with limited circulation and is unlikely to be an environmental issue in open ocean waters [8]. However, in very large, high-density offshore farms, depletion of food within the farm and reduced growth and condition of the stock may be an issue for producers.

Hydrodynamic alteration is another environmental effect that has been documented in sheltered embayments with high-density shellfish culture [39] and has recently been an issue of concern in New Zealand where large-scale open ocean mussel farming is in development. Plew et al. [28] reported significant current and wave attenuation and strong water column stratification at a large (230 longline) mussel farm in Golden Bay, New Zealand. The farm was located in relatively shallow water (10–12 m) and the culture organisms were suspended from the surface to a depth of 8 m, therefore, occupying nearly the entire water column. As it is likely that open ocean development will use submerged culture in much deeper water (30–100 m) with ample space above and below the culture arrays, the severity of flow modifications as observed in this study are improbable.

Mussel Culture, Open Ocean Innovations. Figure 6
Seed collecting rope (*black*) is attached to the backbone of a submerged longline

A legitimate environmental concern for open ocean mussel culture is entanglement of whales and other marine life in seed collection lines [40]. These collectors are either discrete lengths of line or one continuous length of rope suspended from the backbone to provide substrate for settlement of mussel larvae (Fig. 6). As this sector develops, it is important to avoid deployment of seed collection lines in the migratory pathways of endangered marine mammals or to use weak links and electronic alert systems in the farming infrastructure [41].

Future Directions

Developments over the past 2 decades indicate that aquaculture production of mussels in open ocean environments is feasible and that opportunities exist for large-scale production [9, 10]. Conflicts with other uses can be significantly reduced, though they are not totally eliminated [34]. There is also evidence to support the premise that environmental impacts can be reduced by farming in open ocean environments [8, 36]. There is also strong indication that if sites are chosen properly, faster growth and excellent product quality can be achieved [9].

Though some technical challenges remain such as the development of large, purpose built, and highly seaworthy service vessels, obstacles to development of open ocean mussel farming are primarily economic, social, and political in nature. The scale of investment needed to establish and operate large-scale open ocean mussel farms is not well known, though it is assumed that production costs will be higher than for nearshore farming. The additional costs could be partially offset if ocean grown mussels, due to superior quality and greater consumer confidence in product safety can command a higher price [9], however, market prices are subjected to many economic externalities that are difficult to forecast. Space conflicts with the fishing industry may be an issue in some locations, therefore, involvement of local capture fishermen in industry development may be needed to gain acceptance of an alternative use of ocean space. As many countries move toward spatial planning of their territorial ocean waters, it is important to include a future vision of the potential for open ocean mussel farming in the planning process and give due consideration to compatibilities and possible synergies with other uses. Many countries also currently lack the regulatory framework for permitting open ocean farming sites. Until economic and regulatory uncertainties are resolved, entrepreneurs will be reluctant to make the level of investment needed to move this sector forward.

Ideally, development of open ocean farming should take place within the context of overall ocean management and marine spatial planning in order to assure compatibility with other uses and consistency with broader goals to restore and sustain the health, productivity, and biological diversity of the oceans.

Bibliography

1. FAO (2006) State of world aquaculture: 2006. Food and Agriculture Organization of the United Nations, Rome, FAO Fisheries Technical Paper # 500
2. NOAA (2005) Fisheries of the United States – 2003. NOAA, Washington, http://www.st.nmfs.gov/st1/fus/fus03/index.html
3. Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, Jackson JBC, Lotze HK, Micheli F, Palumbi SR, Sala E, Selkoe EK, Stachowicz JJ, Watson R (2006) Impacts of biodiversity loss on ocean ecosystem services. Science 314:787–790

4. Ryan J (2004) Farming the deep blue. Board Iascaigh Mhara Technical Report, pp 82
5. Shumway SE, Davis C, Downey R, Karney R, Kraeuter J, Parsons J, Rheault R, Wikfors G (Dec 2003) Shellfish aquaculture – in praise of sustainable economies and environments. World Aquacult 34(3):15–19
6. Buck BH, Krause G, Rosenthal H, Smetacek V (2003) Aquaculture and environmental regulations: the German situation within the North sea. In: Kirchner A (ed) International marine environmental law: institutions, implementation and innovation, vol 64. Kluwer Law International, The Hague, pp 211–229
7. Ward LG, Grizzle RE, Irish JD (2006) UNH OOA environmental monitoring program, 2005. CINEMar/open ocean aquaculture annual progress report for the period from 1/01/05 to 12/31/05. Final report for NOAA grant No. NA16RP1718, interim progress report for NOAA Grant No. NA04OAR4600155, Submitted 23, Jan 2006. http://ooa.unh.edu
8. Langan R (2007) Results of environmental monitoring at an experimental offshore farm in the Gulf of Maine: environmental conditions after seven years of multi-species farming. In: Lee CS, O'Bryen PJ (eds) Open ocean aquaculture – moving forward. Oceanic Institute, Waimanalo, pp 57–60
9. Langan R, Horton CF (Dec 2003) Design, operation and economics of submerged longline mussel culture in the open ocean. Bull Aquac Assoc Can 103-3:11–20
10. Buck BH, Thieltges DW, Walter U, Nehls G, Rosenthal H (2005) Inshore-offshore comparison of parasite infestation in *Mytilus edulis*: implications for open ocean aquaculture. J Appl Ichthyol 21(2):107–113
11. Jeffs AG, Holland RC, Hooker SH, Hayden BJ (1999) Overview and bibliography of research on the greenshell mussel, *Perna canaliculus*, from New Zealand waters. J Shellfish Res 18(2):347–360
12. Island Institute (Sept 1999) The maine guide to mussel raft culture. Island Institute, Rockland
13. Scott N, Tait M (1998) Mussel farming – an expanding industry in shetland North Atlantic fisheries college. Fisheries information note no. 1, October 1998
14. Bonardelli J (1996) Longline shellfish culture in exposed and drift-ice environments. In: Open Ocean Aquaculture: proceedings of an International Conference, Portland, 8–10 May 1996, Marie Polk editor. New Hampshire/Maine Sea Grant College Program Rpt. #UNHMP-CP-SG-96-9, pp 235–253
15. Michler-Cieluch T, Krause G (2008) Perceived concerns and possible management strategies for governing wind farm-mariculture integration. Mar Policy 32(6):1013–1022
16. Brenner M (2009) Site selection criteria and technical requirements for the offshore cultivation of blue mussels. Dissertation. Jacobs University Bremen, Bremen
17. SubSea Shellfish (Dec 2004) Biology and innovation primer project 2004. http://www.freepatentsonline.com/EP1476011.pdf
18. Stanley S (2005) development of a submersible raft for shellfish aquaculture. US Department of Commerce National Oceanic and Atmospheric Administration Small Business Innovation Research (SBIR). Abstracts of Awards for Fiscal Year 2005, pp 12
19. Buck BH (2007) Experimental trials on the feasibility of offshore seed production of the mussel *Mytilus edulis* in the German Bight: installation, technical requirements and environmental conditions. Helgol Mar Res 61(2):87–101
20. Holmyard J (2008) Potential for offshore mussel culture. Shellfish News, 25, Spring Summer 2008
21. Buck BH, Buchholz CM (2004) The offshore-ring: a new system design for the open ocean aquaculture of macroalgae. J Appl Phycol 16:355–368
22. SB Mariculture (2008) http://www.sbmariculture.com/
23. Brehmer P, Gerlotto F, Guillard J, Sanguinède F, Guénnegan Y, Buestel D (2003) New applications of hydroacoustic methods for monitoring shallow water aquatic ecosystems: the case of mussel culture grounds. Aquat Living Resour 16(2003):333–338
24. Van Nieuwenhove K, Delbare D (2008) Innovative Offshore Mussel Farming in the Belgian North Sea. www.vliz.be/imisdocs/publications/132623.pdf
25. Jeffs AG (2003) Assessment of the potential for mussel aquaculture in Northland NIWA client report: AKL2003-057, NIWA Project: ENT03101. National Institute of Water and Atmospheric Research Ltd, Auckland
26. Thompson NW (1996) Trends in Australasian Open Water Aquaculture. In: Open ocean aquaculture: proceedings of an International Conference. May 8–10, 1996, Portland, ME. Marie Polk editor. New Hampshire/Maine Sea Grant College Program Rpt. #UNHMP-CP-SG-96-9: pp 223–234
27. Stevens C, Spigel R, Plew D, Fredricksson D (Mar/Apr 2005) A blueprint for better mussel farm design. NZ Aquac 04:8–9
28. Plew DR, Stevens CL, Spigel RH, Hartstein ND (2005) Hydrodynamic implications of large offshore mussel farms. IEEE J Ocean Eng 30(1):95–108
29. Lovewell MA (2008) The fisherman. Martha's Vineyard Gazette. 28 Aug 2008. http://www.mvgazette.com/article.php?18129
30. Maine DMR (2009) The blue mussel in maine. Maine Department of Marine Resources. http://www.maine.gov/dmr/rm/bluemussel.html
31. Lander T, Barrington K, Robinson S, Mac Donald B, Martin J (2004) Dynamics of the blue mussel as an extractive organism in an integrated multi-trophic aquaculture system. Bull Aquac Assoc Can 104-3:19–29
32. Langan R (2004) Balancing marine aquaculture inputs and extraction: combined culture of finfish and bivalve molluscs in the open ocean. Bull Fish Res Agency Jpn Suppl 1:51–58
33. Richards JB, Trevelyan GA (Dec 2001) Mussel culture. In: Leet WS, Dewees CM, Kleingbeil R, Larson EJ (eds) California's living marine resources: a status report. California Department of Fish and Game, Yountville

34. Buck BH, Krause G, Rosenthal H (2004) Extensive open ocean aquaculture development within wind farms in Germany: the prospect of offshore co-management and legal constraints. Ocean Coast Manage 47(3–4):95–122
35. Michler-Cieluch T, Krause G, Buck BH (2009) Reflections on integrating operation and maintenance activities of offshore wind farms and mariculture. Ocean Coast Manage 52(1):57–68
36. Hatcher A, Grant J, Schofield B (1994) Effects of suspended mussel culture (*Mytilus spp*.) on sedimentation, benthic respiration and sediment nutrient dynamics in a coastal bay. Mar Ecol Prog Ser 115:219–235
37. Fabia G, Manoukian S, Spagnoloa A (Apr 2009) Impact of an open-sea suspended mussel culture on macrobenthic community (Western Adriatic Sea). Aquaculture 289(1–2): 54–63
38. Prins TC, Smaal AC, Dame RF (1997) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquat Ecol 31(4):349–359
39. Grant J, Bacher C (2001) A numerical model of flow modification induced by suspended aquaculture in a Chinese Bay. Can J Fish Aquat Sci 58:1003–1011
40. Lloyd BD (2003) Potential effects of mussel farming on New Zealand's marine mammals and seabirds: a discussion paper. Department of Conservation, Wellington, Vii: 34 p
41. Paul W (1999) Reducing the Risk of Open Ocean Aquaculture Facilities to Protected Species. In: NOAA (Ed.): National Strategic Initiative Project, Summaries 1999, Aquaculture Information Center – DOC/NOAA. 1–11

Nuclear Transfer to Produce Transgenic Mammals

Alexander Kind, Angelika Schnieke
Chair of Livestock Biotechnology, Technical University of Munich, Freising, Germany

Article Outline

Glossary

Cell cycle The sequence of stages between one cell division and the next. These are: gap 1 (G1) DNA synthesis (S), gap 2 (G2), and mitosis (M).

Chimera An organism composed of a mixture of genetically distinct cells that originate from two individuals.

Chromatin The natural form of genomic DNA within the nucleus. A highly structured multi-coiled fiber composed of a complex of DNA, RNA and proteins.

Clone (a) Molecular clone, an isolated DNA fragment propagated artificially in bacteria. (b) Cell clone, a group of genetically identical cells descended from a single individual. (c) Animal clone, an animal produced by embryo splitting or nuclear transfer.

Cytoplasm The portion of a eukaryotic cell outside the nucleus.

Differentiation A process by which a cell takes on a more specialized role or function.

Epigenetic regulation Regulation of gene expression by modification of chromatin not involving changes to the DNA sequence, e.g., by methylation of cytosine bases.

Locus A defined position in the genome.

Metaphase A stage of cell division when chromosome pairs are condensed and ready to be divided between the daughter cells.

Pluripotent The ability of a single cell to generate all cell types of the body. Pluripotent cells are distinct from totipotent cells in that they cannot make extraembryonic structures, such as the amniotic sac or the placenta.

Promoter trap A method of enriching gene-targeted cell clones against a background of random integrants. Expression of a selectable marker is conditional on integration at the desired location and driven by the promoter of the target gene.

Pronuclei Two structures formed from the sperm and oocyte genetic material following fertilization. Pronuclei later fuse to form the nucleus of the zygote.

Reprogramming A general term describing a radical change in the pattern of gene expression by a nucleus. This may be in response to a change in cytoplasmic factors, for example, after transfer of a nucleus from a somatic cell into an oocyte.

Somatic cell Cells of an organism other than the germ line.

Transcription The first stage in the expression of a gene, in which an RNA copy, or transcript, is made from a DNA template.

Transfection Term covering a variety of chemical, electrical and mechanical methods of introducing nucleic acids into cells.

Undifferentiated Relating to a cell that is capable of differentiation but has not generated specialized characteristics.

Zygote The one-cell embryo formed after fusion of the male and female pronuclei.

Definition of the Subject

Over the last 3 decades, researchers in molecular genetics and developmental biology have generated a repertoire of powerful biological techniques that allow genes to be isolated, analyzed, modified at will, then transferred and studied in cultured cells and live animals. This technology has revealed the functions of thousands of genes and dramatically advanced the knowledge of normal and disease states, providing the basis for numerous practical benefits especially in biomedicine. This entry tracks one strand within this interconnected and ever-broadening field: the use of nuclear transfer to generate transgenic mammals.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Transgenic animals were originally defined as those that contain additional DNA, such as a gene introduced from another species. The term "transgenic" is now commonly used to encompass a range of experimentally engineered genetic modifications including DNA deletion, rearrangement, and replacement. Here the word is used in this broader sense.

Nuclear transfer is the replacement of the nuclear genetic material of an oocyte or zygote with the nucleus of another cell. The technique has been applied in three main areas:

1. Therapeutic cloning – the generation of embryos and stem cells from differentiated cells.
2. Reproductive cloning – the replication of animals from cells, for example, where sexual reproduction is difficult or impossible, or to preserve valuable genetic material.
3. Cell-mediated transgenesis – the production of transgenic animals from genetically modified cultured cells.

The production of transgenic animals was a principal motivation for the development of nuclear transfer in mammals and is the focus of this entry.

This entry is structured as follows: Section "History and Introduction" gives some historical background to the conception and development of nuclear transfer. Section "Nuclear Transfer" outlines the current state-of-the-art and related transgenic technologies. Section "Future Directions" describes possible developments in the near future. This entry can however provide only an overview of this diverse and expanding subject. Interested readers can gain more detailed information by accessing the key papers, reviews, and books cited.

History and Introduction

Cloning animals by nuclear transfer is not a new idea. The first recorded reference is attributed to Yves Delage, a French marine biologist who wrote in 1895: "if, without any deterioration, the egg nucleus could be replaced by the nucleus of an ordinary embryonic cell, we should probably see this egg developing without changes" [1]. There is however no evidence that Delage actually carried out such an experiment. Hans Spemann described the first nuclear transfer in 1928 [2]. In a remarkable piece of microsurgery using micro-tweezers and a loop of hair from his baby daughter, he constricted a single-cell salamander embryo into two parts, one of which contained the cell nucleus. Left to develop, the portion with the nucleus divided and formed an embryo, while the other side remained a pouch of clear cytoplasm. As the embryo developed further, Spemann allowed a single nucleus to pass back into the empty cytoplasm; this reconstructed single cell then developed into another normal embryo.

In the early 1950s, Robert Briggs and Thomas King performed a series of nuclear transfer experiments with frogs. They removed the nucleus from an activated frog oocyte using a glass needle. A single cell dissected from a later stage embryo was then drawn up into a fine glass pipette connected by rubber tubing to a syringe. The cell broke open as it was squeezed within the pipette, and the free nucleus was then injected into the enucleated egg. Culturing the reconstructed embryos further, they found that cell nuclei from blastula stage embryos could support normal development to tadpoles. But nuclei from later stage embryos, in which the major embryonic cell lineages were already established, were unable to do so.

Transfer of nuclei following this basic scheme was continued by various researchers for several decades. The work was at first restricted to the large eggs of amphibians, but in the late 1970s, embryo culture and micromanipulation techniques improved sufficiently to use the far smaller and more vulnerable eggs of mammals. The overwhelming finding was that the developmental capacity of transplanted nuclei decreased with the age and extent of differentiation of the donor cell. Nuclei of very early embryonic cells had equivalent potential, but at some stage in development, it seemed that the fate of different cells became determined, "hard wired" in some way. The mechanism was unknown, but some form of irreversible modification or loss of nuclear DNA was viewed as a likely explanation. The concept of cell determination thus became widely accepted among developmental biologists. In 1984, James McGrath and Davor Solter seemed to put an end to the possibility of nuclear transfer in mammals. They systematically transferred nuclei from one-, two-, four-, eight-cell and blastocyst stage mouse embryos into enucleated one-cell stage embryos, zygotes. Nuclei from one-cell embryos supported

development to blastocysts, but success dropped off sharply using two-cell stage nuclei and failed entirely with later stages. They interpreted this as a rapid loss of nuclear capability during development and concluded their paper with the categorical statement: "the cloning of mammals by simple nuclear transfer is biologically impossible" [3].

However, strict cell determination remained difficult to reconcile with the well-known capacity for organ regeneration shown by various vertebrates, including most fish and some types of amphibian. If for example a newt loses a limb, cells from surrounding tissues migrate into the wound and undergo a process of reverse development, changing their identity to form a mass of rapidly dividing undifferentiated cells termed a blastema. Cells within the blastema then organize and undergo processes similar to those in embryo development to form a replacement limb [4]. This was good evidence that some adult differentiated cells are not determined in their fate and can radically change their identity. So, despite the repeated failure of experimental nuclear transfer, there remained the tantalizing possibility that the limitations were essentially technical rather than biological. This provided sufficient motivation for some researchers to continue testing.

In retrospect, it was unfortunate that early efforts focused on mice, because they are now known to be one of the more difficult species to clone by nuclear transfer. So, somewhat unusually, the major breakthroughs were made with livestock. The first indication that nuclear transfer from later stage cells might be possible came ironically just a few months before McGrath and Solter's paper was published. Steen Willadsen produced three Suffolk sheep by merging single cells from eight-cell embryos with enucleated unfertilized eggs [5]. It later emerged that the reason for the discrepancy with the mouse work was because McGrath and Solter had used enucleated zygotes for nuclear transfer, because mouse oocytes are too fragile to survive nuclear transfer. Willadsen had been able to use unfertilized oocytes, which are more robust in sheep. Years of work have subsequently shown that unfertilized oocytes are successful recipients for nuclear transfer in numerous species, while zygotes can only be used at a very particular stage.

In the decade that followed Willadsen's achievement, nuclear transfer remained restricted to cells of the very early embryo. While this was potentially useful in increasing the number of embryos of valuable animals such as prize cattle, there were few other practical applications. However, developments in transgenic technologies over the same period led to increasing pressure to improve nuclear transfer.

In 1980, it was reported that naked DNA microinjected into the pronuclei of fertilized mouse eggs could stably integrate into the host genome [6]. Microinjection by the same procedure was extended to livestock 5 years later [7]. A host of practical applications for transgenic livestock species were soon envisaged, including enhanced production characteristics, disease resistance, and the production of pharmaceutical proteins. However as time went on, it became apparent that DNA microinjection suffered numerous drawbacks. The process was inefficient and offered no control over where a transgene would integrate in the host genome. Randomly integrated transgenes exhibited a very wide range of expression, and this was a major problem for biotechnology companies seeking to make useful transgenic products, such as pharmaceuticals.

A quite different means of generating genetically modified animals, based on the use of cultured cells, was also developed during the 1980s. In 1981, Martin Evans and Gail Martin independently isolated embryonic stem (ES) cells from early mouse embryos [8, 9]. ES cells can be grown indefinitely in culture and then be incorporated back into a developing embryo. In 1984, it was found that these cells could also contribute to the germ line and produce functional sperm or oocytes [10]. This provided a means of establishing mouse strains derived from the ES cell genotype, including any experimental modifications. In 1987, a method of introducing predefined alterations into genes in situ, originally developed in tumor cell lines [11], was applied to ES cells by Thomas and Capecchi [12]. This technique, termed gene targeting, made it possible to engineer and study precise genetic alterations in whole animals. The phenomenal power of gene targeting in ES cells has provided an abundance of knowledge about the function of genes in mammals, and was recognized by the 2007 Nobel Prize for Medicine, awarded jointly to Mario Capecchi, Martin Evans, and Oliver Smithies.

Mice are vital for basic research, but by the late 1980s, it was apparent that many practical applications

in areas such as biomedicine and animal agriculture required ES technology or a functional equivalent in other species, particularly livestock. Despite considerable efforts, fully functional ES cells had not (and still have not) been derived from livestock. Despite the difficulties, transfer of nuclei from "ordinary" somatic cells was seen as a possible alternative means of transferring engineered genetic modifications from cells to whole animals. However, this would not be possible if nuclear transfer was restricted to cells obtained directly from early embryos. Genetic modification and powerful techniques like gene targeting require cells to be cultured for several weeks.

The breakthrough in nuclear transfer came in the 1990s, when it became clear that the cell-cycle stage of the nuclear donor cell must be correctly matched to the state of the recipient cytoplasm. Keith Campbell and Ian Wilmut of the Roslin Institute near Edinburgh made the key insight that only nuclei in G1 phase (prior to DNA replication) would support normal development in unfertilized oocytes. Transfer of nuclei at other stages in the cell cycle leads to aberrant chromosomal replication and inviable embryos. The method they developed and which is still widely used is to block cycling in the donor cells before DNA replication by reducing the amount of serum in the culture medium.

In 1995, two live lambs, Megan and Morag, were produced by transfer of nuclei from cells that were derived from a day 9 sheep embryo and then grown in culture for 6–13 passages [13]. The following year a similar experiment was performed that also included cultured adult sheep mammary cells. On 5 July 1996, a lamb was born from these cells and named Dolly [14]. Her birth showed that previous difficulties with nuclear transfer had indeed been technical. Adult cell nuclei were not irreversibly determined in their developmental potential and could be radically reprogrammed to adopt a new fate. Shortly after, transgenic and gene-targeted animals were produced, demonstrating that nuclear transfer from somatic cells was a practical means of generating genetically modified animals [15, 16].

Nuclear Transfer

Choice of Transgenic Methods

As mentioned in the introduction, the development of nuclear transfer in livestock mammals was largely inspired by the power of ES cell–mediated transgenesis in mice. The aim was to gain more precision and control over the type of genetic modifications that could be carried out. This section reviews nuclear transfer within the overall context of transgenic technology.

Methods of producing transgenic mammals can broadly be divided into two categories:

1. Direct transgenesis – the transfer of transgene DNA directly into embryos. Methods include DNA microinjection, viral transduction, transposon-mediated transgenesis, and sperm-mediated DNA transfer.
2. Cell-mediated transgenesis – the engineering of genetic alterations in cultured cells that are then converted to whole animals. Nuclear transfer falls within this category. The other major method is the incorporation of ES, or induced pluripotent stem cells, into developing embryos to produce chimeric animals.

Methods of direct transgenesis are more straightforward than cell-mediated transgenesis. However, at present these allow only transgene addition. Earlier techniques, such as DNA microinjection, are also very inefficient. Although not a significant drawback in mice, this is an important factor in livestock because gestation and maturation times are longer and maintenance costs far higher. For example, cattle have a generation interval between 2 and 3 years and each cow bears either one or two calves, while the generation time of mice is 3 months and litter size is between 6 and 12 pups. There has therefore been a strong incentive in livestock programs to reduce the number of animals gestating non-transgenic fetuses. Transgenic fetuses can be identified in utero by analysis of cells shed by the developing fetus and obtained by amniocentesis or allantocentesis. However, these procedures carry a significant risk of inducing abortion. Some efforts have also been made to detect and analyze fetal cells or DNA in the maternal circulation, but with little success. Definitive analysis of the integrated transgene must therefore be carried out in animals shortly after birth using small samples taken from blood, tail, or ear tips.

Methods of cell-mediated transgenesis share the important feature that genetic manipulation and analysis of the transgenic genotype are carried out in cells in the laboratory, rather than in animals "on the farm." These cells are then used to transfer the modified

genotype to whole animals. While cell-mediated transgenesis is more labor intensive than direct transgenesis, it offers significant advantages. Cells can be analyzed in detail so that only those with the desired genotype are converted to whole animals. Far larger numbers of independent transgene integration or other events can be screened and investigated in cells than in whole animals. This allows the selection and isolation of cells carrying rare integration events resulting from homologous recombination, the basis of gene targeting.

Nuclear transfer is currently the only practical form of cell-mediated transgenesis in mammals other than mice.

Nuclear Transfer Basics

The standard nuclear transfer procedure is to prepare a recipient cytoplast by removing the genomic DNA from an unfertilized oocyte. This is usually carried out by microsurgical withdrawal of a portion of cytoplasm containing the second metaphase plate using a micromanipulator. A low-tech option is to split the oocyte under a microscope using a thin blade, dye is then used to identify the half containing the metaphase chromosomes, a procedure termed "hand made cloning." The donor nucleus, or a whole cell, is then introduced into the enucleated oocyte by microinjection or electrofusion. Reconstructed embryos are activated with an electrical pulse to simulate fertilization, cultured if possible to identify viable embryos, and then transferred to the oviduct of foster mothers to complete gestation. Readers interested in detailed protocols are referred to a book by Verma and Trounson [17].

As mentioned in the introduction, the key insight that led to the success of nuclear transfer from a wide variety of cells was recognizing the importance of matching the cell-cycle stage of the donor nucleus and the oocyte cytoplasm. Oocytes of most mammalian species pause twice during the meiotic cell divisions that form the gametes: once before the first meiotic metaphase, and again at the second metaphase, at which stage the oocyte is mature and can be fertilized. Oocyte maturation and arrest are induced by a high level of a protein complex termed maturation-promoting factor (MPF). Fertilization causes a chain of events that result in proteolytic cleavage of MPF, breaking the arrested state and allowing the fertilized oocyte to complete meiotic division.

The level of MPF in the oocyte has a profound effect on the outcome of nuclear transfer. If a nucleus is transferred into oocyte cytoplasm with high MPF, the nuclear envelope breaks down and chromatin undergoes chromosome condensation, followed by nuclear reformation and DNA replication. A nucleus from a cell in G1 phase of the cell cycle will undergo normal DNA replication and can support normal development. However, a donor nucleus in S, or G2, phase undergoes aberrant re-replication of DNA, causing chromosomal damage or an abnormal chromosome number with consequent failure of development. Nuclear transfer into unfertilized oocytes can therefore be improved by synchronizing donor nuclei in G1 phase. This can be achieved by depriving donor cells of serum, blocking the cell cycle before DNA replication.

It has been proposed that the key to successful reprogramming of a transferred nucleus is the free availability in the recipient cytoplasm of factors necessary for regulating the program of gene transcription [18]. These are normally associated with DNA within the nucleus, but dissociate from the genome during cell division, and are released into the cytoplasm when the nucleus breaks down, ready to be distributed with the chromosomes into the new daughter nuclei. These factors reassociate with the DNA in G1 phase to reestablish the transcriptional program. Unfertilized oocytes have an abundance of such free factors, being paused part way through meiotic metaphase. Consistent with this model, if the genome of a zygote is removed during cell division, the resulting cytoplast can successfully reprogram an incoming cell nucleus to embryonic gene expression, but if the zygote nucleus is removed during interphase, it cannot [18].

Nuclear transfer from somatic cells opened a new route to produce transgenic livestock, and lifted the requirement for ES cells for cell-mediated transgenesis. Cells such as primary fetal fibroblasts can be obtained in large quantities, manipulated in culture, and then converted into whole animals by nuclear transfer. The initial work carried out in Edinburgh was inspired by the possibility of producing large numbers of genetically modified animals without conventional breeding,

termed "instant flocks," for applications such as biopharmaceutical production in milk [19]. The first large animal produced in this way was a Poll Dorset sheep "Polly" that carried a human clotting factor IX transgene, randomly introduced into the genome by in vitro transfection of fetal fibroblasts [15]. Shortly after, an α1-antitrypsin transgene was placed by gene targeting into a site chosen as likely to favor expression [16]. Somatic cell nuclear transfer has since been used to generate transgenic and gene-targeted animals in several other species.

From the outset, comparative data indicated that nuclear transfer requires fewer experimental animals than DNA microinjection to produce a useful transgenic animal [15], reducing costs and benefiting animal welfare. Techniques continue to improve gradually; for example, bovine nuclear transfer data from 1998 to 1999 showed an efficiency of 6.3% calves born per reconstructed embryo, while in 2003–2004 this increased to 15% [20].

Fetal and perinatal mortality and morbidity are the most serious issues that face nuclear transfer. The severity of the problem varies between species, cell types, and experimental regimes and is unrelated to genetic manipulation of the cultured cells. Rather, it is believed to be a consequence of defective epigenetic reprogramming of the donor nucleus and possibly incompatibility between the cell-derived nuclear genome and the oocyte-derived mitochondria. Cumulative data for cattle collected up to 2005 indicate that more than 1,500 cloned calves were born, of these, 60–70% survived normally to adulthood. The performance of these, including reproduction, was similar to non-cloned animals. Evidence is also accumulating that ill effects are limited to the first generation. Offspring, including those from two nuclear transfer parents, exhibit no increased morbidity or mortality.

Nuclear Transfer with Differentiated Cells

Transgenesis by nuclear transfer began with the use of abundantly available somatic cells such as fetal fibroblasts, mainly as a matter of convenience [15]. It is desirable to characterize as fully as possible cell clones used to generate whole animals. However, most primary cell types have a short lifespan in culture, allowing little time for transfection, selection, and the expansion of individual cell clones. Furthermore, prolonged growth in culture risks genetic and epigenetic alterations that may reduce their ability to support normal development.

A number of precautions can be taken to minimize the total time cells spend in culture. Genetic manipulation should ideally be carried out using primary cells at very early passage. Samples of each cell clone can be cryopreserved and retained as a frozen stock for nuclear transfer, while a replicate sample is expanded for further analysis. Determination of the modal chromosome number of cell clones is also useful because it allows those that have undergone gross changes in chromosome complement to be excluded. Frozen samples of cell clones identified as most suitable as nuclear donors can be thawed and used to generate whole animals. The viability and lifespan of primary cells can vary considerably between different isolates and are affected by factors such as the age of the donor animal and the method of preparation. It is therefore important to identify a suitable cell isolate before commencing a transgenic project.

The problem of short lifespan can also be overcome by "rejuvenating" cells by nuclear transfer and re-derivation from resulting fetuses. This allows successive rounds of in vitro genetic manipulation to be carried out relatively quickly, but does introduce a possible risk that genetic aberrations occur in the cultured cells, but remain undetected until animals are born. Serial nuclear transfer has been used to carry out multiple genetic manipulations in large animals. Three successive rounds of cloning were used to inactivate both alleles of the α1,3-GT gene in pigs [21]. In cattle, serial nuclear transfer has been used to inactivate immunoglobulin genes and the prion protein gene PrP [22, 23]. Some researchers have described a progressive decrease in the efficiency of development over successive clonal generations (e.g., [24]). However, there has been a recent report of 15 successive generations of recloned mice produced with no decrease in success rate using a chromatin-modifying reagent trichostatin A [25], suggesting that animal cloning can in principle be repeated indefinitely.

Nuclear Transfer and Gene Targeting

As mentioned in Section "History and Introduction," a major motivation for the development of nuclear

transfer in mammals was to circumvent the need for embryonic stem cells and enable gene targeting in species other than mice [16] In brief, gene targeting exploits the ability of a cell to support recombination between an exogenous DNA molecule and chromosomal DNA at regions of shared homology, as part of the DNA repair process. It can be used to inactivate individual endogenous genes by insertion or deletion, replace whole genes, precisely place transgenes in the host genome, introduce subtle gene modifications, and carry out large-scale modifications such as the deletion of megabase-sized DNA fragments. There are many variations and technical refinements, and the reader is referred to a review by Capecchi [26] for further details. Typically, cells are transfected with a DNA construct carrying an engineered modification flanked by DNA "arms" homologous to the target locus. At a certain frequency, homologous recombination occurs between the construct and the target gene and the engineered modification is seamlessly incorporated. The frequency of homologous recombination events varies by locus, vector, and the cell type used. Identifying cell clones carrying a targeted event among a large background of random integrants is key to success. Various methods have been devised to aid their isolation, the most powerful of which is to use a drug resistance marker gene that is expressed only if the vector recombines correctly at the chosen target site. Variations of this technique are referred to as "promoter trap," "intron trap," or "polyA trap" selection.

Nuclear transfer is currently the only practical method of producing gene-targeted livestock; however, it has become clear that gene targeting in somatic cells is considerably more difficult than targeting in mouse ES cells, and relatively few gene-targeted large animals have been generated. Genes so far targeted in livestock are: *COL1A1* in sheep [16], *PRNP* in cattle, sheep and goats [22, 27, 28], *GGTA1* in pigs [29, 30], *IGH* in cattle [22], and *CFTR* in pigs [31]. The main problems are the short lifespan of primary somatic cells in culture, as discussed above; the overall lower frequency of homologous recombination relative to random integration events in somatic cells; and the difficulty of targeting genes not expressed in the host cells.

Although direct comparisons are hard to find, it is generally understood that homologous recombination events are less frequent in somatic cells than in mouse ES cells [32]. Methods such as promoter trapping can enrich rare cell clones bearing targeted events, but lack of transcriptional activity in the host cell precludes their use. There is also evidence that spontaneous homologous recombination events are particularly rare for non-expressed genes [33]. This has made targeting of many potentially important genes difficult. There are however promising strategies to enhance the proportion of homologous recombination events in somatic cells, such as the synchronization of cells in late S phase so that vector transfection can be optimally timed [34], but these have yet to be fully evaluated. Current progress and a review of the field are provided by Laible and Alonso-González [35].

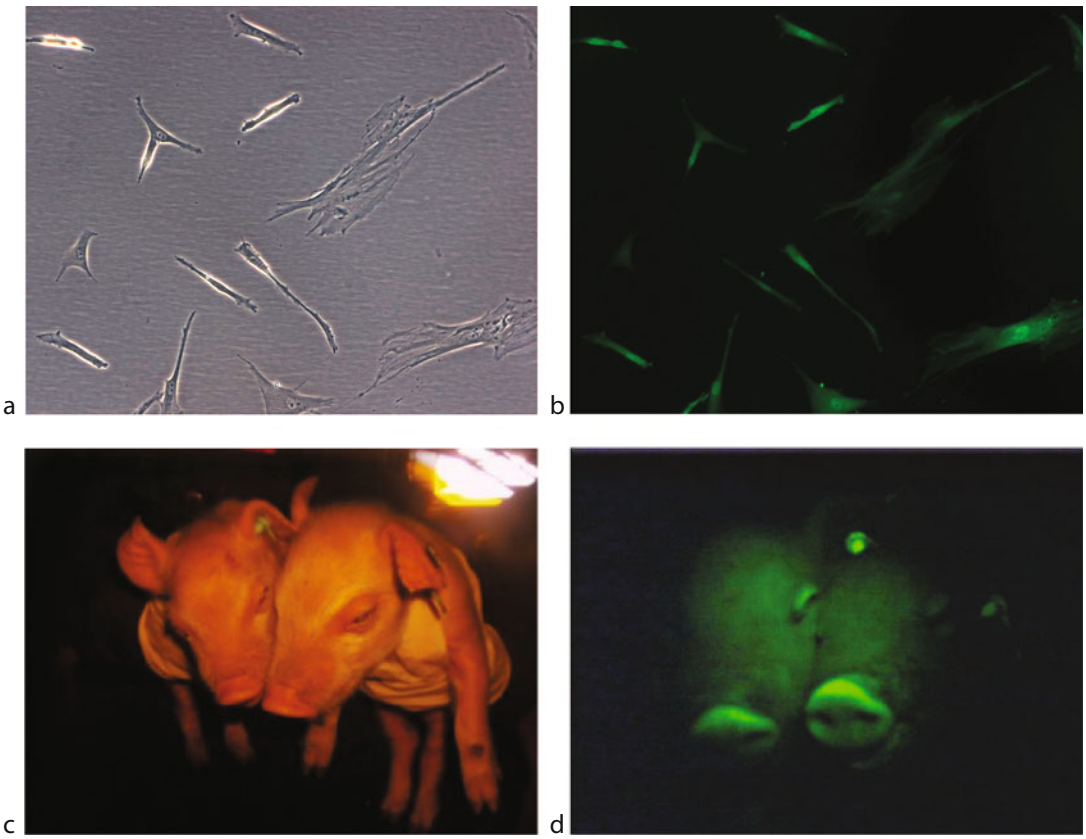
An entirely new approach to mammalian gene targeting is now emerging, based on the use of highly specific nuclease enzymes. This promises to overcome many of these problems and possibly supplant nuclear transfer for some applications, as discussed in Section "Beyond Nuclear Transfer – Direct Manipulation of Early Embryos."

Multipotent and Pluripotent Stem Cells as Nuclear Donors

The main advantage of multipotent cells, such as mesenchymal stem cells (MSCs), and pluripotent cells, such as ES cells, over more differentiated cell types is their longer lifespan and greater stability in culture. This facilitates genetic manipulation procedures and the isolation of cell clones for the generation of transgenic animals.

One of the defining characteristics of ES cells is that they can grow indefinitely as undifferentiated cells in culture while retaining the ability to form all tissues of the body. However, experience gained in mice is not always applicable to other species. From the few species investigated, it is clear that not all ES cells are as easy to handle as those from mouse. For example, human ES cells survive electroporation and dissociation to single cells poorly and this has made derivation of stable transfected human ES cell clones difficult [36]. Despite considerable efforts over more than a decade, few gene-targeted human ES lines have been reported [37].

However, the main problem is that ES cells are simply not available for most mammals. Artificial induction of pluripotency by the expression of key transcription factors [38, 39] offers an alternative to



Nuclear Transfer to Produce Transgenic Mammals. Figure 1
Legend (**a**) gfp-transfected porcine MSCs; (**b**) gfp-transfected porcine MSCs under UV light; (**c**) nuclear transfer piglets generated from gfp MSCs; (**d**) nuclear transfer piglets under UV light

classical ES cells that is rapidly being extended to other species. Induced pluripotent stem (iPS) cells can be cultured for long periods, and mouse iPS cells have already been used for nuclear transfer [40]. At the time of writing, the detailed characteristics of iPS cells from most species however remain unknown. It is perhaps cautionary to note that although candidate pig iPS cells have been reported by several authors, in each case, the reprogrammed pluripotent state was not stable in culture and relied on continued expression of exogenous transcription factors.

Multipotent mesenchymal stem cells (MSCs) are attractive alternatives to ES cells for generating transgenic animals. MSCs can be readily derived from bone marrow or adipose tissue. They proliferate well for long periods in culture and have been used successfully for nuclear transfer in cattle and pigs [41–44]. MSCs can be transfected and retain their multipotent identity and support preimplantation development of nuclear transfer embryos at rates similar to non-manipulated MSCs [42, 43]. Figure 1 shows an illustrative transgenic experiment in which a green fluorescent protein reporter gene was introduced into pig MSCs and whole animals derived by nuclear transfer.

Studies of reconstructed embryos produced by nuclear transfer indicate that an important cause of developmental failure is incomplete reprogramming of the transferred nucleus. This leads to deficient expression of early embryonic genes and failure to establish a normal embryonic pattern of epigenetic regulation and chromatin structure. It has been suggested that the epigenetic state of the donor nucleus influences reprogramming efficiency, and cells with a high degree of developmental plasticity, such as

multipotent and pluripotent stem cells should be more successful nuclear donors than differentiated cell types (e.g., [45]). In mice, a higher fraction of embryos derived from ES cells were found to develop to term than those from differentiated donor cells [46]. Evidence from other species is however less clear, although some studies in pigs describe higher rates of development to blastocyst with bone marrow MSCs than fetal fibroblasts [47, 48]. These findings were supported by analysis of gene expression, which revealed that porcine MSC-derived cloned embryos resembled normal embryos more closely than did those from fetal fibroblasts [48].

As mentioned in the previous section, another advantage of stem cells is that they may support homologous recombination more efficiently than other cell types, enabling gene targeting. There is insufficient data regarding MSCs, but evidence from mice indicates that iPS cells support homologous recombination at rates similar to ES cells [49]. Human ES cells also seem to support homologous recombination at a rate similar to mouse ES cells [50].

Beyond Nuclear Transfer: Direct Manipulation of Early Embryos

Sequence-specific DNA endonuclease enzymes that recognize long target sequences are new tools that promise to revolutionize genetic manipulation in many species. Enzymes of this type include naturally occurring homing endonucleases, often termed meganucleases, and artificially engineered enzymes such as zinc finger nucleases (ZFN), please see review by Cathomen and Joung [51]. Recent years have seen particular interest in the use of ZFNs in mammals.

ZFNs are artificial enzymes produced by fusing the cleavage domain of the FokI restriction endonuclease with a series of DNA-binding domains known as zinc finger motifs. Each ZFN is a heterodimer that can be produced to recognize a specific DNA target sequence of 18–24 base pairs. An appropriately designed ZFN can create a double-strand break at a single predetermined site in the genomic DNA. In eukaryotes, the error-prone non-homologous end-joining DNA repair pathway is often mutagenic, creating small insertions and deletions at the break site. Thus, careful choice of break site provides a means of inactivating a chosen gene, an alternative to gene knockout by classical gene targeting. ZFNs must be designed and custom made for each individual target site. A critical goal of enzyme design is to ensure that it does not produce double-strand breaks at sites other than that intended. Such off-target activity is likely to be toxic or mutagenic.

Perhaps the most important feature of this technology is that the frequency of gene inactivation can be so high that selection or enrichment of cells carrying the targeted event is unnecessary, effectively removing the need for cell culture. In 2008, ZFN-mediated gene inactivation was achieved directly in vertebrate embryos. Viable gene-targeted zebra fish were created by injecting mRNAs encoding ZFNs directly into one-cell embryos [52, 53]. This approach has now been extended to mammals, with the report of rats carrying inactivated IgM and Rab38 genes produced by ZFN targeting in embryos [54].

At present, ZFNs are expensive and have yet to be widely tested, but on present evidence, it seems likely that this approach could replace somatic cell nuclear transfer as a means of generating knock out animals in many mammal species. The exception being mice, where ES cells already provide a simple means of producing gene-targeted animals. ZFNs also promise to facilitate gene-targeted insertion or substitution of defined sequences in cultured somatic cells. As mentioned in Section "Nuclear Transfer and Gene Targeting," the low frequency of homologous recombination is a problem, and some genes have been difficult to target. Evidence indicates that a double-strand break at the target site dramatically increases the frequency of targeting events [55]. It has recently been shown that ZFNs facilitate gene targeting by homologous recombination in human ES and iPS cells, which had proved difficult [56]. It remains to be seen whether gene-targeted insertions or substitutions can also be achieved directly in embryos, this is a fascinating possibility that will undoubtedly be explored in the near future.

Future Directions

Science can be very unpredictable, and history is littered with authoritative, but inaccurate forecasts of future progress. So these comments are necessarily tentative.

What is already clear is the attention that nuclear transfer has brought to questions of cell identity and fate. The year 1997 marked a radical change in experimental biology; the concept of cell determination was

overturned and replaced by the possibility of complete reprogramming of cell identity. This inspired many talented people to embark on projects that were previously inconceivable, with perhaps the most notable outcome being the derivation of induced pluripotent stem cells. The production of primitive stem cells essentially at will, coupled with increasing knowledge of the normal development of many cell types, is fuelling intense research activity in regenerative medicine and will undoubtedly bring significant advances in medicine and benefit to human health.

The efficiency of nuclear transfer has improved, but advances have been incremental rather than dramatic. There is still considerable mortality during gestation and around birth. First generation nuclear transfer animals sometimes face health difficulties, probably as a consequence of epigenetic defects. These may be resolved or mitigated by the use of pluripotent stem cells as nuclear donors, but supporting evidence has so far been restricted to mice. At least for a while, somatic cell nuclear transfer remains the preferred option for the production of genetically modified livestock, and the next decade will likely see gene-targeted animals of many kinds being generated, especially for biomedicine. However, while nuclear transfer remains technically difficult and inefficient, alternative methods will continually be sought. It is possible that efficient methods of embryo incorporation such as laser-assisted injection [57] could supersede nuclear transfer, with iPS cells used to generate 100% chimeras.

Another notable new technology is the use of zinc finger nucleases in early embryos. It already seems likely that this approach might take over from nuclear transfer for the production of gene knockout animals. There is also the promise that the use of ZFNs in combination with gene replacement vectors in cultured cells will significantly improve the efficiency of conventional gene targeting and give access to more difficult genes.

The availability of an enabling technology is in itself insufficient reason to produce genetically modified animals. Any transgenic project requires an objective risk to benefit analysis in which the well-being of the animal is given a high priority. But, where there are real long-term benefits to the environment, or for animal or human health, then nuclear transfer and its successor technologies can offer powerful and precise tools.

Bibliography

1. Beetschen JC, Fischer JL (2004) Yves Delage (1854–1920) as a forerunner of modern nuclear transfer experiments. Int J Dev Biol 48:607–612
2. Spemann H (1928) Die Entwicklung seitlicher und dorsoventraler Keimhälften bei verzögerter Kernversorgung. Zeitschr für Wiss Zool 132:105–134
3. McGrath J, Solter D (1984) Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226:1317–1319
4. Gilbert SF (2006) Developmental biology, 8th edn. Sinauer, USA, pp 573–579
5. Willadsen SM (1986) Nuclear transplantation in sheep embryos. Nature 320:63–65
6. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 77:7380–7384
7. Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
8. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
9. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78:7634–7638
10. Bradley A, Evans M, Kaufman MH, Robertson E (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309:255–256
11. Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS (1985) Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature 317:230–234
12. Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51:503–512
13. Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66
14. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813
15. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KHS (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130–2133
16. McCreath KJ, Howcroft J, Campbell KHS, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066–1069
17. Verma PJ, Trounson A (2006) Nuclear transfer protocols: cell reprogramming and transgenesis. Humana Press, New Jersey

18. Egli D, Birkhoff G, Eggan K (2008) Mediators of reprogramming: transcription factors and transitions through mitosis. Nat Rev Mol Cell Biol 9:505–516
19. Kind A, Schnieke A (2008) Animal pharming, two decades on. Transgenic Res 17:1025–1033
20. Heyman Y (2005) Nuclear transfer: a new tool for reproductive biotechnology in cattle. Reprod Nutr Dev 45:353–361
21. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL (2003) Production of alpha 1, 3-galactosyltransferase-deficient pigs. Science 299:411–414
22. Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, Tomizuka K, Ishida I, Robl JM (2004) Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet 36:775–780
23. Kuroiwa Y, Kasinathan P, Sathiyaseelan T, Jiao JA, Matsushita H, Sathiyaseelan J, Wu H, Mellquist J, Hammitt M, Koster J, Kamoda S, Tachibana K, Ishida I, Robl JM (2009) Antigen-specific human polyclonal antibodies from hyperimmunized cattle. Nat Biotechnol 27:173–181
24. Kubota C, Tian C, Yang X (2004) Serial bull cloning by somatic cell nuclear transfer. Nat Biotechnol 22:693–694
25. Thuan NV, Kishigami S, Wakayama T (2010) How to improve the success rate of mouse cloning technology. J Reprod Dev 56:20–30
26. Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6:507–512
27. Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, de Sousa P, Travers A, Wilmut I, Clark AJ (2001) Deletion of the alpha(1, 3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat Biotechnol 19:559–562
28. Yu G, Chen J, Yu H, Liu S, Chen J, Xu X, Sha H, Zhang X, Wu G, Xu S, Cheng G (2006) Functional disruption of the prion protein gene in cloned goats. J Gen Virol 87:1019–1027
29. Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL (2002) Targeted disruption of the alpha1, 3-galactosyltransferase gene in cloned pigs. Nat Biotechnol 20:251–255
30. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS (2002) Production of alpha-1, 3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295:1089–1092
31. Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS (2008) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest 118:1571–1577
32. Arbonés ML, Austin HA, Capon DJ, Greenburg G (1994) Gene targeting in normal somatic cells: inactivation of the interferon-gamma receptor in myoblasts. Nat Genet 6:90–97
33. Thyagarajan B, Johnson BL, Campbell C (1995) The effect of target site transcription on gene targeting in human cells in vitro. Nucleic Acids Res 23:2784–2790
34. Zaunbrecher GM, Dunne PW, Mir B, Breen M, Piedrahita JA (2008) Enhancement of extra chromosomal recombination in somatic cells by affecting the ratio of homologous recombination (HR) to non-homologous end joining (NHEJ). Anim Biotechnol 19:6–21
35. Laible G, Alonso-González L (2009) Gene targeting from laboratory to livestock: current status and emerging concepts. Biotechnol J 4:1278–1292
36. Costa M, Dottori M, Sourris K, Jamshidi P, Hatzistavrou T, Davis R, Azzola L, Jackson S, Lim SM, Pera M, Elefanty AG, Stanley EG (2007) A method for genetic modification of human embryonic stem cells using electroporation. Nat Protoc 2:792–796
37. Elefanty AG, Stanley EG (2009) Reshaping pluripotent stem cells. Nat Biotechnol 27:823–824
38. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
39. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
40. Kou Z, Kang L, Yuan Y, Tao Y, Zhang Y, Wu T, He J, Wang J, Liu Z, Gao S (2010) Mice cloned from induced pluripotent stem cells (iPSC). Biol Reprod (Epub ahead of print)
41. Kato Y, Imabayashi H, Mori T, Tani T, Taniguchi M, Higashi M, Matsumoto M, Umezawa A, Tsunoda Y (2004) Nuclear transfer of adult bone marrow mesenchymal stem cells: developmental totipotency of tissue-specific stem cells from an adult mammal. Biol Reprod 70:415–418
42. Bosch P, Pratt SL, Stice SL (2006) Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. Biol Reprod 74:46–57
43. Colleoni S, Donofrio G, Lagutina I, Duchi R, Galli C, Lazzari G (2005) Establishment, differentiation, electroporation, viral transduction, and nuclear transfer of bovine and porcine mesenchymal stem cells. Cloning Stem Cells 7:154–166
44. Faast R, Harrison SJ, Beebe LF, McIlfatrick SM, Ashman RJ, Nottle MB (2006) Use of adult mesenchymal stem cells isolated from bone marrow and blood for somatic cell nuclear transfer in pigs. Clon Stem Cell 8:166–173
45. Oback B, Wells D (2007) Donor cell differentiation, reprogramming, and cloning efficiency: elusive or illusive correlation? Mol Reprod Dev 74:646–654
46. Rideout WM, Eggan K, Jaenisch R (2001) Nuclear cloning and epigenetic reprogramming of the genome. Science 293:1093–1098
47. Kumar BM, Jin HF, Kim JG, Ock SA, Hong Y, Balasubramanian S, Choe SY, Rho GJ (2007) Differential gene expression patterns in

porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. Dev Dyn 236:435–446
48. Jin HF, Kumar BM, Kim JG, Song HJ, Jeong YJ, Cho SK, Balasubramanian S, Choe SY, Rho GJ (2007) Enhanced development of porcine embryos cloned from bone marrow mesenchymal stem cells. Int J Dev Biol 51:85–90
49. Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R (2007) Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science 318:1920–1923
50. Cathomen T, Joung JK (2008) Zinc-finger nucleases: the next generation emerges. Mol Ther 16:1200–1207
51. Zwaka TP, Thomson JA (2003) Homologous recombination in human embryonic stem cells. Nat Biotechnol 21:319–321
52. Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol 26:702–708
53. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA (2008) Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat Biotechnol 26:695–701
54. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R (2009) Knockout rats via embryo microinjection of Zinc-finger nucleases. Science 325:433
55. Smih F, Rouet P, Romanienko PJ, Jasin M (1995) Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. Nucleic Acids Res 25:5012–5019
56. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 27:851–857
57. Poueymirou WT, Auerbach W, Frendewey D, Hickey JF, Escaravage JM, Esau L, Doré AT, Stevens S, Adams NC, Dominguez MG, Gale NW, Yancopoulos GD, DeChiara TM, Valenzuela DM (2007) F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. Nat Biotechnol 25:91–99

Ocean Farming and Sustainable Aquaculture Science and Technology, An Introduction to

Barry A. Costa-Pierce
University of New England, Marine Science Center, Biddeford, ME, USA

Aquaculture is closely tied to enhanced fisheries; indeed, marine fisheries enhancement by using the "aquaculture toolbox" is happening around the world and in some countries on a massive scale, e.g., in China. The science of marine enhancement is still in its infancy compared to other fields of fisheries science, but now shows potential to (1) increase fishery yield beyond that achievable by exploitation of the wild stock alone, (2) help restore depleted stocks, (3) provide protection for endangered species, and (4) provide critical information on the natural ecology, life history, and environmental requirements of valuable marine species (► Marine Fisheries Enhancement, Coming of Age in the New Millennium). The key to successful use of stocking is to plan enhancement programs from a fisheries/resource management perspective, using a broad framework and a transdisciplinary scientific approach where stakeholders are engaged from the outset in planning new programs.

Marine fisheries enhancement is a powerful tool that requires careful and interdisciplinary planning to control its effects. Marine enhancement will come of age in this new millennium as agencies and stakeholders align in truly group efforts. Few marine fisheries enhancement programs have enlisted all of the key elements of the Responsible Approach to Marine Stock Enhancement and the Code of Responsible Conduct for Marine Stock Enhancement. But these principles are now well described and laid out in a systematic manner. It is reasonable to expect that future, more sustainable fisheries enhanced by aquaculture will be successful as long as dedicated attention is focused on applying each of the key elements of the Approach and Code.

Polyculture is a sophisticated ecological precept illustrating the "niche concept." In aquaculture, it is ancient practice originating in China where the production of two or more noncompetitive species are cultured in the same physical space at the same time, often with the objective of producing multiple products from one system, and thus multiplying production and adding value. Polyculture is used primarily to enhance total production within an aquaculture facility while maintaining, and in many cases, enhancing water quality. Species used may be a combination of animals and plants, or aquatic and terrestrial species. In the modern context, polyculture systems have evolved to incorporate hydroponic systems (aquaponics).

Aquaculture may be an ancient practice, but until recently, the field has developed mainly on a trial and error basis. Modern aquaculture developments are organized principally in a traditional, compartmentalized manner designed to maximize financial returns. However, macroeconomic factors are not the sole drivers of success in aquaculture. Advancing sustainable aquaculture science and technology requires innovation in multiple fields, plus must incorporate innovative participatory processes that engage scientists, extension professionals, policy-makers, and the public. Aquaculture has advanced rapidly in many economically developing countries due to the formation of multidisciplinary teams that have worked together over 10–20-year periods not only to incorporate the latest biophysical and engineering advances but also to evolve innovative, socioecological, and ecosystem governance approaches to management and civil society (► Aquaculture, Sustainability Science in). In the twenty-first century, the environmental and social costs of aquatic food production cannot be externalized, and "social profit" is as important as economic. It is well recognized that the development of sustainable aquaculture is dependent not only on the technical needs for hatcheries to produce seed and feed mills to produce feeds but also on markets, equipment, and the overall capabilities of the entire "seafood infrastructure." Aquaculture is evolving rapidly and has a real chance to be a shining example of sustainable foods production for millions of people both rich and poor. A new development paradigm is needed, one that uses the principles and practices of ecology to break through disciplinary bounds and tie together real-world

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

knowledge, combining the findings of disparate, applied research disciplines in the ecological and social sciences in order to create a new, knowledge-based infrastructure and social-ecological support system (► Aquaculture, Ecological). FAO has developed guidelines for an ecosystems approach to aquaculture which can be used to design "aquaculture ecosystems" at many scales. Until recently, there were few commercial examples of successful models of these advanced aquatic food ecosystems, especially in marine waters, but the basis and content of two commercial operations are described here (► Aquaculture, Integrated Multi-trophic (IMTA), and ► Sustainable Ecological Aquaculture).

IMTA and SEA systems incorporate innovative engineering and ecological design components that demonstrate a clear transition from single to multiple species aquaculture production in the ocean. The concept is simple, but the practices are elegant. The farming of aquatic species from different trophic levels in proximity allows one species' uncontaminated (no toxicants used), uneaten feed and nutrient wastes to be recaptured and converted into fertilizers, feeds, and energy for other aquatic crops. IMTA and SEA systems combine "fed aquaculture" (e.g., finfish) with "extractive aquaculture," which utilizes inorganic waste nutrients (absorbed by seaweeds) and organic waste nutrients (filtered by shellfish) from the fed aquaculture system. The aim is to design and operate a balanced farming ecosystem for improved environmental sustainability, greater economic profit (improved outputs, lower costs, product diversification, reduction of risks, value added, ecolabeled products), and societal acceptability (job creation, improved governance, safe products, no contaminants).

Production of finfish species from coastal aquaculture systems has been limited until recently to the use of open, net pen cage systems. These systems are the most available (inexpensive in terms of cost/volume) and, thus, have proliferated where the regulatory environment has allowed their use inshore. Surface gravity cage systems are also the most controversial of all engineered aquaculture systems. They can be more easily destroyed in storms. And they are capital and labor intensive, requiring large and expensive hydraulic and feeding equipment for management, maintenance, net handling, washing, and cleaning.

In the Mediterranean, coastal finfish aquaculture in surface gravity cages has spread throughout the region. Progress has been made, but there is still a poor understanding of the environmental interactions of aquaculture in such a unique sea. Scientists remain unable to calculate the carrying capacity of areas of the sea for aquaculture (► Marine Aquaculture in the Mediterranean). There are many different types of habitats and ecosystems within the Mediterranean Sea so that it is essential that ecological and socioeconomic research address regionally specific issues. There is a special urgency to improve the understanding of fish pathology in Mediterranean aquaculture systems, especially in view of EU policies concerning the reduction of chemical use in the aquatic environment. Sustainable aquaculture within the Mediterranean Sea may be achieved by combining therapeutic treatments with health management strategies as breeding of tolerant fish, improving water quality, and vaccination.

Aquaculture in the open ocean or in high-energy coastal areas is predicted to grow rapidly in the twenty-first century due to space and resource conflicts in crowded coastal areas where most of the world's population resides. There are few commercial examples of successful models of well-studied commercial finfish cage operations in marine waters off islands, so the example of 5 years experience at the Kona Blue farm site, the evaluation of actual data, and observations recorded there are globally important (► Environmental Impacts of an Open Ocean Mariculture Operation in Kona, Hawaii). Baselines and evaluation of impacts of commercial aquaculture at such sites, their context, and their interpretations give invaluable insights into the future trajectory of sustainable open ocean aquaculture for many tropical nations.

Escaped fish may impact wild fish through competition, predation, habitat displacement, gene pool dilution, etc. In an attempt to reduce the numbers of escapees, rapid progress is being made throughout the world in the design of new, submersible containment cages that are easier to manage in difficult environments and are more storm resistant. One globally important aquaculture engineering innovation is the development of a modular "Aquapod™ cage system" which utilizes individual triangular net panels fastened

together to construct a rigid sphere as a secure enclosure. The Aquapod™ panels are made of simple structural members (plastic lumber from mainly recycled bottles) and wire mesh netting. Some individual panels or groups of panels have other functions, such as access, feeding, fish transfer and grading, harvest, mooring, and mortality recovery. Other individual panels may have pneumatically controlled flotation devices which allow an almost infinite orientation of the Aquapod™ in the water (► Aquapod Systems for Sustainable Ocean Aquaculture).

The rationale placement of aquaculture systems in the ocean is now being considered as an essential part of coastal and marine spatial planning (CMSP) efforts, and for some countries, the movement toward development of offshore renewable energy systems (wind, tidal, wave) has been viewed as a opportunity to examine closely the opportunity to site multiple, compatible uses in the ocean, such as the integration of offshore aquaculture with renewable energy systems. Analyses and field experiments have been conducted that merge submerged finfish cages and shellfish growing structures with offshore wind energy structures, and some important logistical, economic, and environmental advantages have emerged (► Aquaculture and Renewable Energy Systems, Integration of). Offshore windmills use the wind above the surface to produce energy, and their fixed pylons, concrete foundations, metal jackets, tripods, or tripiles offer excellent potential for a wide range of aquaculture systems. Pioneering studies conducted in Germany have examined the colocation of offshore wind farms and off-bottom offshore aquaculture for shellfish and algae. In addition, a broad-based stakeholder process involving all concerned in the multifunctional uses of offshore wind farms for aquaculture has been conducted which has helped dispel the many mistaken perceptions, concerns, and doubts about these ideas.

Engineering advances for open ocean shellfish aquaculture worldwide, initially adapted from deep water suspended scallop culture systems in Japan, push the frontier of shellfish production away from the multiple user conflicts of the world's coast and offer enormous potential for the production of low trophic level grazing shellfish species that could provide massive amounts of new, high protein, and fatty acid rich seafood for humanity. Advances have not been limited to new types of lines, moorings, buoys, but also in shellfish management and innovative extension methods to move capture fishermen into shellfish aquaculture (► Mussel Culture, Open Ocean Innovations). Offshore deepwater shellfish farming technologies have been shown to be effective for mussel production in very high-energy open ocean conditions (e.g., significant wave heights >10 m) in the northeast USA, and at a test site in the German Bight with significant wave heights >8 m and current velocities up to 1 ms^{-1}.

Shellfish and seaweed aquaculture are considered by many marine stakeholders to be best choices due to the elimination of many of the environmental concerns over feeds plus their beneficial impacts on particulate and nutrient pollution (► Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems). The sustainable expansion of seaweed aquaculture is also being driven by the needs for additional sources of high quality, nutrient-dense human foods (sea vegetables with high levels of omega-3's). Expansion of seaweed aquaculture is being considered in offshore and coastal areas but also in land-based systems. Land-based cultivation of seaweeds reduces the pressure on wild harvests, particularly those which are unsustainable and ecologically damaging, assists in the evaluation of numerous species that are unsuitable candidates for traditional, open water systems, and allows for increased environmental and input controls. High levels of control in land-based settings provide the necessary traceability, security of supply, high quality standards, and safety standards required not just for human consumption but also for nutraceutical and pharmacological applications (► Mariculture Systems, Integrated Land-based).

Shellfish aquaculture is one of the fastest growing sectors of the food industry, raising concerns about the influence of the activity on the environment. The sustainability of shellfish aquaculture and assessments on its trajectory toward sustainability involve a detailed review of the ecological impacts – both positive and negative – on nutrients, particles (filtration), exotic species, biodiversity, structure (habitats), and opportunities for incorporating best practices and developing innovative culture systems (► Shellfish Aquaculture, Methods of Sustainable). The concept of "acceptability" of impacts is critical to determining the

sustainability of shellfish aquaculture in the marine environment. Since the term "acceptable" is governed primarily by social values, the social carrying capacity of aquaculture should be the basis of a sustainability program to assess the sum of activities, not only including aquaculture, within a defined area. The mechanism toward identifying sustainable activities in the marine environment will progress when clear policies are elucidated and an inclusive approach is adopted reflecting legislative requirements and the views of all stakeholders.

Sustainable shellfish aquaculture will depend upon a strong but reasonable regulatory system, responsible farmers adhering to best management practices so that they raise do not deplete resources in a given area to such an extent that bivalve production is decreased, and a society that judges if shellfish aquaculture has an acceptable impact on the environment and is sustainable. Since there is intense competition for space and use in many coastal zones, the siting of shellfish aquaculture farms can be contentious. Thus, many organizations have stressed the importance of determining the carrying capacity of different areas for shellfish aquaculture. There are a number of ways that "carrying capacity" may be defined, including physical, production, ecological, and social, and the first three categories are to lesser or greater degrees related to social expectations and standards. A number of methods have been developed to calculate these different categories of carrying capacity for bivalve culture. Advances to estimate the different categories of carrying capacity have been made (► Carrying Capacity for Sustainable Bivalve Aquaculture). Scientific advances in modeling for shellfish aquaculture have encouraged the rapid development of a sustainable shellfish aquaculture industry, with excellent case studies from throughout the world (► Carrying Capacity for Aquaculture, Modeling Frameworks for Determination of).

There have been concerns that aquaculture has been moving away from its global responsibility to be more sustainable and to realize its altruistic goals of providing net benefits (additional foods) for a protein-hungry planet. Whether the word sustainability has become overused or not, it has catalyzed a forum for oversight of the growth and development of sustainable aquaculture on a global scale. The challenges to aquaculture sustainability are numerous from the biological, environmental, economic, technological, engineering, regulatory, and societal perspectives. Appropriate species need to be selected based on their biology, growth, and harvesting technologies, all adapted to local environmental conditions. Cost-effective, safe, and full containment engineering innovations will be required. Growing multiple species in an ecosystem sense will require aquatic farmers to develop additional "skill sets" since multiple farming practices are oftentimes completely different activities. There is one view that high value markets will have to be found for these additional "janitorial" species to justify their culture. There are also issues with permitting and regulatory authorities and not so trivial opposition from coastal and riparian land owners.

Sustainable aquaculture will also have to incorporate at the outset, and not as an afterthought, planning for not only the sustainable production of aquatic foods but also for innovative participatory social processes, community development, and the wider social, economic, and environmental contexts of aquaculture at diverse scales, both large and small. To judge the sustainability of new models of ecological aquaculture will require the development and use of new, more comprehensive models for decision-making for the evaluation of aquaculture production, such as life cycle assessments, an ISO-standardized accounting framework that allows for multicriteria environmental and social performance assessments. Such comprehensive methods will help a new cadre of innovators see the opportunities to evolve the next generation of sustainable practices in aquaculture. With an increase in ecological innovations coming to aquaculture, a higher level of sustainable intensification could be achieved. Important flows of natural resources will be increasingly understood, measured, used, and allocated more efficiently globally, regionally, and locally, which could result in the reallocation of resources more consciously into the most efficient animal and plant production systems for humanity. Such a sustainable intensification of ecological aquaculture will be essential in order to maintain and preserve aquatic ecosystems while providing additional high-energy aquatic foods for people by 2050.

Pig Breeding for Increased Sustainability

Pieter W. Knap
PIC International Group, Schleswig, Germany

Article Outline

Glossary

Allele Allele is one of the several possible forms of the DNA sequence at a particular locus.

BLUP (best linear unbiased prediction) BLUP is a method to estimate breeding values by taking account of the pedigree relationships among the individuals instead of assuming uncorrelated residuals as in ordinary least squares methodology.

Corticosteroids Corticosteroids are steroid hormones such as cortisol, produced in the adrenal cortex. They are involved in energy metabolism, immune response, stress response, and many other functions.

Cryoconservation Cryoconservation is the conservation of living material by freezing.

Effective population size Effective population size is a measure of the genetic variability of a population. It is defined as the number of breeding individuals in a stable population with a 1:1 sex ratio, no overlapping generations, and random mating and reproduction, that would lead to the same rate of inbreeding as what occurs in the population under study.

Fitness constraint Fitness constraint is a reduction of the health and strength of an individual.

Microsatellite Microsatellite is a repeating sequence of a few DNA base pairs, highly variable and therefore used as molecular markers in genetic analyses. Typically neutral, i.e., not associated with functional genes. The alleles differ in terms of the number of repeats, so there can be many.

SNP (single nucleotide polymorphism) SNP is a mutation of a single DNA base pair at a specific locus, possibly in a functional gene. SNPs are used as molecular markers in genetic analyses. They usually carry two alleles.

Stereotypy Stereotypy is a repetitive, apparently purposeless, behavior such as pacing, rocking, chewing, and licking during psychological distress. This behavior is seen in captive animals, often caused by the lack of options to exercise *other* instinctive behavior patterns because their required substrate (e.g., space or companions) is not available.

Definition of the Subject

The sustainability of farm animal production depends largely on strategies for animal management, health care, and nutrition, and on strategies for processing and marketing the products (e.g., meat, eggs, milk, and manure). Strategies for animal *breeding* exploit genetic and reproductive technology to better match the next generation of production animals to what the market requires. Breeding creates gradual changes in the animal species, providing a way to support sustainable development: better match the next generation of production animals to what enhanced sustainability requires. The technological challenge is to consider the balance among the various sustainability elements (profitability, human nutrition, environmental load, resource management, animal welfare), and to design genetic strategies to support that balance.

Introduction

Gamborg and Sandøe [1, 2] write about "applying the notion of sustainability" in animal breeding. They first explain why this is relevant at all: "animal breeding [. . .] is a largely unnoticed, yet economically vital part of the agriculture and food sector. But despite remarkable advances in productivity [it has] negative impacts: for example, on animal health and welfare, and on genetic diversity. This raises the question of what limits

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

to acceptable practice we should set in this area." This is about societal regulation of industry practice: about a *license to breed*.

These authors notice that "discussions of sustainability may open up dialogue on ethical issues and [may] help to set an agenda," and describe projects where "breeders were required [...] to develop a definition of sustainable farm animal breeding [...]. They were asked to identify their key concerns and priorities, to characterize any resulting dilemmas, and to suggest ways towards a meaningful operationalization.

The [...] breeders [could describe and clarify] the concerns they considered relevant [...]. But they found it harder to identify the concerns they had chosen to *exclude* [...] Most difficult [...] was the prioritization of potentially conflicting concerns. Roughly speaking, there are two ways to overcome conflict here:

1. technological solutions, in which relevant conflict is resolved through technological changes in breeding practice
2. increased transparency, in which clear statements about the relative priorities [...] are essential."

The rest of the above text focuses on point (2).

By contrast, the present chapter deals mainly with point (1), focusing on pig breeding while borrowing from poultry and cattle breeding where relevant. Prioritization of the relevant, and potentially conflicting, concerns is indeed difficult, but essential in any concrete case. Such a prioritization cannot be attempted here, as it will always depend on that concrete case.

The classical "triple bottom line" of Elkington [3] is extended here with a fourth element, leading to the sustainability targets

People — Pigs — Planet — Profit

The possible contribution of the technology of pig breeding and genetics to two of these targets is discussed here: *Pigs* is about animal welfare, *Planet* deals with biodiversity and pollution.

People is about social justice, with little connection to pig breeding technology. A possible case would be biopiracy which is more a political and economic issue than a technological one, and more relevant in the plant breeding sector – but see [4] for a case study of the immigration of Meishan into western pig populations, a commercial failure due to unforeseen technological developments. Influencing *Profit* by pig breeding has been covered intensely since selection indexes were designed – there is no need to repeat that here.

It must be borne in mind throughout that "sustainability will always be a matter of more or less: it can never be an absolute goal" [2].

Biodiversity

FAO's *State of the world's animal genetic resources for food and agriculture* [5] mentions 140 known extinct and 599 non-extinct pig breeds. Of the 599, 90% are *local breeds* (LB, occurring in one country only) and 6% are *international transboundary breeds* (ITB). Of these 599 breeds, 22% are "at risk" based on population size: roughly, populations with less than 1,000 breeding females or 20 breeding males are considered to be at risk, genetic survival being endangered. The risk status of 38% of the breeds is unknown, 40% are "not at risk."

Livestock breeds become endangered in many ways. FAO [5] gives three categories: (1) emergencies: drought, flooding, earthquakes, famine, war; (2) epidemics and zoonosis eradication campaigns; (3) most important: livestock sector trends, described earlier [6–8] in terms of displacement by other breeds, indiscriminate crossbreeding with exotic germplasm, overfocus on a single trait, no sustained breeding program, changes in production systems or producer preferences, and technology development. To this can be added the reduction of demand for the breed's products – for pigs, since about 1950, such a product would typically be fat. Most pig breeds have evolved as an intrinsic part of a production system, catering for demands for particular products. When the production system (or the demand for its products) disappears, its associated breeds (e.g., lard-type pigs) will disappear with it – unless they find an alternative niche.

Extinction of a livestock breed can be undesirable for several reasons. A pressing one is (A) when it contributes to "maintaining the identity of human communities" [9], e.g., pigs in the South Pacific, or even more when the livelihood of a human group depends on it; this typically involves ruminant breeds. Simianer [10] gives two other categories: (B) "the insurance

argument": "genetic diversity can be seen as an insurance against future changes, [with] the objective [. . .] to maintain sufficient genetic diversity to be able to adapt to the challenges that are ahead" – bearing in mind that those challenges are increasingly unpredictable in times of global climatic change and intensifying global trade; and (C) cultural arguments: "farm animal breeds must be seen as a man-made good with a long history, often parallel with [human] cultural development [. . .] and therefore similar arguments for conservation apply as for other cultural assets [such as historical] buildings or artwork".

All these arguments call for genetic conservation, most pressingly with regard to category (A). With regard to category (C), the question is how to objectivize the cultural *value* of a livestock breed [11], which will be necessary when the costs of its conservation are being budgeted.

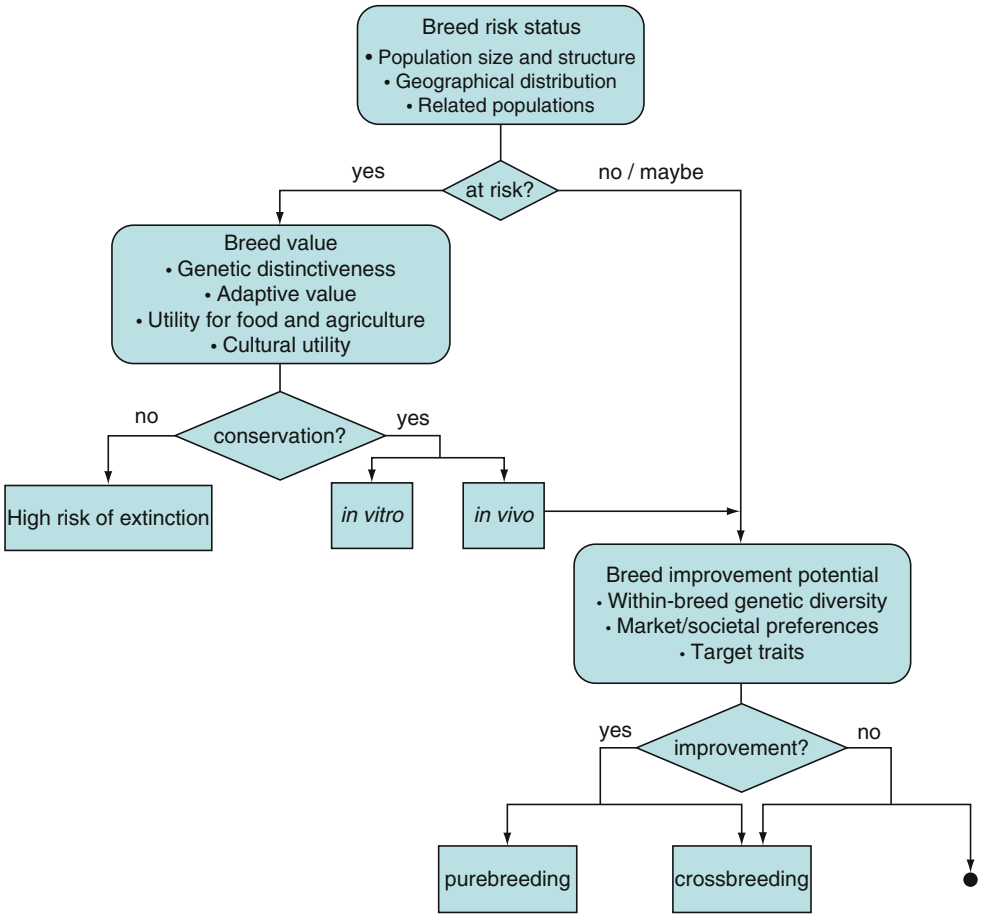
With regard to category (B), the main question is what kind of future challenges might require the breeding sector to exploit genetic material that previously has been unsatisfactory enough to become endangered; why [12] "preserve animals that farmers have abandoned"? The interest must then be in traits that were not important in mainstream breeding, e.g., meat quality traits (covering unexpected changes in consumer preferences) or robustness traits that facilitate adaptation to previously uncommon conditions, covering unexpected changes in options for health or climate control, or nutrition.

There is serious doubt among geneticists whether this insurance argument for breed conservation is realistic at all. To quote [13, 14], rearranged for consistency: "little use is made of conserved populations in mainstream commercial production of livestock; modern populations [. . .] are so far ahead of conserved strains in production traits, that adaptation of [the modern populations] offers far more opportunity than crossing back to far out of date stocks; even in countries where highly adapted breeds have evolved, typically these breeds are not perceived as having sufficient immediate utility to make them commercially viable; there are no present-day commercial animal-improvement companies or organizations that feel the need to invest in conservation as an insurance; the main perceived insurance benefit [. . .] is the conservation of adapted [sets of] alleles that are confined to one or a few breeds; there is [. . .] no reason to assume that there is [. . .] little variation in fitness associated traits in livestock populations simply due to selection; another important justification for conservation [. . .] is the value [. . .] for the increasingly powerful genomics analyses that have the potential to shed much light on the basic biology of adaptive traits; with moves towards genomic selection [. . .] the emphasis moves more towards best utilization of the large amounts of variation present in [. . .] commercial populations." Much earlier, Dempfle [15] wrote "only in very exceptional cases would a geneticist interested in improving [a leading] breed consider going to another breed to exploit interbreed variation, since most likely this would result in lowering of the mean. This is not likely to change very much, even with future technology."

Despite such skeptical points of view, endangered breed conservation is a current political commitment and an actively promoted reality, so it is valid to consider its technical issues.

Category (B) above centers around disappearance of (possibly) useful alleles – emphasizing within-species genetic diversity rather than particular breeds. From a technological point of view, in vitro cryoconservation would be adequate. So, allowing for the 38% "unknown" risk status breeds mentioned above, the technological challenge is to conserve the useful alleles carried by 130–360 pig breeds. Most of these are LBs.

Conservation of animal genetic resources is expensive, logistically complicated, and its worldwide funding is limited and fragmentary. Conservation of all genetic diversity is therefore not feasible and choices will have to be made with regard to funding the conservation of particular breeds, and not other ones: *global genetic resource management*. FAO [5] presents criteria (the breed's status, value, and potential for improvement) to support decision-making around conservation and genetic improvement actions, see Fig. 1. It holds several items where animal breeding and genetics technology can usefully contribute: (1) genetic distinctiveness; (2) population size and structure; (3) utility for food and agriculture, including adaptive traits. On a higher level, the balance between distinctiveness, risk status, and utility: (4) the ultimate priority level of the breed. Further elements are (5) target traits for genetic improvement and (6) genetic



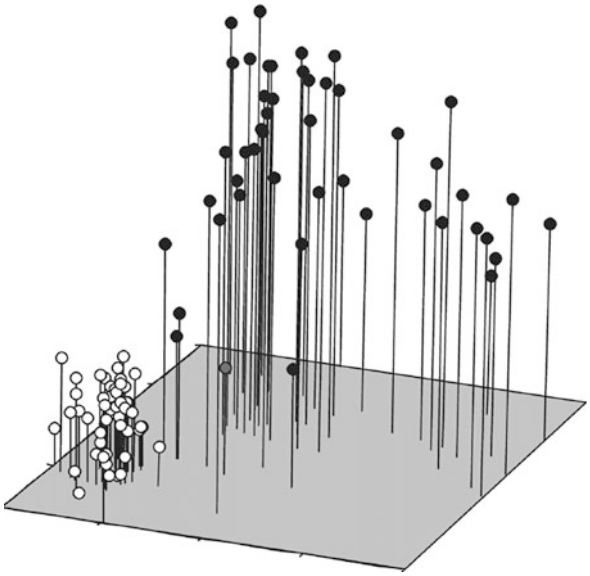
Pig Breeding for Increased Sustainability. Figure 1
Information required to design strategies for global genetic resource management (Modified from [5])

improvement programs. Sections "Genetic Distinctiveness" to "Genetic Improvement Programs" deal with these items in this order.

Genetic Distinctiveness

Megens et al. [16] describe the genetic diversity among 46 Chinese and 51 European pig breeds. Microsatellite marker genotypes were converted to genetic distance estimates among breeds, which can be worked into phylogenetic trees (dendrograms) and other cluster representations; see [17] for background information about various techniques. The results of this analysis are in Fig. 2, which shows genetic distance estimates among these breeds through three-dimensional scaling. This reveals a marked difference between the Chinese breeds (strongly diverse in all three dimensions, falling apart into five geographic clusters) versus the European breeds which form a separate cluster, much tighter when compared on the same scale. The European dendrogram (not shown here) gives more detail, suggesting distinct groups of English and south-European LBs. The authors relate their results in beautiful detail to the domestication history of the breeds.

An earlier study of roughly these same European DNA samples [18, 19] measured the *allelic richness* of these breeds, in terms of the mean effective number of alleles per marker (2.3–3.5 in the ITBs, 1.9–4.0 in the LBs, for microsatellites), and the number of *private alleles* (found in one breed only) – zero to five, with an outlier at 15.



Pig Breeding for Increased Sustainability. Figure 2 Estimated genetic distances among 46 Chinese (*black circles*) and 51 European (*white circles*) pig breeds, and one Sino-European synthetic (*gray circle*). The genetic distance between two breeds is quantified here by the physical distance between their two circles (From [16])

Many similar analyses have been reported since 1995 ([20]; four Belgian breeds). Recent ones involve three Brazilian LBs, one ITB and an industrial synthetic [21], and twelve North and South American LBs, three ITBs, and three wild varieties [22].

Obviously, the accuracy of such analyses depends on the number of markers per breed. Alves et al. [23] show a diminishing-returns pattern for the accuracy of clustering six pig breeds and wild boar, as a function of numbers of microsatellites analyzed – with ~24 markers required for 95% overall clustering accuracy, and ~35 to cluster the "less divergent populations." The accuracy of clustering parameter estimates is commonly quantified by bootstrapping. Felsenstein [24] notices that such resampling techniques are particularly valuable when the function to be estimated (e.g., a dendrogram) is algebraically complicated so that its variance cannot be derived analytically. An example of analytical derivation is in [25] where the standard error of the genetic distance estimate was obtained from observed allele frequencies. Contrary to data-specific procedures such as the bootstrap, analytical forms allow for algebraic rearrangement of terms so that data volume and structure required for a particular accuracy level can be obtained – as done empirically in [23].

To support the decision-making process of Fig. 1, quantification of genetic distinctiveness of a breed must consider within-breed and between-breeds diversity. The breed's (possibly unique) alleles will be of future interest more likely (1) when it shows a clear genetic distance to other breeds in the between-breeds diversity as in Fig. 2, and (2) when it shows a larger within-breed diversity (carries a larger number of different alleles). Also, a successful breeding program will be easier to implement for breeds with larger within-breed diversity, see the section on "Genetic Improvement Programs". Therefore a breed's genetic distinctiveness should be quantified by some combination of (1) genetic distance to other breeds and (2) within-breed genetic variability. Their relative weighting will depend on the value of parameters such as the proportion of total diversity due to diversity between breeds (Wright's F_{ST}, estimated at <0.3 in the above studies, naturally dependent on the sample's breed composition); the "standard" approach [26] is to weight item (1) by F_{ST} and (2) by $(1 - F_{ST})$. It will also depend on strategic issues like the breed's intended purpose. For example, the between-breeds component would count more if the breed must play a role in a terminal crossbreeding program [27] and less if the breed must be merged into a synthetic for subsequent genetic improvement. Approaches are described, discussed, and/or illustrated by [28–31] and sources referenced therein. A practical suggestion [32] is "to consider how much diversity the breed adds to a core set constituted by commercial lines or breeds that are already subject to successful conservation."

All the above presumes a random approach: no specific traits are targeted and genetic diversity is valued as a neutral entity. Neutral markers like microsatellites are appropriate if future needs are indeed unknown: "since we need to maintain the genetic capacity to cope with challenges not even known today, this can be best accomplished by maintaining neutral genetic diversity" [10].

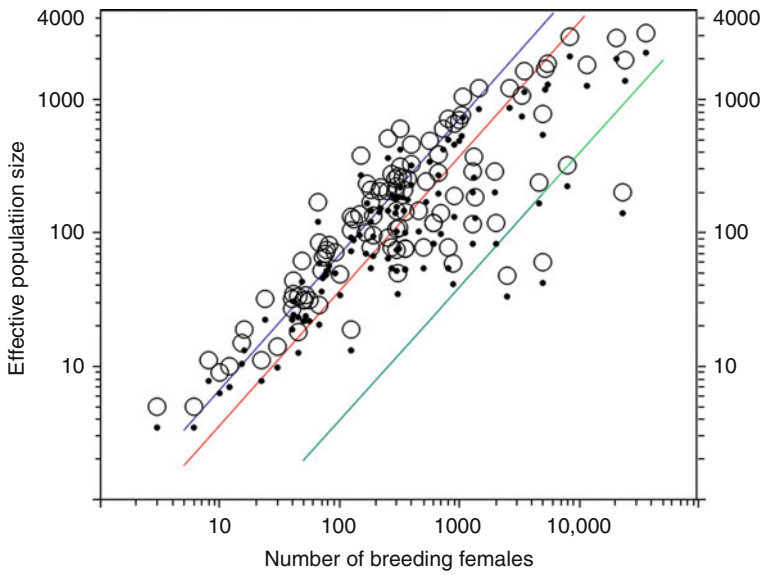
Nevertheless, some studies have deliberately used markers associated with specific traits. Ciobanu et al. [33] argue that "the relationship between variability at

neutral marker loci [. . .] and adaptation or individual fitness is still unclear" and "to characterize a breed not only in terms of genetic distance [. . .] but also in terms of variation at interesting loci associated with phenotypes, [. . .] will give more opportunity to elaborate an efficient strategy for conservation of breeds, maintaining their 'useful' genetic diversity and providing important resources for possible new unique traits." Likewise, half of the markers typed by Iannuccelli et al. [34] were SNPs "chosen for their position close to interesting QTLs." They mention that in addition to comparisons between breeds, they have "focused on the diversity within genomic regions containing genes that influence economically important traits, the variability of which is supposed to have evolved under the influence of artificial selection. [This has] allowed us to reveal regions where artificial selection favored certain alleles. For some SNPs where both alleles were found in all breeds, the frequencies were very different between breeds, suggesting different selection histories" (translated). A method to combine neutral diversity and diversity due to selection into a single criterion is presented in [35].

Population Size and Structure

There are three ways to estimate effective population size (N_e). Two make use of quantitative genetics theory [36].

First, from census counts of breeding males and females (N_m, N_f), typically through approximations developed for scenarios without selection, such as $N_e \approx 4\times(N_m\times N_f)/(N_m+N_f)$, possibly expanded with information on variation in family structures. This method has been applied to Romanian, Japanese, and Croatian pig breeds [37–39]. The European Farm Animal Biodiversity Information System (EFABIS; http://efabis.tzv.fal.de) holds data on livestock breeds, including N_e derived this way. Figure 3 shows its estimated N_e values for 111 pig breeds, in relation to their N_f values. These datapoints are not on a straight line because of variation in the (N_f/N_m) ratio; the reference lines indicate where N_f equals 5 (*top*), 10, or 100 (*bottom*) times N_m. Therefore, datapoints above the two highest reference lines represent situations with less than five (or ten) recorded breeding females per recorded breeding male – not very feasible scenarios in pig breeding,



Pig Breeding for Increased Sustainability. Figure 3
Reported numbers of breeding females (N_f) in 111 European pig breeds, and the associated N_e values from $4\times(N_m\times N_f)/(N_m+N_f)$; N_m is the number of breeding males. The reference lines indicate where N_f equals 5 (*top*), 10, or 100 (*bottom*) times N_m. Open circles, raw data; small dots, multiplied by 0.7 in an attempt to adjust for the effects of selection (Data from EFABIS, December 2009)

certainly at N_f values above 100. This only illustrates the difficulty of obtaining consistent census counts, and the limitations to deriving credible N_e estimates from them.

Second, through the rate of inbreeding (ΔF) as calculated from pedigree analysis, exploring the fact that $N_e = 1/(2\,\Delta F)$ under random mating. The main resource required here is a complete pedigree. This method has been applied to Japanese, American, German, and Finnish pig breeds [38, 40–42]. The approach can be taken an important step further by focusing on its across-breeds distribution characteristics [43].

A third approach makes use of molecular genetics technology, with various ways of analyzing marker data – for an overview see [44]. Álvarez et al. [45] analyzed the full pedigree of a small fragmented sheep population and genotyped microsatellites. They conclude that co-ancestry coefficients as estimated from pedigree data and from molecular data become rapidly correlated when pedigree depth increases, so that the expense of recording "molecular information in well established conservation programs may not be justified"; on the other hand, for small populations with a shallow pedigree "neither [pedigree] nor molecular information by themselves are sufficient [...]; each available parameter offers partial information."

For the correlation between pedigree-based and molecular measures of diversity "to be substantial, a considerable number of loci is required and, more importantly, a high variance of the [pedigree-based] inbreeding values should be present; [...] it should be preferable to use pedigree information whenever available, and limiting the use of markers to verify, correct, complete or even implement pedigree recording" [32].

Of course, in livestock breeds, deep and complete pedigrees are almost as scarce as dense DNA marker data, so "markers could be most useful in cases where little information on population history is available" [46]. Toro et al. [47] give a beautiful example of two LB varieties with complete 20-generation pedigree data, but these were maintained on an experimental farm – not a common situation.

Therefore, the molecular approach is widely seen as potentially more powerful than classical quantitative methods. Aspi et al. [48] write: "Owing to variation in family size and overlapping generations [...] N_e is [...] difficult to estimate from demographic field surveys. [...] Genetic methods may provide more effective ways for estimating N_e." They genotyped microsatellites and estimated N_e at ~40. Moreover, from those same data "large genetic variation was found in the population despite a recent demographic bottleneck. No spatial population subdivision was found, even though a significant negative relationship between genetic relatedness and geographic distance suggested isolation by distance." This is about a wolf population; similar quantification of N_e, demography, and subdivision would be very useful in livestock genetic resource management.

An effective population size of 40 would be regarded as dangerously low. Meuwissen [49] mentions a "critical effective size" (below which fitness steadily decreases) of 50–100, at least in populations not selected for traits negatively correlated to fitness. Ollivier et al. [50] transform N_e into the *extinction probability* P_{ext}, operationally defined as the expected level of inbreeding (accumulating deleterious mutations, which eventually leads to extinction) after 50 generations: $P_{ext} = 1 - e^{-50/(2N_e)}$. With a pig generation interval of 1 year, this goes back to [51] where risk of extinction is based on cumulative inbreeding over 50 years. The above $N_e = 40$ works out as a dangerous 46% probability that the population will not survive 50 generations. A slightly different approach is the *degree of endangerment* described by [52]. The relationships among P_{ext}, N_e, and ΔF are described in more detail in [53–55], at different levels of complication. The latter source also considers the estimate's accuracy, which will be useful once such estimates are to be used in practice.

Effective population size influences the extent of genetic drift, which affects the development of linkage disequilibrium (LD), i.e., the correlation between genotypes at different loci. Loci that are closer together are less often separated by recombination – so they are more strongly correlated, with higher LD values. In terms of data analysis of biallelic loci, the relevant relationships can be generalized [56] in terms of what is actually happening (due to N_e) and of what can be observed (due to sample size) as

$$E(r^2) \approx \frac{1}{\alpha + kcN_e} + \frac{1}{n} \qquad (1)$$

$E(r^2)$ is the expectation of the square of the abovementioned correlation, a common parameter to

quantify LD. Parameter α equals 1 in the absence of mutation, and 2 if mutation is accounted for; k equals 4 for autosomes, and 2 for the X chromosome; c is the recombination rate between the markers being analyzed (their genetic distance from each other, in Morgan); n is sample size.

For an analysis of many autosomal markers, ignoring mutation, this works out as

$$N_e \approx \frac{1-r^2}{4cr^2} + \frac{1}{4cr^2(nr^2-1)} \quad (2)$$

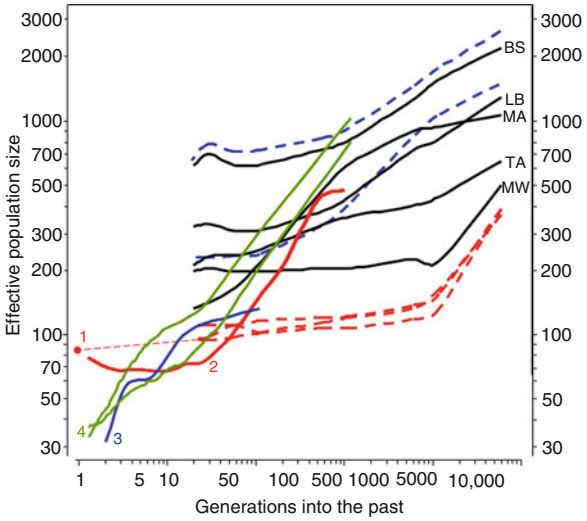
Hayes et al. [57] introduced *chromosome segment homozygosity* (CSH), an alternative parameter to r^2 for quantifying LD. CSH has a smaller sampling variance than r^2, but the same approximate expectation (Eq. 1). When population size changes linearly over time, the effective population size of 1/(2 c) generations ago can be approximated as (1 – CSH)/(4 c CSH), the same form as Eq. 2 when n is large.

Accordingly, when many individuals are genotyped for many biallelec DNA markers varying widely in their mutual distance c, then the (supposedly linear) history of N_e can be traced by plotting $\frac{1-CSH}{4cCSH}$ against $\frac{1}{2c}$.

Amaral et al. [58] estimated LD decay (reduction of r^2 with increasing c) on SNP markers in ten European and ten Chinese pig breeds (subsets of the breeds in Fig. 1). Figure 4 shows those data reworked into Hayes's relationship, using r^2 instead of CSH, for the European breeds ($N_e \geq 10{,}000$ for the Chinese breeds). Comparable data on commercial pig lines [59, 60, 226] has been added.

Figure 4 illustrates that this method can reveal interesting information about a population's demography. Such information would be most relevant to livestock genetic resource management when it covers recent generations, as [59, 60, 226] do. This requires large c values: from the equation above, the situation of two and five generations ago is represented by LD among markers 0.25 and 0.10 Morgan apart. The maximum distance between Amaral's markers was 0.03 Morgan.

This methodology is statistically demanding. England et al. [61] report on "simulations to show that [the most widely used LD] estimator is strongly biased when sample size is small [...] and below true N_e. This is probably due to [LD] generated by the sampling process itself." They also proposed



Pig Breeding for Increased Sustainability. Figure 4
Time trends in effective population size for 14 European pig breeds, estimated from linkage disequilibrium decay patterns according to [57]. Blue dashed line, transboundary breeds; black solid line, local breeds (British Saddleback, Large Black, Tamworth, Middle White, Mangalica); Others, commercial lines: "1" from [67]; "2" from [59]; "3" from [60]; "4" from [226] (All other data from [58])

"a way to determine whether a given sample size exceeds population N_e and can therefore be used for computation of an unbiased estimate." Waples [62] confirmed this by describing how Eq. 1 above is inappropriate for low values of n and N_e, particularly when $n < N_e$.

The accuracy of the N_e estimator of Eq. 2 depends not only on the numbers of individuals and markers involved, but also on N_e and c. Based on [44], for a system with n individuals genotyped for m pairs of markers, each with a within-pair distance of c Morgan, an approximation of the standard error of estimated N_e is

$$\mathrm{stderr}(N_e) \approx \left(N_e + \frac{2c(2-c)}{(1-c)^2+c^2} \times \frac{N_e^2}{n} \right) \times \sqrt{\frac{2}{m}} \quad (3)$$

From this equation, estimates of $N_e = 100$ or $N_e = 200$ for five generations into the past (c = 0.1 Morgan) would require n = 100 individuals genotyped for

m = 400 or m = 750 marker pairs to achieve a standard error of 10% of the estimate (i.e., 100 ± 10 or 200 ± 20). Similar accuracies for one generation into the past (c = 0.5 Morgan) would require m = 3,000 or m = 10,000 marker pairs. N_e estimates for more recent situations require more data for a given proportional accuracy. However, high accuracies (low standard errors) are easier achieved for lower N_e levels – often the more interesting ones from a resource management point of view.

Other molecular approaches to estimate N_e trace changes in allele frequencies over time (the *temporal* method), or derive (small) population size from (large) sampling errors of observed heterozygote proportions deviated from Hardy–Weinberg proportions (the *heterozygote excess* method). Schwartz et al. [63] give a review; further developments are described in [64–66]. The latter paper introduces the immigration rate into the equation – a useful issue in livestock scenarios with their common introgression of extraneous germplasm. Abdallah et al. [67] also take immigration into account when analyzing 12 of the breeds of Fig. 2. One of these was also analyzed by Amaral [58]; Fig. 4 gives Abdallah's estimate as the end point of the Hayes approach to Amaral's data.

Relative Utility

Comparisons of pig lines are difficult to organize and expensive to run: (semi)-governmental versions of such tests have run for several decades as *commercial product evaluation* in western Europe, focusing on growth and carcass traits. Reproductive traits are sometimes included by across-farm analysis of routine field data, with its inevitable and unpredictable bias. Robustness traits are very rarely included, not surprisingly given the demanding nature of categorical trait analysis. Such a test was designed [68] for growth and carcass traits to detect differences between genotypes of 0.25 trait standard deviations at 95% significance, with a statistical power of 75%; this led to a scheme with, on average, 67 sires per genotype, 2.6 litters per sire, and 1.8 tested pigs per litter. Subsequent tests also covered reproduction and longevity traits; these schemes specify 65 sires, 3 litters per sire, and 2.7 tested daughters per litter [69]. The statistical significance of breed differences must be tested against the variation among sires within breed, which requires inconvenient sampling schemes.

Gibson et al. [70] (condensed here) discuss characterization for production and robustness traits: this can only be genetically meaningful when the production environment is properly accounted for; environmental factors are so complex that records from different locations or times cannot be validly compared; valid breed comparisons are possible, first, when breeds are recorded simultaneously at the same location under identical management [as in the previous paragraph]; second, through meta-analyses linking records from different locations or times through overlapping breeds, statistically adjusting for environmental effects; such meta-analyses are powerful, but only valid when genotype×environment interactions are negligible (as in controlled confinement conditions); it will remain problematic that lifetime productivity traits are extremely difficult to record.

These authors stress that the functionality of information systems "must be greatly increased to allow extraction and customized analysis of phenotype and molecular genetic data within and between data sources; [. . .] breed information can be linked to [. . .] environment and production system mapping, allowing [. . .] disease resistance and adaptation traits to be predicted from past and current breed distribution and use." They conclude that "these are substantial but fully achievable functions."

Tixier-Boichard et al. [46] emphasize characterization for robustness: "local breeds survive in harsh environments and this needs to be better understood; epidemics are major threats for all animal genetic resources across the world; climatic change is likely to increase the spread of tropical diseases to temperate areas. [Scientific evidence] that local breeds are adapted and resistant [. . .] has been obtained in several instances [. . .], well documented for parasitic diseases [. . .], with local breeds maintaining a better performance in the presence of parasites and/or exhibiting lower levels of parasite infestation [tolerance]". These authors take a utilitarian position: "data on production systems, phenotypes and molecular markers should be used altogether in an integrated approach to characterization. [. . .] Decisions regarding conservation should incorporate all descriptors. Conserving without documenting would be useless."

In summary, a livestock breed's direct-use utility value should be assessed in terms of production traits and robustness traits like disease resistance/tolerance, adaptation to unfavorable conditions, and lifetime productivity. This assessment must use any available phenotypic and molecular data (the latter element comes back to the work of Ciobanu [33] and Iannuccelli [34], section "Genetic Distinctiveness"). All this will require extensive information systems, difficult to achieve but not impossible. Without such functionality, utility-directed breed conservation is not feasible.

Of course, phenotypic and molecular characterization can only focus on known characteristics. The main drawback is that the traits of true future interest (covering unexpected changes in consumer preferences, or in options for health or climate control or nutrition) cannot, by definition, be defined or measured. This makes it difficult to combine the utility issue with the insurance argument for conservation: it calls for option values. Viral diseases (e.g., PRRS, PMWS, influenza) are expected to break out on a wide scale every few years [71, 72], and any breed that happens to carry full resistance would suddenly have a very high utility value. This would require fast and widespread testing, and challenging logistics to distribute the relevant alleles throughout the worldwide pig industry – which may well become feasible with further development of genomics and reproductive technologies. But that particular breed's utility is likely to drop dramatically again, as soon as the next outbreak (of a different virus) occurs.

Urgency, Importance, and Feasibility: Priority Level of the Breed

To quote Wikipedia, Covey et al. [73] introduced "a framework for prioritizing work that is aimed at long-term goals, at the expense of tasks that appear to be urgent but are in fact less important." This is about time management, but the same is relevant in global genetic resource management. Sections "Genetic Distinctiveness" to "Relative Utility" describe different features that might make a livestock breed a candidate for conservation – but limited funding requires prioritization. Obviously, highest conservation priority should be given to breeds that (1) have a great utility, (2) are strongly distinct from other breeds with much within-breed variation, and (3) are strongly endangered due to inadequate population size or structure. Items (1) and (2) are about *importance*; item (3) is about *urgency*. This requires integration of these issues, preferably quantitatively so that priority levels can be ranked and funding allocated. Another element to include is then (4) the cost of conservation – introducing the issue of *feasibility*: "identified benefits could be quantified so that society has some sense of how much the conservation is worth. Society can then determine how much they would want to spend on a conservation effort" [74].

A comprehensive way to deal with the above elements [75] defines the genetic distinctiveness of a breed (calculated with the relevant emphasis on between-breeds and within-breed diversity) as D, its utility value (for all relevant purposes) as U, recalls its extinction probability P_{ext} (based on its N_e and possibly on additional parameters) and defines the cost of reducing it (through any relevant conservation action) by ΔP_{ext} units as C. Then the priority ranking R for such a conservation action would simply be

$$R = \frac{(D + U) \times \Delta P_{ext}}{C} \quad (4)$$

This would be calculated for every breed in the conservation portfolio, assuming that all their D, U, P_{ext}, and C values can be directly compared – which requires D and U to be expressed in the same unit, and everything to be calculated using the same algorithm and parameter definitions across breeds. Breeds with higher R values get a higher priority for conservation – because their conservation is more important (D, U), more urgent (P_{ext}), and/or more feasible (C). The probability P_{ext} takes values from 0 to 1, and ΔP_{ext} from 0 to P_{ext}: reducing the extinction probability by its full value (i.e., $\Delta P_{ext} = P_{ext}$) comes down to safeguarding the breed entirely.

These authors notice that the costs of the most complicated in vivo, in situ conservation schemes would likely be proportional to conservation effort, i.e., $\Delta P_{ext}/C$ is roughly constant and ranking is based on D and U. By contrast, the costs of the simplest in vitro cryoconservation schemes might vary only little, i.e., C is roughly constant and ranking for complete safeguarding is based on the *cryoconservation potential* $(D+U) \times P_{ext}$. Real-life conservation programs would fall between these extremes.

Simianer et al. [76] compared three forms of the actual relationship between C and ΔP_{ext}, applied these to a set of breeds characterized in terms of genetic distances and extinction probabilities, and found that "conservation funds should be spent on only three to nine of the 23 breeds, depending on the model used." This approach was further formalized [77] into a comparison of *maximum-risk*, *maximum-diversity*, and *maximum-utility* strategies to determine the optimum set of breeds to conserve, which favors the latter strategy which combines diversity and utility – although it obviously requires the quantification of U, which makes it difficult to implement in practice. The latter two approaches quantify the *expected conserved diversity* or the *expected conserved utility* of possible sets of breeds that may be successfully conserved at some point in the future, and then calculate the *marginal diversity* or *marginal utility* of each breed by differentiating with respect to the breed's P_{ext}. These are then multiplied by P_{ext} to obtain the breed's conservation potential, similar to the term $(D+U)\times P_{ext}$ of Eq. 4.

As argued at the end of the section on "Relative Utility," utility is the weakest element here: conservation aims at the future, and future utility cannot be predicted. All the other elements can in principle be dealt with by a complete pedigree and/or dense DNA samples from ~100 individuals. One of the options would be to drop U from the equation and rank conservation priorities on D only (the *maximum-diversity* option of above). This reasoning is taken to its logical extreme by suggesting to "devote the majority of present conservation budgets to freezing [...] samples from existing breeds [...] concentrate on ova and sperm from abattoir material, and somatic cells, e.g., ear clips (the latter in anticipation of the increasing effectiveness of somatic cloning)," because future "genomic tools will open up completely novel means of exploiting genetic resources" [14]. In line with this, the USA has "invested in the establishment of an in vitro conservation program and a genebank [covering 18 local pig breeds and one ITB at the time of reporting]. Collections are being built up very quickly, in close collaboration with the industry. Breeding companies use the genebank as a backup of their breeding work. In Canada, a program for in vitro conservation [...] will be implemented in the near future" [5], as later documented by www.ushrl.saa.ars.usda.gov/SP2UserFiles/Place/54020500/documents/update%208-10-02.pdf and dsp-psd.pwgsc.gc.ca/collection_2008/agr/A52-88-2008E.pdf.

Target Traits for Genetic Improvement

Livestock breed improvement requires a breeding goal and a selection strategy best suited to the needs of the production system and the market that it supplies – this is one of the elements of *population-level genetic resource management*. Animal breeding technology has a long tradition in this field, but improvement of an endangered breed may require substantially different goals and strategies than in mainstream industry breeding programs. FAO [5] write: "the most appropriate strategies for managing these breeds may involve only limited genetic change [...] to maintain adaptation to the local environment and disease challenges, and [...] to maintain the level of a production trait [...] if this is currently [near] an optimum level." Of course, the insurance argument for breed conservation assumes that the breed may, at some time, support a different production system than its original one, because it happens to carry alleles (likely adaptive ones) of large utility, then and there. This adaptive utility must be balanced with production utility, which will likely be much lower than in commercial lines. The actual uptake of the breed as a source of adaptive alleles will be much easier when the production-related lag is limited. So for the insurance argument, genetic improvement of production traits is desirable – but not at the expense of adaptive quality: possible genetic antagonisms between production traits and anything else require specific attention. "The genetic basis of population differentiation for fitness traits will be non-additive, with different adaptive gene complexes evolved in each breed. Genetic improvement programs therefore should start with an adapted population, with selection then for production traits" [78].

Another argument in favor of improvement of production traits is that many endangered breeds are endangered precisely because they lag behind other breeds in terms of production traits. The common idea that improvement of production traits inevitably leads to a reduction of robustness is false – it just requires a sensible breeding program, see the section on "Robustness."

Genetic Improvement Programs

As argued above, any livestock breed with a future must support an agricultural production system. Rege [79] writes: "the most rational and sustainable way to conserve animal genetic resources is to ensure that indigenous breeds remain functional parts of production systems, that is, *conservation through use*. This is possible only if economically important attributes of indigenous breeds are identified, studied and incorporated in breed improvement programmes." Genetic improvement will always be required – unless the production system is completely static, without any interactions with the world around it. A useful concrete example is from [80]: "during the past few years the Limousin pig, endangered and neglected during the 1970s and 1980s in favor of the better-performing large breeds, has become popular with consumers looking for quality. Today it is victim to its own success with supply being lower than demand, so that it has become necessary to develop its productivity" (translated).

This requires a breeding goal and a selection strategy best suited to the needs of the production system and the market that it supplies, as discussed in the Section on "Target Traits for Genetic Improvement." A checklist of items that play a role here, from definition of the product and the market to evaluation of the breeding program's profitability, is in [81].

The breed also has to be *maintained* – preserving as much of its genetic variation as is feasible. And its qualities must be *exploited* in an optimal way, making efficient use of other resources.

Livestock breed *maintenance* requires strategies and tools to keep N_e sufficiently high. This comes down to keeping inbreeding under control, usually by minimizing co-ancestry in the breeding population. There is a possible antagonism with the previous point: selection in a population reduces N_e, so a balance will have to be found and maintained. There are many rules-of-thumb to delay inbreeding (e.g., *keep one son from every sire* or *maximize generation intervals*); an example of implementation in commercial lines is in [82]. Many of these rules perform well on the short term but have unexpected long-term effects, and they usually reduce genetic improvement unpredictably, leading to uncertain genetic resource management. N_e can be affected by age at first breeding and culling policy in quite counterintuitive ways [83], so that "the general tendency is contrary to the expectation that [N_e] would increase with increasing [longevity]." High longevity increases generation length but reduces genetic drift; the combined effect favors a short productive lifetime, so that N_e can actually be increased by early culling.

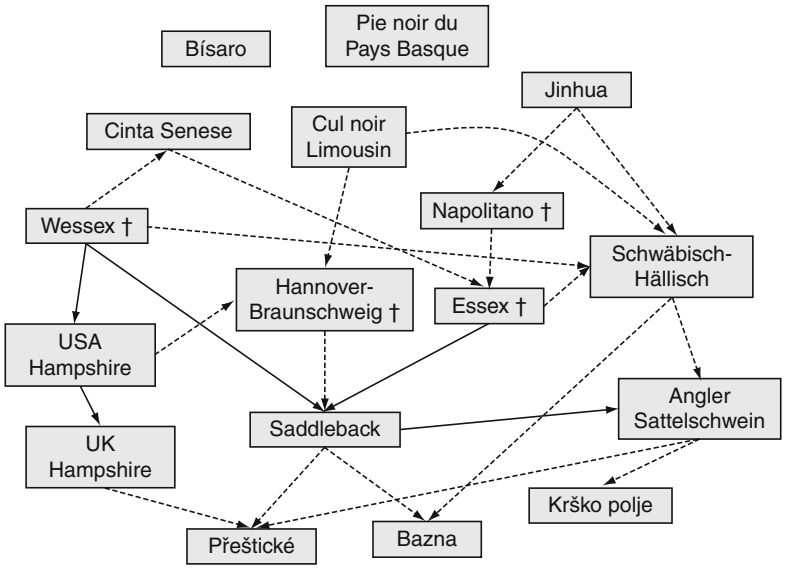
A better solution is to apply co-ancestry management and mate selection based on optimum contribution theory or similar frameworks. The principles are covered in detail in [49, 84, 85]. The latter source's *mate selection index* (MSI) is an optimized criterion, based on an objective function such as [86]:

$$\mathrm{MSI} = \mathrm{EBV} - \lambda_1 \mathbf{x}' \mathbf{A}\mathbf{x} - \lambda_2 \overline{F} \qquad (5)$$

Here, (1) EBV holds estimated breeding values; (2) $\mathbf{x}' \mathbf{A}\mathbf{x}$ represents average co-ancestry in the system: A holds the additive relationships among all animals, weighted by their contributions x (numbers of progeny) to the next generation; (3) $\overline{F}$ is their average inbreeding coefficient, calculated from A; λ_1 and λ_2 are positive weighting factors. This is a cost-benefit equation, with element (1) representing the benefit and (2) and (3) representing the (genetic) cost: EBV, A, and $\overline{F}$ are known, so the system can be solved to deliver the contributions x that give the best (genetic) cost-benefit for particular values of λ_1 and λ_2. The result of this is a list, based on x, with animals to select and animals to mate to each other (the optimum selections and optimum matings that lead to optimum contributions).

Proper genetic resource management can lift populations much smaller than the FAO threshold of 1,000 breeding females to the "not at risk" level. Many industrial pig lines are maintained at far less than that population size (e.g., see Fig. 4) with a secure genetic future. Careful immigration and admixture is regular practice in the breeding industry, but it is often avoided by breed societies and similar structures for chauvinistic reasons.

For example, Fig. 5 shows an intensive network of historical genetic connections among the European black-belted and saddled pig breeds, with a genetically useful loop with Hampshire (an ITB). Many of these populations have a herd size far lower than 1,000 sows, and most of those focus on the same set of



Pig Breeding for Increased Sustainability. Figure 5
Genetic connections from ~1,770 (Jinhua) to present, among black-belted and saddled pig breeds. Consecutive series of arrows do not imply any chronological development. Solid arrows represent the main source of a breed; dashed arrows imply the existence of other sources not included here. (Data from [16, 37, 222–225]; Lenoir H, 2010, IFIP Institute du Porc, Le Rheu, France, Personal communication; www.elbarn.org; www.besh.de; efabis.tzv.fal.de; dad-training.fao.org/cgi-bin/EfabisWeb.cgi; www.thepigsite.com/info/swinebreeds.php)

characteristics (meat quality and robustness, and of course coat color) when describing their distinctiveness in the associated Web sites. This would suggest a clear case for the quantification of the various elements of Eq. 4 above, followed by regular and careful genetic exchange to make resource management easier.

Exploitation: A common negative term in the literature on genetic resource management is *indiscriminate crossbreeding*. For example, "very often, crossbreeding has been indiscriminate and the local breeds that underpin the crossbreeding program have been lost because of a lack of understanding [...] that these pure breeds must be maintained to support the system" [87]. This is clearly a management issue. On the other hand, these authors mention systems where crossbreeding is "used for gradual breed replacement with [...] the controlled [...] formation of composites [...] for specific production systems," as "a rapid method of introducing desirable traits into local well-adapted breeds," and "as a way out of a narrowed genetic base in commercial breeds." The logical way of making use of exotic germplasm without any risk of endangering the LBs is "structured cross-breeding systems, such as 'terminal crossing' where [F_1] animals are slaughtered or where specialized crossbred dam lines are used." Pig examples are from Germany where Angler Sattelschwein, Schwäbisch-Hällisch, and Bunte Bentheimer are crossed with Pietrain; and from Spain where various Iberico strains are crossed with Duroc, all to optimize meat quality versus meat quantity in the terminal F_1 product.

This chapter is about science and technology. But "logistics, not science, is the underpinning of a successful breeding policy. Without a system for handling the details of livestock identification, classification and movement, the science is of little avail" [88]. This certainly holds for endangered populations, typically managed by fragmented groups of independent-thinking people. Nimbkar et al. [87] stress the importance of "structures to organize the keepers of animals and help motivate communal efforts [...] allowing livestock keepers better access to information, [...] extension services, facilitating the organization of training, and improving [...] marketing. In Europe,

there are strong farmer cooperatives and breeding organizations that go back a century." Indeed, successful breeding programs were founded on equally fragmented conditions in many European countries and also in Canada, starting out with breeds with similar production performance as the ones from that same area that are now endangered or extinct. Apart from the clear need for incentive and for institutional backing, there are two prerequisites for such development. First, technically: an efficient system for ensuring genetic connections among farms, e.g., through regular exchange of males or across-farm AI – everything else of a technical nature will have to build upon that. Second, organizationally [89]: employment of professional genetic expertise by the breeders' organization – so that the system does not have to rely on fragmented and unstable governmental service, and can arrange for effective feedback between breeders' objectives and technological options.

Pollution

FAO's *Livestock's long shadow: environmental issues and options* report [90] gives an overview of the amount of nitrous oxide (N_2O) released from livestock manure and urine, worldwide, in 2004. Of the total emission of 3.69×10^9 kg, 12% was due to pigs – just over half of that from Asia. N_2O is an effective greenhouse gas (GHG), also involved in the depletion of the ozone layer. Other nitrogen compounds that enter the environment from livestock excreta are ammonia (NH_3) and nitrogen oxides (NO and NO_2), involved in acidification or, indirectly, in global warming.

Technology

The FAO report [90] devotes much text to options for reduction of nitrogen emission, most of which involve manure management and improved animal nutrition. For example (p. 122): "An important mitigation pathway lies in raising the low animal nitrogen assimilation efficiency [...] through more balanced feeding (i.e., by optimizing proteins or amino acids to match the exact requirements of individual animals or animal groups). Improved feeding practices also include [...] improving the feed conversion ratio [FCR] by tailoring feed to physiological requirements. However, even when good management practices are used to minimize nitrogen excretion, large quantities still remain in the manure." This is quantified in another table in the report (p. 137) with typical values for nitrogen intake, retention, and excretion in cattle, pigs, and poultry. According to these numbers (which go back to [91]), across these species, only about 19% of nitrogen ingested in "less productive situations" is retained in meat, eggs, and/or milk – in "highly productive situations," this goes up to 30%. Likewise nitrogen retention rates (N_{ret}) of about 34% for pigs were reported for the 1995 "highly productive situations" of France, Denmark, and the Netherlands [92].

These retention rates are indeed low, but the difference between the above productivity levels is considerable, suggesting scope for increase by "improved feeding practices". Dourmad et al. [93] state that "the ultimate reduction of N excretion can be reached when multi-phase feeding is combined with a perfect balance of essential amino acids and [...] optimization of the supply of non-essential amino acids" – ideally on a daily basis. They refer to a 1995 experiment [94] where the use of a single diet over a growing period from 26 to 101 kg body weight was compared to such an optimized multiphase feeding strategy, and where N_{ret} values at 34% (single diet) and 50% (optimized regime) of the ingested nitrogen were found.

Later studies in laboratory conditions have achieved higher retention rates. De Lange et al. [95] studied the effect of dietary amino acid levels on protein deposition rate in pigs from 39 to 77 kg liveweight, and present results that lead to $N_{ret} = 61\%$ at complete amino acid availability – "at a more typical protein digestibility, this would become 56%" (cf. de Lange, personal communication, 2010). Similarly, Buraczewska et al. [96] measured N_{ret} of up to 57% in 35-kg and 45-kg pigs of a "high lean gain potential" genotype, after optimization of the dietary amino acid composition. In more practical conditions, Pomar et al. [97] fed pigs from 25 to 105 kg liveweight according to a "traditional three-phase feeding program" or with "individually tailored diets," obtaining $N_{ret} = 37\%$ and 48%, respectively.

Curiously, the FAO report [90] pays no attention to a logical alternative to "improving [FCR] by tailoring feed to physiological requirements," i.e., improving it by tailoring these physiological requirements

themselves, through animal breeding [98]. When nitrogen excretion data of growing (Landrace × Large White) and (Hampshire × Duroc) pigs were adjusted for body weight and feed intake, significantly different values were obtained between the genotypes – which reveals genetic variation so that "genetic selection may be an effective method for altering nutrient utilization and output" [98]. Heritabilities for laying-hen excretion traits such as dry excreta weight, excreta humidity rate, and the ratios of dry excreta and nitrogen excreta to feed intake (0.25–0.46) and for dairy cow methane production (0.12) were reported in [99, 100].

Improvement of traits like litter size, sow feed intake, growth rate, and FCR reduces nitrogen excretion (N_{excr}) from sows and growing pigs [101]. The 1988–2007 genetic trends for growth rate (+8.5 g/day/year), FCR (−0.02 kg/kg/year), and litter size (0.16 pigs/litter/year) in the UK pig sector were estimated to cause 0.8% annual reduction of the associated global warming potential of GHG emission [102]. In that study, the genetic reduction of FCR explains about 70% of the reduction in N_2O emission; the genetic increase of growth rate explains about 70% of the reduction in NH_3 and methane emission. The genetic increase of litter size (which reduces the sow herd with its emission, for a fixed number of slaughter pigs) explains about 20% of all three elements. The future scope for emission reduction is underestimated in that study, for two reasons. First: genetic trend of lean content (which was substantial in the UK during this period, probably about 0.5%/year) was not taken into account. Second: future trends in all these traits may be expected to be stronger than the historic values, due to further development of genetic technology and of the "uptake rate of improved genetics by the commercial level" [102].

The reduction of N_{excr} due to genetic improvement of production traits is quantified by the simulation results in Fig. 6, obtained with the model of [103]. The time trends of model parameters such as maximum protein deposition rate (PD_{max}) that were described for six pig sire lines [104] can be used to model N_{ret} and N_{excr} at the nucleus level throughout the 1969–2004 period. The simulations involve these six progressively advanced genotypes, grown from 20 to 120 kg body weight. Each of these genotypes was fed ad libitum on each of seven three-phase (20–50, 50–80, and 80–120 kg) diet specifications, targeting overall lean tissue growth rates (LTGR) from 275 to 425 g/day in steps of 25 g/day [105]. This involves diets with a fixed digestible energy content (DE = 14.2 MJ/kg) and varying levels of crude protein (e.g., from 12.2% to 15.5% in phase 3) and essential amino acids (e.g., lysine from 0.525 to 0.765% in the diet, ditto).

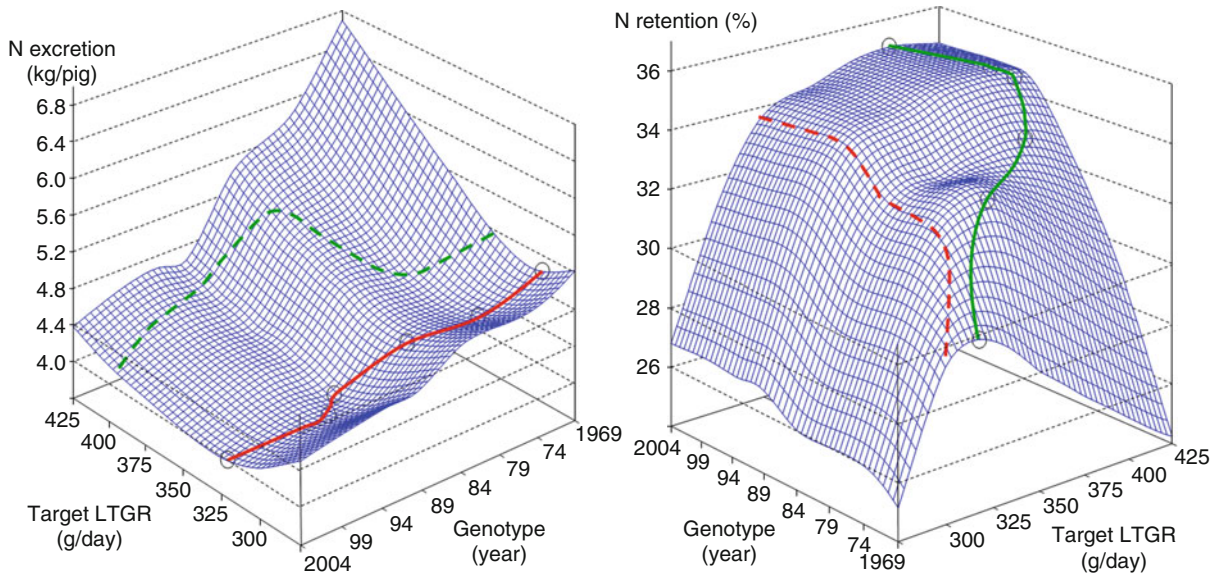
The older genotypes in this simulation do not have the potential to achieve the higher LTGR targets, with the consequence of low N_{ret} and high N_{excr} levels. Low LTGR targets obviously lead to low N_{ret} as well, more so in the older genotypes. Figure 6 shows clear optimum trajectories across genotypes and feeding strategies, for both N_{ret} and N_{excr}. It also shows that these optimum trajectories follow different paths for both characteristics, particularly in the more advanced genotypes.

Along these optimum trajectories (i.e., when fed to achieve maximum N_{ret}, or minimum N_{excr}, within the limits of the three-phase feeding program) the 2004 genotype shows a proportionally 19% higher N_{ret} or, alternatively, a 20% lower N_{excr} than the 1969 genotype. Deviations from these optimum diet composition settings have much stronger effects in the older genotypes than in the more advanced ones.

The more advanced genotypes in this simulation show a clear diminishing-returns pattern for N_{ret} with a very flat asymptote around N_{ret} = 35%. But the simulated PD_{max} levels (i.e., genetic potential for N_{ret}) increase progressively throughout the 1969–2004 period covered here – so the expression of that potential must be constrained by the three-phase fixed-diet program employed here. This comes back to the real-life results of Pomar et al. ([97]; above) who obtained N_{ret} = 37% with a three-phase fixed-diet program and N_{ret} = 48% with "individually tailored diets." The latter strategy involved a daily analysis of the performance-to-date of each pig, to predict today's individual body weight, growth rate, nutrient requirements, and ad libitum feed intake. Each pig was then fed a mixture of basic rations via automated feeders to match these predictions.

It follows that the more advanced genotypes require more advanced feeding strategies to bring their more sustainable performance potential to expression.

This was further explored by Morel and Wood [106], who used simulation modeling to quantify nitrogen flux in growing pigs of low, medium, and



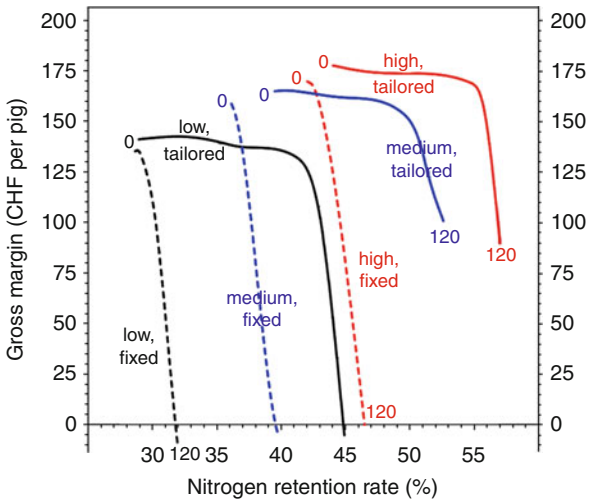
Pig Breeding for Increased Sustainability. Figure 6

Nitrogen excretion (*left*) and retention rate (*right*) in simulated growing pigs of six genotypes (representing sire lines from 1969, 1976, 1984, 1990, 1993, and 2004; From [104]). Each genotype was fed from 20 to 120 kg liveweight on three-phase diet specifications targeting [275, 300,. . .,400, 425] g/day lean tissue growth rate (LTGR), according to [105] – the higher LTGR targets are beyond the potential of the older genotypes, and the more advanced genotypes do not realize their potential due to inadequate nutrient supply. The blue response surfaces are spline interpolation plots through the 6×7=42 simulated datapoints. The red and green trend lines represent minimum excretion and maximum retention, respectively, for each genotype. Each broken trend line mirrors the solid one of the same color (with its datapoints as black circles) in the other graph, approximately

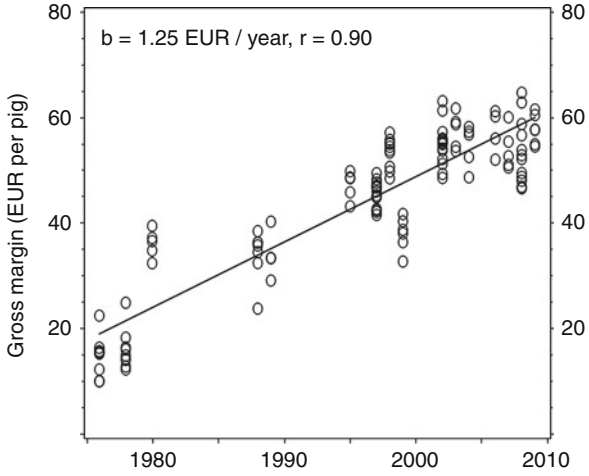
high LTGR genotypes, on a variety of dietary regimes. Their simulated high LTGR genotype achieved N_{ret} = 57% when fed daily individually tailored diets (similar to Pomar et al.'s [97] diets mentioned above) with a strong focus on the minimization of N_{excr}. By contrast, the simulated low LTGR genotype achieved N_{ret} = 29% on a three-phase fixed-diet program with all focus on gross margin (i.e., carcass return minus feed costs). So the various N_{ret} results of these simulations (see Fig. 7) span the relevant range of commercial and high-tech conditions described above.

These authors conclude that although "a reduction in nitrogen excretion is mainly achieved through a reduction in nitrogen intake" (as in Fig. 6), "genotypes with a high lean growth potential can be more profitable [in terms of gross margin] *and* have improved nitrogen excretion." A simple ANOVA of their results of gross margin and N_{ret} across the simulated scenarios (supplementary data from Morel PCH, 2010, personal communication) quantifies this. The low versus high LTGR genotypes show least-square means for simulated gross margin at 102.0 versus 150.0 CHF per pig, and for N_{ret} at 34.1% versus 46.8%, respectively. Their "three-phase fixed-diet" versus "individually tailored" feeding regimes show least-square means for gross margin at 116.3 versus 138.6 CHF, and for N_{ret} at 36.7% versus 45.0%, respectively. So Morel's genetic scenarios [106] are *more* effective (because further apart) than his feeding regimes, for improving both gross margin and N_{ret}. With some generalization, this can be put into perspective as follows.

With the 1969–2004 trend of PD_{max} in pig sire lines from [104] (above), the PD_{max} input values of Morel's simulated genotypes (120, 160, and 200 g/day) can be located in time at 1970, 1987, and 1997, respectively – so his low and high LTGR genotypes are 27 years of sire line genetic improvement apart. The simulated gross



Pig Breeding for Increased Sustainability. Figure 7
Gross margin (from growth rate, backfat depth, and feed intake) in relation to nitrogen retention rate (N_{ret}) in simulated growing pigs of three genotypes (low, medium, and high lean tissue growth rate), fed three-phase fixed diets or individually tailored rations. The labels "0" and "120" indicate the extremes of a range of weighting [N_{ret}: margin] from [0:1] to [120:1] in the objective function for diet optimization. CHF: Swiss Francs (Data from [106] and Morel PCH, 2010, personal communication)



Pig Breeding for Increased Sustainability. Figure 8
Gross margin (from growth rate, estimated lean content, and feed intake) in slaughter pigs of commercially available genotypes, as recorded 1976–2009 in public Commercial Product Evaluation trials. The trend line is the linear regression. EUR: European Euros (Data from [107])

margin of those genotypes (three-phase fixed-diet, full focus on gross margin) differs by 35.7 CHF (i.e., about 23 EUR).

Time trends of growth and carcass traits in commercially available slaughter pig genotypes [107] can be converted to gross margin trends as in Fig. 8. The average range for a contemporary comparison of seven genotypes (the most common configuration in this data, and also a fair representation of practical local availability of genotypes) is 10 EUR per pig.

This provides an (under)estimate of the range in gross margin among the various slaughter pig genotypes that are commercially available at any point in time (an underestimate, because these CPE trials do not include *all* available genotypes, particularly not the less advanced ones). This number is equivalent to 10/23 = 43% of the difference between Morel's [106] low and high LTGR genotypes. Combined with the above surmise that "Morel's genetic scenarios are more effective [. . .] than his feeding regimes are, both for improving gross margin and for improving N_{ret}," it follows that a well-informed choice of the most appropriate slaughter pig genotype *available at any point in time* will have almost half of the influence on N_{ret} (and on margin) that full implementation of individually tailored diet optimization will have.

Strategies

The above section shows that pig breeding in general makes a considerable contribution to the reduction of nitrogen emission from growing pigs. Up to now, these effects have not been incorporated into formal breeding goals, so they are not under control and cannot be credited, as such. Many governments have an increasingly active policy of pollution reduction, and the global warming potential of the excreta from livestock production is under increasing scrutiny. This has led to a series of economic studies into the effectiveness of taxation of nitrogen (and other chemicals) emission on the farm level, focusing on countries as politically (and productively) different as Italy, Switzerland, and the Netherlands [108–110].

A central element in the associated econometric approaches is the *shadow price* of nitrogen emission. Paul et al. [111] describe the "shadow values for the bad outputs" of an agricultural production system as "the marginal amount that producers [. . .] would be willing to pay for unrestricted use of the environment" to dispose of those bad outputs – e.g., for the right to increase their bad outputs in a situation where legislation attempts to reduce these.

Operationally, shadow prices are estimated as the partial derivative of the producer's profit equation with respect to the bad output factor – equivalent to marginal economic values for production traits in animal breeding. These are demanding statistics. Key et al. [112] studied the productivity of the USA slaughter pig sector, and note that estimating the "shadow price of manure [. . .] with the data available would require making a set of assumptions that would likely introduce substantial error [. . .], an accounting of hog farm output that includes manure is left for future research."

The shadow price of nitrogen emission from growing pigs, sows, and dairy cows in the Netherlands was estimated at 2.7, 10.8, and 6.5 NLG/kg, respectively [110, 113].

The UK government has included the shadow price of GHGs as a structural element of its cost-benefit evaluation of any policy that it funds or supports, using values based on [114] where a shadow price of CO_2 is derived that can be converted to a shadow price of nitrogen (as a component of N_2O) at 9.25 GBP/kg.

Obviously, such shadow prices depend on practically every feature of the production system and its surrounding conditions. Using again Paul et al.'s [111] description above, the amount that producers would be willing to pay for the right to increase their bad outputs in a situation where legislation attempts to reduce these, would depend on (1) how strongly this legislation attempts to enforce the reduction, and (2) the impact of such a reduction on the remaining elements of the producer's profitability. Point (1) is largely a political factor; the UK government was criticized by environmental NGOs for the supposedly artificially low value of its abovementioned shadow price of CO_2. Point (2) may well lead the producer to decide to *not* reduce his bad outputs, because that is still more profitable – as was the case in Paul's study which focused on pesticide usage: her shadow price estimates were negative.

Wall et al. [115] notice the equivalence of the shadow price of a bad output and the marginal economic value of a production trait, in the sense that both can be used to weight their characteristic into a breeding goal. They refer to the EU-ETS Emissions Trading Scheme that was set up to support the EU to meet its commitments to the Kyoto Protocol (ec.europa.eu/environment/climat/emission/index_en.htm), which will effectively determine the shadow price of GHG emission in the EU. They write "suppose [. . .] that agriculture is forced into an [ETS] and that farmers must hold valuable permits either through initial allocation or by purchasing in the ETS. [This] will immediately move GHG mitigation traits from a public to a private breeding objective [. . .]; the prevailing emissions price becomes the relevant economic weight that should be incorporated in any breeding index that includes mitigation potential." The demand for "breeding indexes that include mitigation potential" will become very concrete, once pig producers have to deal with such a scheme – in line with this, the total costs of 2007 environmental government policy in the Netherlands were estimated at "around 0.11 EUR per kg [carcass] weight, of which 0.08 EUR was for manure disposal. In 2013, these costs will be 0.02 EUR higher as a result of the ammonia emission reduction policy" [116].

Figures 6 and 7 show that such a pig breeding policy is technically feasible: Nitrogen retention rate is favorably correlated with the conventional production traits and can easily be included into breeding goals and selection strategies.

Animal Welfare

In 1976, the member states of the European Community ratified the *European convention for the protection of animals kept for farming purposes*, regulating that livestock must be properly housed, fed, and cared for. In 1992, the following text was added (condensed here for clarity): "Breeding procedures which may cause suffering or injury to animals shall not be practiced. No animal shall be kept unless it can be expected, on the basis of its phenotype or genotype, that it can be kept without detrimental effects on its health or

welfare." Five years later again, the EC's Scientific Veterinary Committee recommended that "no selection should occur without reference to the effects of that selection on welfare of [pigs]. The continuation of new genetic lines in which the welfare of the animals is, on average, worse than that of existing lines should not be permitted" [117].

Such statements leave the impression that animal breeding may be bad for the animals involved. This goes back to the late 1970s when animal breeding technology became much more powerful than before, due to improved data recording and processing (BLUP and, above all, computing power), and improved reproductive technology. Simultaneously, animal production in the western world experienced strong intensification connected to a long period of low, volatile, and unpredictable farm profitability [118]. The production sector therefore developed a strong and focused demand for animals with improved production performance.

This led to a strong and effective focus on production traits in livestock breeding in that period, with less attention for animal robustness traits. This lack of balance has caused fitness constraints in pigs, particularly in environmental conditions inadequate to support the improved production potential, and particularly before this problem was being dealt with in modern pig breeding.

Intensification of animal production has also led to housing and management conditions that shelter the pig from climatic, nutritional, parasitic, and predatory challenges – but compromise much of the expression of its instinctive behavioral repertoire. This deprivation leads to frustration, with welfare problems for the affected individual and for its penmates.

Fitness constraints and deprivation are among the "criteria for determining the ethical limit for genetic selection" [119]. A third issue is formed by the routine invasive treatments that have been common, worldwide, since the onset of animal domestication – in pigs mainly tail docking and castration. These aim at a pragmatic (but unrefined and reoccurring) reduction of undesirable features that can also be dealt with through animal breeding – more complicated, but permanent and less physically invasive.

While worldwide demand for pig meat increases, it will become more and more relevant to resolve problems with pig welfare in intensive production systems. It is unlikely that this increasing demand will be met by any other production system than intensive ones, particularly in Latin America, Russia, and Asia. In accordance with FAWC's [120] plea for "a greater emphasis in breeding programs on traits associated with good welfare," animal breeding and genetics technology can then contribute in three areas: (1) robustness, (2) deprivation, and (3) avoidance of invasive treatments. In practice, each of these areas raises (4) ethical arguments, sometimes intense enough to dominate the issue – so although this chapter is about technology, these will be addressed as well. Sections "Robustness" to "Ethical Aspects" deal with these areas in this order.

Robustness

When animals of high-performance genotypes are kept in production systems that are inadequate to provide the resources they need to express their potential, the animals commonly show disturbed resource allocation [121] and functional disorders of the skeletal and cardiovascular systems, muscle physiology, the reproductive system, or immunocompetence [122]. For pigs, obvious indicators of reduced animal welfare in this respect are (1) increased mortality rates and reduced sow longevity, (2) disease (morbidity), and (3) lameness.

The issue here is one of environmental sensitivity. There are two ways to deal with the problem: make the environment more resource providing or make the genotype less sensitive. There are two strategies for the latter: (1) direct selection for robustness traits and (2) selection against environmental sensitivity as measured through reaction norms.

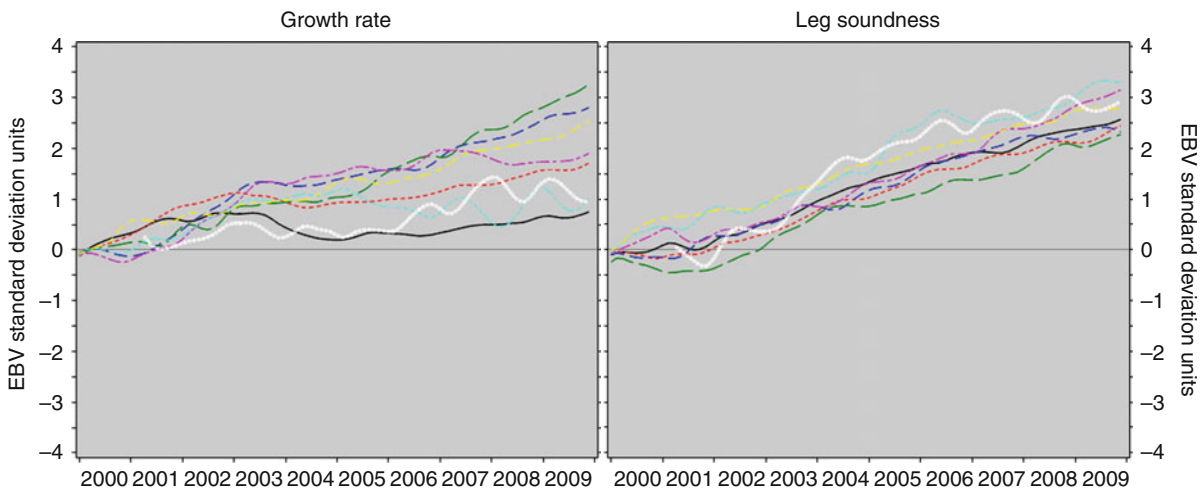
Selection for Robustness Traits Livestock robustness can be defined as "the ability to combine a high production potential with resilience to stressors, allowing for unproblematic expression of a high production potential in a wide variety of environmental conditions" [123]. The classical problem with this ability is in genetic antagonisms between production traits and resilience [121] – natural selection is not powerful enough to maintain (or improve) animal robustness in intensive production systems; it must be supported

by artificial selection. Genetic antagonisms can be neutralized by using adequate selection criteria to select for an adequate breeding goal. Earlier breeding goals were inadequate in this respect, as they did not include robustness traits [124]. Following Gjedrem [125], a breeding goal should include all heritable traits that have an impact on profitability – and mortality, morbidity, and lameness certainly do. Knol et al. [126] discuss this in detail and stress that "simplicity and straightforwardness of the breeding goal has to be weighted against completeness and complexity." Such traits can be included in the profit equation for pig production [127]; this provides marginal economic values, required for inclusion in the breeding goal.

The various strategies for genetic improvement of piglet vitality and survival, leg weakness and longevity, stress sensitivity, and disease resistance are summarized in [128]. These are hard-to-measure traits, mostly categorical with low incidences and relatively low heritabilities, so that large data volumes from adverse environments are required for meaningful breeding value estimation [129]. Their genetic improvement has benefited considerably from BLUP and will benefit just as much from MAS [130, 131]. Figures 9–11 show realized genetic improvement of leg soundness and mortality traits, coinciding with improvement of production performance, in eight pig lines.

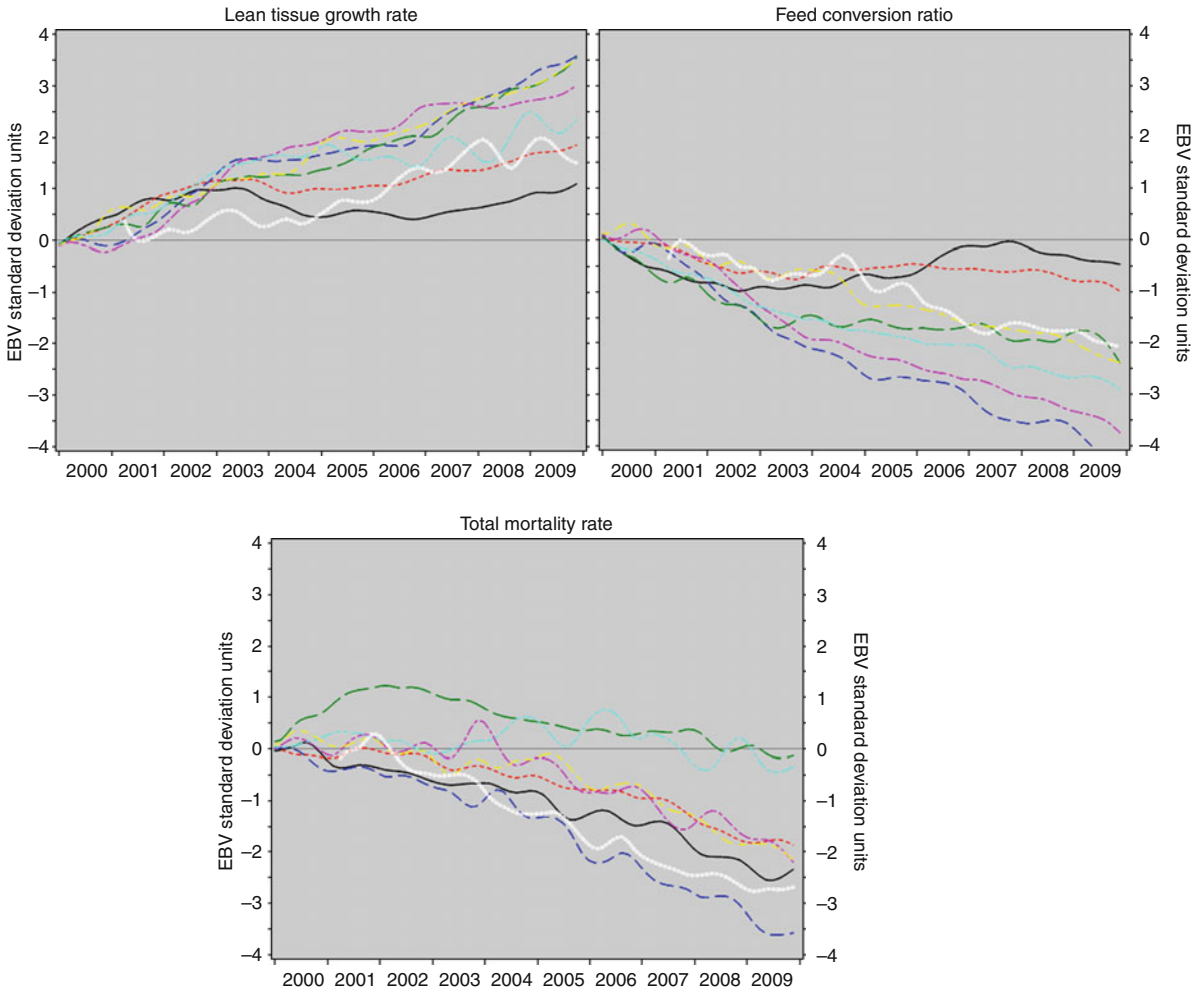
SVC [117] write: "The criterion for selecting animals for use in breeding has been an increase in economic performance and this has often not coincided with improved animal welfare. Hence the term *genetic improvement* is misleading since, in some cases, the changes are not improvements for the animal but may make the life of the animal more difficult." The genetic trends of Figs. 9–11 show that this can be overcome: antagonistic correlations do exist, but genetic improvement can be achieved in production traits and robustness traits simultaneously – neutralizing genetic antagonisms by adequate selection. This effect will depend on the emphasis given to each trait in the breeding goal of each line.

Reaction Norms When progeny of specific sires are (1) identified as such, (2) spread across a wide environmental range (usually through artificial insemination), and (3) recorded for a production trait, their production performance can be regressed on a descriptor of the environment (e.g., a herd-year-season effect). This produces a positive slope overall: better environments lead to better production. When



Pig Breeding for Increased Sustainability. Figure 9

Simultaneous genetic trends of growth rate and leg soundness in eight pig lines. Each trait was scaled by the standard deviation of its estimated breeding values; all trend lines for each trait were forced through the same origin in 2000 to make the trends comparable across lines

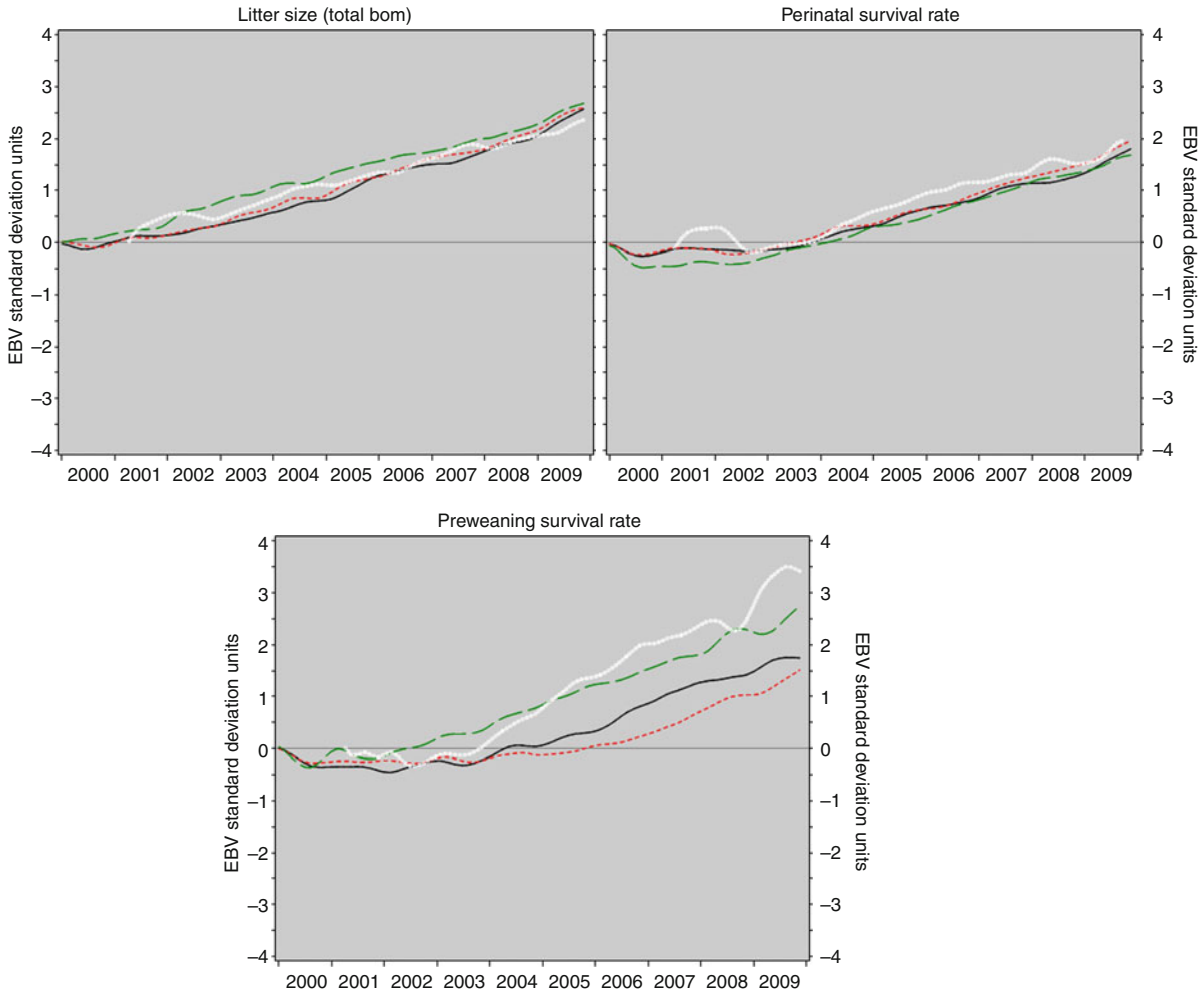


Pig Breeding for Increased Sustainability. Figure 10

Simultaneous genetic trends of lean tissue growth rate, feed conversion ratio, and total mortality from birth to slaughter in eight pig lines (same formatting as in Fig. 9)

the regression is performed separately for sire progeny groups, and if there is genetic variation in environmental sensitivity of the trait's production potential, regression lines are produced ("reaction norms" in population genetics) with different intercepts and slopes for different sires. The intercepts are equivalent to the conventional estimated breeding values for the trait. The slopes quantify an animal's requirements for environmental support of its genetic potential; they detect robustness as defined above.

Environmental sensitivity in pigs was analyzed this way in [132, 133]. Friggens and Van der Waaij [134] discuss how selection for increased production levels (i.e., for high reaction norm intercepts) will cause a gradual increase of environmental sensitivity (i.e., of the slopes). This was confirmed [133] in terms of a strongly positive genetic correlation between intercept and slope of their reaction norms for litter size. The slopes have a very low heritability in that data, so the increase of environmental sensitivity would be very slow. This presents another example of genetic antagonisms, which can be neutralized by including both the intercept and the slope of the reaction norm of each production trait in the breeding goal and in the selection criterion. A way to calculate their marginal economic values is in [123].



Pig Breeding for Increased Sustainability. Figure 11

Simultaneous genetic trends of litter size, perinatal survival rate, and preweaning survival rate in four pig damlines (same formatting as in Fig. 9)

These are demanding statistics. Knap and Su [133] analyzed their data in three consecutively larger subsets. The medium subset held more than 50,000 sows – comparable to the largest litter size datasets in the scientific literature. Still, its parameter estimates for the reaction norm slopes differed considerably from those from the largest dataset with more than 120,000 sows, and the accuracy of its slope estimates was too low to be useful in practical breeding. The smallest subset held more than 30,000 sows, but its parameter estimates were clearly unrealistic and not significantly different from zero. Similar issues hold for the required environmental range in the data.

A single trait's reaction norm is equivalent to "a single-trait definition of robustness" and, as such, not a simple and foolproof recipe that can be applied without proper care for the system as a whole. An animal "that maintains [. . .] production in the face of decreasing [environmental support] is deemed to be robust, but [it] must be diverting nutrients away from other life functions. [. . .] When robustness can be understood as the ability to cope with environmental challenges [. . .] an animal that maintains milk production by suppressing its immune function is clearly less robust than an animal that maintains milk production by reducing growth rate" [134]. Which means that

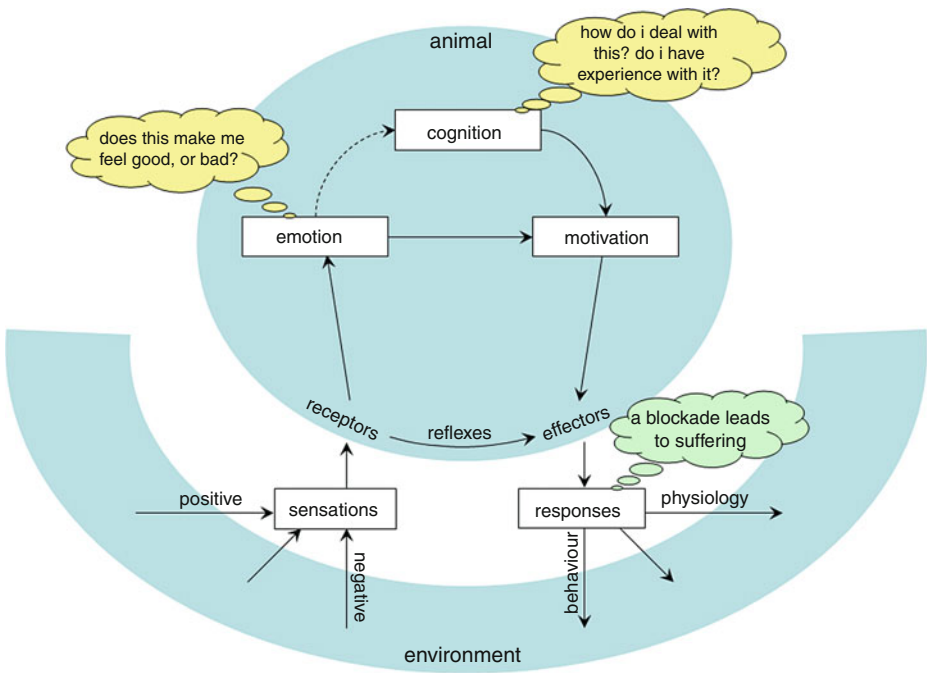
(1) it becomes interesting how the environmental sensitivity rates are correlated among traits, and (2) breeding goals must be designed and monitored with care, as argued above.

Deprivation

Intensive housing and management systems tend to compromise the expression of instinctive behavior patterns ("motivations" [135]) of the pig: foraging, rooting, and exploring in all age classes [117], and nest-building in sows [136] – because intensive housing environments do not provide the required space or substrates. Something similar holds for deprivation of social contact in individually housed sows [137], where the "required substrate" is other pigs. For more detail see [138]. This results in frustration, leading to apparently irrationally redirected behavioral outlet functions: stereotypies or apathy occur when the animal is severely or chronically frustrated [139–141]. Mason and Bateson [142] describe this situation as "internal states of deprivation [...] leading to sustained high motivations that the [animal] cannot reduce: it cannot perform the activity that would result in negative feedback." Another outlet function is wide-sense agonistic behavior such as tail biting [143, 144]; see the section on "Harmful Social Behavior."

In sentient species, such frustration leads to suffering. A sentient animal has been described [145] as an animal that has the capacity to suffer when it learns that it is unable to cope with stress: "it may fail to cope, either because the stress is too [intense], or because [the animal] is constrained in such a way that it is prevented from doing what it feels necessary to relieve the stress." This leads to anxiety or depression. Figure 12 illustrates the interdependence of the various components.

Held et al. [138] write: "pigs used today for pork production seem to have retained many of the faculties of their wild ancestors, and may therefore be behaviourally, cognitively and emotionally at odds with the husbanded environment [...]. Understanding the cognitive abilities, behavioural priorities and emotions of commercial pigs therefore lies at the very heart of improving their welfare." Jensen [146] describes



Pig Breeding for Increased Sustainability. Figure 12
Relationships between emotional, cognitive, and motivational information processing in sentient animals (Modified from [145] and [139])

those "faculties of the wild ancestors" as "subtle [behavioural] differences between domestic and wild animals [...] attributed to modified stimulus thresholds, causing some behaviour patterns to become more common and others to be rarer during domestication." Such changes in pig behavior are described in detail in [147, 148].

One of the options for resolving such cognitive and emotional conflicts in an intensive environment would be to further reduce those faculties of the wild boar, further modifying those stimulus thresholds and simplifying what the animal feels necessary to relieve its stress. Kruska [149] sees changes in behavior and brain size as "adaptations to the special ecological niche of domestication." "Modern housing systems [have a] short history compared to the history of the pig as a domestic animal, and it is likely that adaptation has not kept pace with the intensification of pig husbandry" [150]; also, of course, because most housing systems were not designed to match behavioral needs.

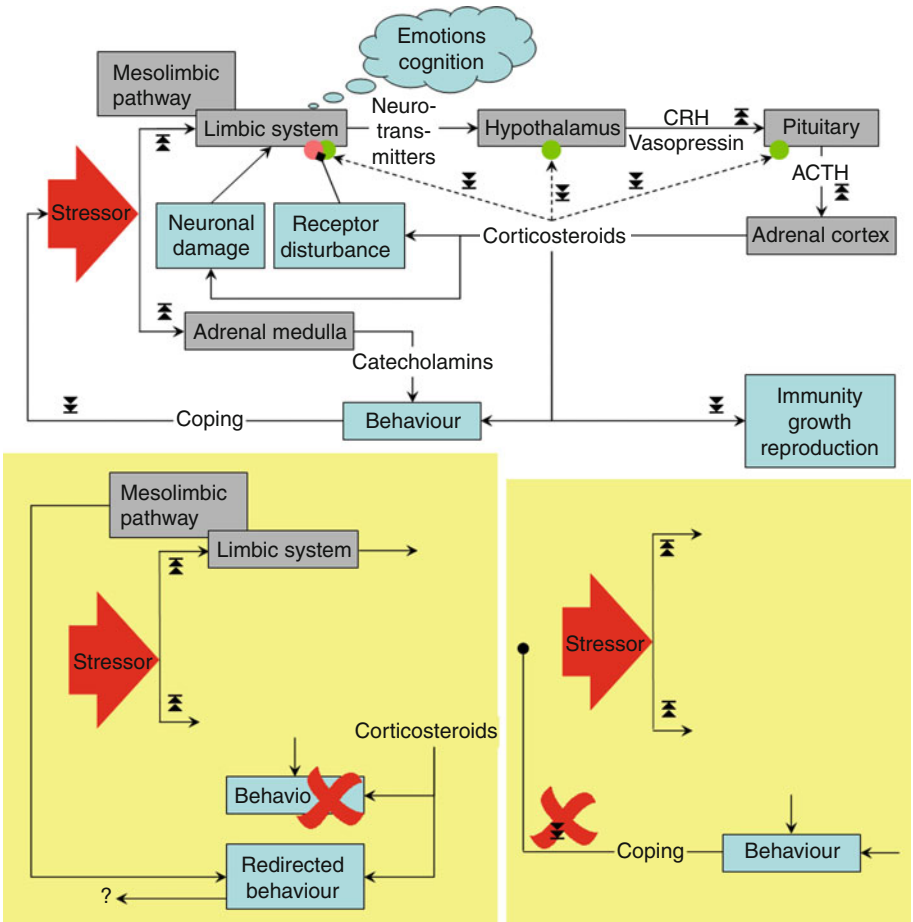
Neuroendocrinology The neuroendocrinological aspects of *coping with stress* (as above) are studied in relation to human conditions such as mental depression [151]. This is a novel area for animal breeding, with legible summaries of the state of the art from [139, 152–154]. Chronic stress affects the hypothalamus-pituitary-adrenal (HPA) axis; it can cause a persistent overproduction of corticosteroids, which can disturb neurotransmitter systems by causing a chronic downregulation and/or an imbalanced activation of the various types of corticosteroid receptors in the brain. Probably independent of the HPA axis (but interacting with its corticosteroids) is the mediation of stress-induced stereotypic behavior by the mesolimbic pathway. Figure 13 presents a simplified model of these systems.

The sympathetic nervous system also influences corticosteroid production, and its interaction with the HPA axis leads to *active* versus *passive* coping strategies. These terms represent the extremes of a continuous distribution of animal-intrinsic behavioral flexibility. This has been described [154–157] as follows: active copers rely on stable conditions, show poor adaptation to changing conditions, and attempt to deal with any challenge through routines and behavior patterns that were successful previously, trying to remove the stressor or move themselves away from it. Passive copers show higher cognitive performance, thrive better in changing conditions, and face challenges by modifying their behavior to deal with a new stressor in its own way, aiming at reduction of the emotional impact of the stress.

This approach has been criticized as an attempt to fit a multidimensional system (reactivity to external factors) into a single (coping) dimension. Ramos and Mormède [158] mention three such dimensions (activity, emotionality, and aggressiveness), and present studies in rodents that quantify these through Principal Components Analysis and similar techniques. Obviously, control of the system through animal breeding would benefit from a detailed focus, so that the various dimensions can be brought under control independently from each other.

These traits are heritable (both coping strategies have natural selective advantages), and rodent populations have been successfully selected into either direction, using various selection criteria [159–161]. Veenema [154] found a higher stress susceptibility in passively coping than in actively coping mice. Her surmise is that this is due to differences in (1) perception of the stressor during acute stress, and (2) coping or habituation during chronic or repeated stress. Under changing conditions, passively coping mice may perform better in terms of dealing with the stressor. But exposure to chronic psychosocial stressors (i.e., living together with a dominant aggressive penmate) induced long-lasting increased activity of the HPA system in Veenema's passively coping animals, which may cause mood disorders like anxiety and depression.

Karman [153] measured the effects of chronic stress (caused by 5 months of individual housing) in gilts that had previously been scored for coping strategy. She characterizes the two strategies in terms of differences in the regulation by the hypothalamus of the pituitary's ACTH production: via CRH in passive copers, and via vasopressin in active copers. In both cases, ACTH levels and corticosteroid production are increased – which comes back to the disturbed neurotransmitter systems of above. Karman concludes that "individual housing is detrimental for the welfare of pigs, independent of coping strategy. Selection of coping strategy [...] will therefore not benefit the welfare of the animals."



Pig Breeding for Increased Sustainability. Figure 13

A simplified model of the neuroendocrine aspects of unsuccessful coping with stress in mammals. *Top*: the full system with its negative-feedback loops that keep the levels of circulating corticosteroids under control. The flow starts with the occurrence of a stressor (*red arrow*). The ⏭ symbol pointing up or down represents upregulation or downregulation, respectively, of the downstream activity. Green and pink circles represent different types of corticosteroid receptors; in the limbic system, these can be disturbed by persistently high corticosteroid levels, obstructing the negative feedback loop. The stressor is neutralized by coping behavior, which terminates its triggering of corticosteroid and catecholamine production. *Bottom*: two options for obstruction of this system. *Left*: an external factor prevents the expression of the required behavior, which leads to redirected behavior (e.g., stereotypies, agonistic behavior) as an outlet function with uncertain consequences. *Right*: an external factor keeps the stressor in place in spite of coping behavior, effectively blocking the stressor's downregulation that would normally follow from coping. In both cases, the stressor is not neutralized and the levels of circulating corticosteroids are out of control; this can result in a reduction of immunity, growth, and reproduction traits, and in damage to the limbic system which brings the system further out of control

If Karman's findings hold, the generic effects of chronic stress in pigs will not be resolved by breeding for an active coping strategy, which would seem to be the obvious approach with Veenema's [154] mice.

This would shift the focus upstream in Fig. 13, aiming at a resolution, through animal breeding, of the effects of chronic stress on the limbic system areas (amygdala, hippocampus) that regulate the hypothalamus. De Kloet [156] makes a distinction between "(1) the core of

the HPA axis with emphasis on dysregulations in the [hypothalamus] and (2) dysregulations in [...] stress inputs to the [hypothalamus] (e.g., from medial prefrontal cortex, hippocampus, amygdala, and brain stem) that are also targets for the stress hormones."

Morris [162] notices that during chronic stress "the hippocampus may become impeded in its role in 'shutting off' HPA axis stress activity. This results in increased secretion of [corticosteroids] and, in a positive feedback cycle with negative consequences, ends up in damaging the hippocampus itself, thereby further reducing [its] ability to regulate the HPA axis. [...] This brain structure is both [i] centrally involved in the neural reaction to aspects of prolonged stress and [ii] itself a target of chronic stress." Loijens [152] reports such findings in tethered sows.

The significant issue here is that the limbic system also regulates emotional and cognitive functions (Fig. 13) – which provides the conceptual connection to Fig. 12. "Sustained activation of [corticosteroid] receptors in the hippocampus [...] may lead to an impairment of declarative (rule-) learning during high levels of chronic stress. [...] The amygdala [...] has been demonstrated to [influence] hippocampal plasticity and hence may be the central link between stress and declarative learning [...]. In farm animals this type of learning occurs during adaptation to [housing] facilities, milking regimes etc." [139].

Selection Objectives "An array of stress-responsive genes has been identified," including genes "related to structural differences in hippocampus of [passively and actively coping] mice. [...] These altered gene patterns can be postulated as markers for *predisposition* for stress-related disorders" [156]. Such gene patterns are being studied in pigs as well, exploring changes of gene expression in the hippocampus, amygdala, and/or frontal cortex due to early weaning and/or social isolation of piglets [163, 164].

"The evidence for a significant genetic contribution to stress responsiveness in vertebrates is overwhelming. [...] Given the complexity of the issues, there is no firm consensus as to whether modification of stress responsiveness can benefit an animal within an intensive rearing environment" [165].

In line with this, the main conclusion from the section on "Deprivation" would be that straightforward selection against specific behavior patterns such as stereotypies and apathy (which would not be difficult to record in intensively housed sows) may be counterproductive. In terms of Fig. 13, such selection might just remove the "redirected behavior" pathway that serves as an outlet for frustrated motivations, and as such forms the animal's final way to deal with the load of the stressor. This would create a system under stress (with its negative consequences for homeostatis and production) without any security valve. D'Eath et al. [166] refer to such animals as *stoics* "because outward signs of suffering appear to be reduced" while the "root cause of the stereotypy" is not changed.

A second conclusion is that the active and passive coping strategies do not seem to offer a useful criterion for pig breeding.

It seems much more useful to look for the abovementioned "adaptations to the special ecological niche of domestication" [149] in terms of changes of the predisposition for stress-related disorders and of perception of the stressor, rather than a change of coping patterns. This would have to target the limbic system, which makes the task much more challenging.

From a very different point of view, Morris [162] quotes Sapolsky: "the body simply has not evolved the capacity or tendency to not secrete [corticosteroids] during a crisis," and adds to this "in effect, evolution has only gotten so far." Clearly, evolution could be moved on (into a more convenient direction) by a much focused targeting of the system that regulates the HPA axis, i.e., the limbic system again.

This amounts to a strategy to modify instinctive patterns so that the motivation for behavior that cannot be supported by the production system is reduced. This would "change the intensity of a behavioral response," which is equivalent to domestication [119]; antipredator responses would be an obvious example. Adaptation of behavior through selection is then effectively an extension of 9,000 years of pig domestication: a process of reducing the animal's drives for exploration, aggression, etc.

Such characteristics form the ultimate example of *hard-to-measure traits* in animal breeding. Genetic improvement will therefore logically focus on marker-assisted selection; phenotypic records will still be required in large volume for marker effect estimation,

but recording can be scheduled on a project basis, and on other animals than selection candidates. As with every trait of livestock species studied up to now, very large numbers of genes are likely to be involved, which makes the candidate gene approach and its search for major genes not very promising – also because of the currently "limited basic knowledge about psychobiological dimensions underlying behavioral trait variability, and the availability of reliable and meaningful measures of these [. . .] free from environmental influences" [167]. The *quantomics* approach that was put forward at www.quantomics.eu seems to offer more power to bring such a system under control: it should "provide the tools to identify rapidly the causative DNA variation underpinning sustainability in livestock, and [. . .] exploit high-density genomic information." Essentially, making use of large numbers of anonymous markers (as in genomic selection) to identify functional elements and their connection to the phenotype (as in QTL studies).

Dominance Aggression Another issue of intensive production systems is avoidance of dominance aggression, particularly when pigs are being mixed into new groups [168, 169] – confinement does not allow for escape from aggressors. Such behavior has significant genetic components [170], possibly but not very clearly connected to coping strategies. The main factor that would complicate selection against such behavior is the difficulty of data recording, which makes marker-assisted selection an interesting option again. QTL associated with such behavior are in the process of being discovered [171, 172].

The ethical aspects of all this are considered in the section on "Ethical Aspects."

Avoidance of Invasive Treatments

There is much societal drive to reduce painful treatments like castration and tail docking of piglets. The relevant issues are then the genetic options to reduce (1) boar taint and (2) tail biting – these form the reasons why those treatments are performed.

Boar Taint Piglet castration needs to be carried out under anesthesia in Norway and Switzerland. The German pig production sector has been recommending castration with analgesia since 2009, and aims at castration-free production on the longer term. The Dutch pig sector aims at castration-free production by 2015; leading Dutch retailers have decided to stop the sales of meat from castrated pigs starting 2011. A logical extrapolation is that by 2020 the European pig production sector will leave most of its male pigs uncastrated. While this provides progress in animal welfare (apart from aggression among entire male penmates), and an advance in gross profitability because entire males grow more efficiently than castrates [173, 174], it causes logistical and technical challenges because of boar taint.

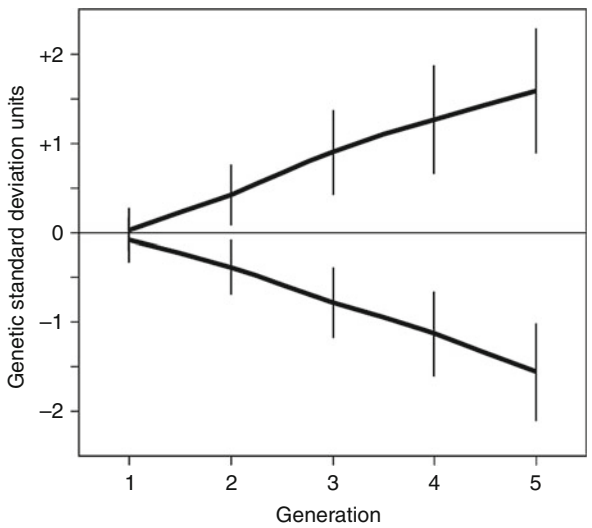
Boar taint is an unpleasant odor of pig meat (occurring in roughly 3–10% of entire males) caused by several chemical components, most importantly androstenone (a sex hormone) and skatole (a metabolite of the gut microflora). The tissue concentrations of both components are variable, line specific, and heritable [175–177], and the genes that influence them are gradually being identified [178, 179] so that it is feasible to select pigs for reduced boar taint levels. Such genetic improvement is a crucial element of the sustainability of sector-wide non-castration: although it is largely uncertain how consumers will react to increased amounts of meat from entire male pigs appearing on the market, boar taint incidence will have to be reduced to manageably low levels to avoid situations where consumer demand for pig meat collapses, or where the processing industry shifts to imported meat from castrates.

Because androstenone is a sex hormone, attempts to reduce its circulating levels may affect similar hormones such as testosterone and the estrogens, with a negative impact on male and female fertility, respectively. This must be counteracted through "balanced breeding" [177], i.e., by simultaneous selection for the relevant fertility traits.

Harmful Social Behavior SVC [117] write about "tail injury caused by biting. Although the motivation of the pig which bites the tail is likely to be investigation, manipulation and perhaps feeding rather than aggression, the consequence for the bitten pig is serious. Bitten tails may attract further biting so that the injury is to the abdomen at the base of the tail after the tail itself has been bitten off." Tail biting is a form of "harmful social behaviour" [180] (HSB; "social"

because it involves other pigs) in intensive animal production [181, 182], possibly a form of redirected outlet behavior (as in the section on "Deprivation") in the absence of rooting substrate such as straw [183]. Other forms are vulva biting in group-housed sows [184], piglet savaging by sows [185], and feather pecking in poultry. Feather pecking and subsequent cannibalistic actions form a major problem in poultry production [186]. In practice, this vice is prevented by beak trimming of the potential actor, whereas tail biting in pigs is prevented by tail docking of the potential recipient; both at a very young age. Both treatments compromise animal welfare, but not performing them may do so too – a circular lose–lose situation to be broken.

Su et al. [187] selected laying hens for increased or reduced feather pecking incidence. Figure 14 shows the genetic trends in their first five generations. The selection trait was the number of pecking bouts delivered by a bird during 3 h, recorded by examining video footage of 250 birds each generation. Behavioral traits are notoriously time intensive to record and therefore a prime candidate for marker-assisted selection (phenotypic records will still be required in large volume for marker effect estimation, but recording can be scheduled on a project basis and on other animals than selection candidates). Motivated by this, "a major dominant allele affecting the [feather pecking] behavior" was identified in the eighth generation of the high-incidence line [188]. More genes with significant associations to feather pecking were found in similar selection lines [189]. Likewise, Quilter et al. [190] report on a search for QTL associated with piglet savaging behavior in sows.



Pig Breeding for Increased Sustainability. Figure 14
Genetic trends of feather pecking incidence in divergent laying-hen selection lines (Data from [187]). The data for each line have been scaled by the within-line genetic standard deviation. Vertical bars represent one standard error each side of the mean value

Social behavior traits involve a recipient and an actor; genetic evaluation should take both into account. Ellen et al. [191] notice that "cannibalism [. . .] differs from conventional breeding traits because it depends on social interactions [. . .]. Selection strategies [. . .] should consider both the direct effect of an individual on its own survival and the social effect of the individual on the survival of its group members (the so-called associative effect)."

In group housing, an individual's phenotype for any trait (growth rate, mortality, etc.) is influenced by its own direct breeding value for the trait and the associative breeding values of its penmates, positive or negative. Hence, if associative effects are significant for the trait, they should be part of the analysis in breeding value estimation – to effectively equip the social environment with a pedigree structure and capture it in more statistical detail. This principle has been worked out in detail for growth rate and feed intake in growing pigs [192, 193]; associative effects contributed the majority of heritable variance in these traits. Intuitively, the same would hold for mortality rates due to cannibalistic HSB.

Muir and Craig [194] discuss *group selection* in laying hens, where "hens of each sire family were housed as a group in a multiple-bird cage and selected as a group" for egg production and survival rate. These hens were housed intensively and were not subjected to beak trimming, allowing for unconstrained expression of HSB. After seven generations of selection, in group housing, the group selection line showed a 20% mortality rate at 58 weeks, compared to 54% in an unselected control line and 89% in a related commercial line (which was selected for egg production and survival rate in individual housing; Muir WM, personal communication, 2010). Plumage scores revealed significantly less HSB in the group selection line. Egg

production in group housing was highest in the group selection line.

These authors conclude that this approach "is effective in improving [welfare] of layers in a relatively short period of time without sacrificing productivity. The way for commercial breeders to develop birds that do not need beak trimming is clear." A study of the endocrine and immune system traits in these lines [195] concluded that "group selection altered the chickens' physiological homeostasis which is reflected in the line's unique coping ability with intensified domestic environments." But the physiological effects of this selection approach are cell-specific [196].

Gunsett [197] describes an application of group selection in pig breeding. He mentions advantages in terms of profitability (improved image of intensive production, increased stocking density, reduced abattoir penalties for damaged carcasses) and animal welfare: (1) reduced incidence of damaged carcasses, i.e., of pigs injured due to HSB, (2) reduced mortality rates, and (3) more docile behavior. He also mentions practical difficulties with the implementation of the method, due to reduced selection intensity and increased rate of inbreeding. Hence the program was changed to a system where the direct and associative breeding values were estimated in an extended BLUP approach [198], which was formally worked out by Bijma [199]. Group selection is then not required anymore. These principles were applied to HSB and its resulting mortality rates in laying hens, leading to the conclusion that "including associative effects in the model will give substantially higher heritable variation than when using the conventional direct effects model [. . .]; prospects for reduction of mortality using the direct-associative effects model are good [. . .]; selection targeting both direct and associative effects is expected to substantially reduce one of the major welfare problems in egg production" [191].

The extension to tail biting in pigs is obvious: Breuer et al. [180] estimated heritabilities of tail biting in two populations (both with an actor incidence of about 3%) at 0.00 and 0.05 on the observed scale, and bravely conclude that "it would be possible to develop a selection index to reduce [. . .] tail-biting behavior through selective breeding" – but any statistical method that delivers substantially higher heritable variation would be very useful here.

Muir and Craig [194] conclude from their selection results that "because group selection is shown to improve well-being in multiple-bird cages, alternatives such as redesigning cage environments, or housing such as floor pens or free ranges, may not be needed." Likewise, Conington et al. [200] write "breeding animals to adapt to their environment, rather than focus on changing environments to match new genotypes (such as altering housing and cubicle design) can minimize the mismatch between them" [200]. Such statements are under debate, following the argument that it would be preferable to adapt the production system to the animal, rather than vice versa, which goes back to Faure [201]. This is in clear conflict with domestication in general, which has always attempted to adapt animals to captivity systems, sometimes by considerable force. The ethical aspects are dealt with in the next section.

Ethical Aspects

Farm animals are there to produce food that people need and want. With an increasing human population and its worldwide purchasing power, there is an equally increasing demand (what people want) for animal products. One of the options of dealing with this increasing demand is to disapprove of it, arguing that the world's carrying capacity does not allow for it [202] and that everyone (particularly in the developed world) should eat less animal products. This raises one of the many arguments in an ethical discussion: to what extent it can be justified to deny people (particularly in the developing world) what they clearly want.

Another (nonexclusive) option is to look for technological solutions. Without doubt, intensive systems will be the norm in worldwide pig and poultry production of the 2020s: in USA, Brazil, the Middle East, Russia, and China – where the enforcement of extraneous norms and regulations is difficult and arguably ethically unjustified. Sections "Robustness" to "Avoidance of Invasive Treatments" show that pig breeding can produce genotypes that are better equipped to fare well in such systems. This would also enhance profitability in such systems (due to lower mortality and morbidity rates), leading to increased worldwide sustainability. The pragmatic approach would then be to

accept this and aim at adapting the pig species to such conditions. There is much debate about this proposition.

Hörning [203] writes: "In general it seems ethically dubious when behavioral problems of intensive production must be reduced by breeding, rather than by changing the management-related causes. On the other hand, selection for certain behavior patterns in extensive production conditions has been recommended by some livestock ethologists: [. . .] maternal behavior of sows in loose housing systems [. . .] or against feather pecking in laying hens in alternative housing systems" (translated).

In line with this, the genetic adaptation of goats, sheep, and beef cattle to marginal (mountainous, wetland, arid) extensive conditions is much explored [204, 205]. Those conditions often lead to severe and persistent violation of the freedom from thirst, hunger, thermal discomfort, parasites, and disease – the breeding of animals that withstand such harsh low-input conditions evokes Kojak's maxim *to survive is a lousy way to live*. The justification of such adaptation strategies is commonly phrased in terms of the economical and/or cultural importance of livestock for such marginal areas (see the section on "Biodiversity," and more specifically pp. 405–419 of [5]), which entirely overlooks animal welfare.

By contrast, animal welfare problems in intensive production systems center around robustness and behavioral deprivation. Strategies to reduce deprivation problems by animal breeding (as outlined in the section on "Deprivation") meet with criticism of an ethical nature, as from Hörning [203], above.

The issue is if *genetic adaptation of livestock species to* the violation of their welfare by thirst, hunger, thermal discomfort, parasites, and disease in extensive conditions (for economic and cultural reasons) would be ethically justified, whereas genetic adaptation to behavioral deprivation in intensive systems (for economic reasons) would be wrong. If it is justified to select poultry against feather pecking in alternative housing systems, the question is valid why such selection in battery cages would be wrong.

There are two elements here: (1) the production system as such and (2) the process of adapting animal species to it through artificial selection. These are difficult to separate, because the argumentation is partly circular.

"Since biology appears to impose few limitations on what is possible, changing the animal to suit the environment raises the question of the ethical acceptability of the environment" [166]. The underlying notion here is that the environment may be intrinsically wrong. Nevertheless, *successful* adaptation of an animal species to any production system would make that system acceptable from the point of view of animal welfare – for that particular species, in that particular system. For anyone who finds such systems unacceptable a priori, such adaptation is therefore undesirable: the circular argument appears here. A housing system that is deemed unacceptable a priori, without taking animal welfare into account, can only make sense from a human perspective.

Artificial Selection Domestication through artificial selection is a human activity, and therefore subject to ethics. By contrast, natural selection just happens.

Natural selection has adapted species to previously hostile conditions: freezing (Antarctic icefish, *Dissostichus mawsoni*), molten sulfur (western Pacific tonguefish, *Symphurus thermophilus)*, cobra venom (mongoose, *Herpestes ichneumon*), high CO_2 and low oxygen levels (naked molerat, *Heterocephalus glaber*), compression (sperm whale, *Physeter macrocephalus*), drought (Arabian camel, *Camelus dromedarius*) and crowding (Mexican free-tailed bat, *Tadarida brasiliensis*), among many others. Natural selection is also adapting *Sus scrofa domesticus* to intensive housing conditions – this is happening now, but so slowly that it is very difficult to notice. Artificial selection can do the same, much faster.

Like natural selection, domestication used to be a slow process – its resulting changes were hardly noticeable with a normal human time horizon. These changes have accelerated considerably since the 1980s, to the extent that they are now measurable within, say, a decade (as in Figs. 9–11) – and many people feel uncomfortable with this. Despite education and popularization of science, the notion of evolution (i.e., genetic change) as a process that is actually taking place today is not widely appreciated. Judeo-Christian culture regards species as fixed entities, which makes

people resist a noticeable change of a species' instinctive repertoire because it is experienced as "unnatural" (which is exactly what it is: domestication, like every other aspect of civilization, is a deliberate move away from nature). This resistance is toward genetic change of the pig *as we know it*. Nine thousand years of domestication have reduced the cephalization ratio (brain size as a proportion of body size) of the domestic pig by 30–40% as compared to the wild boar, the same as the dog-wolf comparison [149, 206]; significantly, the limbic system is most affected, see the section on "Selection Objectives." There is no reason to think that this process has stopped – nor will any dog owner argue that the current situation is wrong. What many people resist is to notice such a process of change actually taking place, if it would be accelerated by more effective artificial selection procedures as in Belyaev's famous fox selection lines [207]. The pig *as we know it* represents one particular stage of an evolutionary continuum, much of which lies in the future. Because this stage is familiar, now, it is experienced as the "natural" one – which it is not: by definition there is no such thing as a natural domestic animal. Accordingly, FAWC [120] stress the distinction between "natural" and "normal" behavior in farm animals.

A common argument is that such further adaptations to the niche of domestication would reduce the animal to a means to an agricultural end, to a commodity – which "embodies an excessively instrumental view on living creatures" [208]; they would violate the animal's *integrity*, "making it in some way less complete than it was previously" [181].

Integrity "Would it be right to produce [. . .] a pig unable to feel pain and unresponsive to other pigs? [. . .] such a pig would not be able to suffer, and its use might lead to significant productivity gains. Someone arguing that [this] would be wrong, would not be able to argue thus on the grounds of animal suffering" [120]. Rather, such argumentation is typically phrased in terms of *integrity*. It is useful to distinguish between two integration levels here: the individual animal, and the species as such.

One view is that violation of an individual animal's integrity is wrong; this is about production systems that keep animals in persistent pain, frustration, or fear – and [209] about breeding that predisposes animals to such conditions.

Quite another view is that it is wrong to breed animals that experience less pain, frustration, or fear in such production systems [210]. This is about species integrity: although the individual pig's integrity is less violated, it would be *less of a pig*, which sounds uncomfortable. Conversely, it can be argued that such a pig would be *less of a wild boar* (*Sus scrofa scrofa*) and therefore *more of a pig* (*Sus scrofa domesticus*) on the evolutionary continuum mentioned above. Importantly, the argument is not about animal welfare but about human values.

Appleby and Sandøe [211] analyze the various schools of philosophical thought on this issue "so that scientists may be more aware of the strengths and weaknesses of their own ideas about animal welfare." Thompson [212] gives an overview of the same; one approach holds that it is important for animals to express their instinctive behavior motivations (see the section on "Deprivation"), as far as "they actually have these [. . .], but whether or not a given animal does or does not have these drives is immaterial. Or put differently, one cannot harm an animal by frustrating a [motivation] that it does not have. Because this view revolves around the [motivations] that individual animals actually have, it does not see anything problematic about producing animals that have different motivations." This goes back to Rollin [213].

An opposing view is that such animals (1) "can be said to have been harmed, even if there is no corresponding adverse affect in terms of animal bodies or animal minds"; this (2) "would regard the use of genetic strategies to address welfare problems as morally problematic" [212]. Gavrell Ortiz [214] disagrees with point (1) but defends point (2) on the grounds of violated animal *dignity*, "even if the modification would improve the animal's welfare."

All this reduces animal welfare to "a subset of human welfare, the animals' preferences and [welfare] having relevance only to the extent that they are important to *us*" [215]. This was extended by Würbel [216]: "it is [. . .] important to distinguish between our intention to protect animals (which may be partly selfish) and true animal protection. Animal protection is

ethically justified by our own human values. What animals need for their protection, however, needs to be justified *biologically* by values that apply to the animals. Only by acknowledging this distinction will we arrive at an ethical and legal framework that satisfies our ethical claims as well as doing justice to the animals" (our emphasis).

The conclusion from this section on "Animal Welfare" is simple and uncomfortable. Intensive pig production systems will expand considerably, particularly in the developing world. Adapting the species to such conditions is technically challenging but feasible; it will improve animal welfare. Argumentation against this serves human moral values, and not animal welfare.

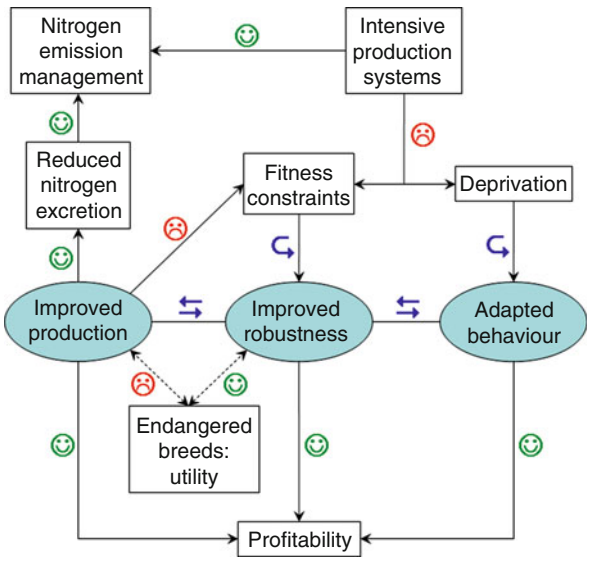
Future Directions

To repeat from the end of the Introduction: *Sustainability will always be a matter of more or less: it can never be an absolute goal.* This can now be made more concrete in terms of conflicting concerns about the various targets of sustainable production (people, pigs, planet, profit), as follows.

De Boer and Cornelissen [217] evaluated three laying-hen housing systems for the sustainability indicators economic performance, ammonia emission, energy use, animal welfare, farmer welfare, and egg quality. These authors notice conflicts such as "improvement of farmer welfare is difficult to achieve in animal-friendly [systems], because unfavorable thoracic dust concentrations [. . .] are a direct result of the presence of litter." With equal weighting to each indicator, the battery cage ranked considerably better for overall sustainability than deep-litter and aviary systems.

Likewise, three scenarios for enhanced sustainability of pig production were studied [218, 219] focusing on (1) animal welfare, (2) pollution, or (3) product quality and safety. Among the reported conflicts are a higher contribution to acidification, a higher greenhouse gas emission, and higher production costs in (1) the animal welfare scenario than in (2) the pollution scenario. "Ranking between the different aspects of sustainability may [. . .] differ between different people and over time. How to evaluate [them] is mainly a political question, and legislation and political decisions can easily change the ranking of the scenarios." This is about production systems, differing in animal management, housing, and feed production strategies. The breeding goals specified in that study were not dramatically different among the scenarios: (1) lean tissue growth rate (LTGR), feed intake, mothering ability, and longevity; (2) LTGR and feed efficiency; and (3) LTGR and meat quality – sets that occur alongside each other in any transnational breeding program, see Figs. 9–11 in the section on "Selection for Robustness Traits," and also Fig. 5 in [107].

However, the inclusion in breeding programs of specific sustainability targets (biodiversity, pollution, animal welfare) will create conflicts that need careful prioritization, as illustrated in Fig. 15. Priorities can be based on economic approaches such as shadow prices (as illustrated for nitrogen excretion in the Pollution section), or benefit functions for animal welfare traits [220]. But the outcome must be a political compromise, and as such will change over time.



Pig Breeding for Increased Sustainability. Figure 15 Elements of pig breeding goals (blue ovals) and their relationships with sustainability issues. The symbols ☹ and ☺ indicate (un-)favorable influences on the downstream element; ↻ indicates that solution of the upstream issue requires the downstream element; ⇆ indicates that a balance must be established. Dotted arrows characterize the sustainability issue in terms of breeding goal elements

A recurring theme of this chapter is: *this technology is statistically demanding*. It follows that sustainable animal breeding equals high-tech animal breeding – just as sustainable animal production must involve precision farming [221] to overcome its inherent conflicts. Earlier breeding programs delivered at lower sustainability levels not only because of incomplete breeding goals (focusing on narrow-sense profitability) but also because the required data recording and processing methodology was not available.

Future directions will have to be set by the production sector and the society that it is part of. The commercial pig breeding sector does not have its own agenda: breeding goals are set based on what the market for breeding stock demands, and those demands are influenced by society – e.g., through legislation, or market regulation, around pollution and animal welfare. This chapter shows that the genetic technology to meet such demands is available, and can be exploited.

Acknowledgments

Thanks are due to Gé Backus, Andrea Doeschl-Wilson, Eildert Groeneveld, Irene Hoffmann, Robert Hoste, Bas Kemp, Cees de Lange, Herveline Lenoir, Asko Mäki-Tanila, Elżbieta Martyniuk, Dave McLaren, Marnie Mellencamp, Patrick Morel, Bill Muir, Candido Pomar, Rainer Roehe, Lotta Rydhmer, Montse Torremorell, Simon Turner, Eldon Wilson, and Hanno Würbel.

Bibliography

1. Gamborg C, Sandøe P (2003) Breeding and biotechnology in farm animals: ethical issues. In: Levinson R, Reiss MJ (eds) Key issues in bioethics: a guide for teachers. RoutledgeFalmer, London, pp 133–141
2. Gamborg C, Sandøe P (2005) Applying the notion of sustainability – dilemmas and the need for dialogue. In: Gunning J, Holm S (eds) Ethics, law and society. Asghate, Aldershot, pp 123–130
3. Elkington J (1999) Cannibals with forks – the triple bottom line of 21st century business. Capstone, Oxford
4. Knap PW, Neeteson-Van Nieuwenhoven AM (2006) Private and public roles in conservation. In: Options and strategies for the conservation of farm animal genetic resources: report of an international workshop and presented papers (CD-ROM). CGIAR Biodiversity International, Rome, pp 62–67
5. FAO (2007) In: Rischkowsky B, Pilling D (eds) The state of the world's animal genetic resources for food and agriculture. Food and Agriculture Organization, Rome. ISBN 978-92-5-105762-9
6. Rege JEO (1999b) The state of African cattle genetic resources. 1: Classification framework and identification of threatened and extinct breeds. Anim Genet Res Info 25:1–25
7. Rege JEO, Gibson JP (2003) Animal genetic resources and economic development: issues in relation to economic valuation. Ecol Econ 45:319–330
8. Tisdell C (2003) Socioeconomic causes of loss of animal genetic diversity: analysis and assessment. Ecol Econ 45: 365–376
9. Gandini GC, Oldenbroek JK (2007) Strategies for moving from conservation to utilisation. In: Oldenbroek JK (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 29–54
10. Simianer H (2005) Decision making in livestock conservation. Ecol Econ 53:559–572
11. Gandini GC, Villa E (2003) Analysis of the cultural value of local livestock breeds: a methodology. J Anim Breed Genet 120:1–11
12. Ollivier L (2005) Economic relevance of animal diversity conservation. In: Rosati A, Tewolde A, Mosconi C (eds) Animal production and animal science worldwide. Wageningen Academic Publishers, Wageningen, pp 271–279
13. Hill WG, Zhang XS (2009) Maintaining genetic variation in fitness. In: Van der Werf JHJ, Graser HU, Frankham R, Gondro C (eds) Adaptation and fitness in animal populations. Springer, Berlin, pp 59–82
14. Nicholas FW (2009) Discussion. In: Van der Werf JHJ, Graser HU, Frankham R, Gondro C (eds) Adaptation and fitness in animal populations. Springer, Berlin, pp 233–234
15. Dempfle L (1990) Conservation, creation, and utilization of genetic variation. J Dairy Sci 73:2593–2600
16. Megens HJ, Crooijmans RPMA, SanCristobal M, Hui X, Li N, Groenen MAM (2008) Biodiversity of pig breeds from China and Europe estimated from pooled DNA samples: differences in microsatellite variation between two areas of domestication. Genet Sel Evol 40:103–128
17. Eding H, Bennewitz J (2007) Measuring genetic diversity in farm animals. In: Oldenbroek JK (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 103–130
18. San Cristobal M, Chevalet C, Haley CS, Joosten R, Rattink AP, Harlizius B, Groenen MAM, Amigues Y, Boscher M-Y, Russell G, Law A, Davoli R, Russo V, Désautés C, Alderson L, Fimland E, Bagga M, Delgado JV, Vega-Pla JL, Martinez AM, Ramos M, Glodek P, Meyer JN, Gandini GC, Matassino D, Plastow GS, Siggens KW, Laval G, Archibald AL, Milan D, Hammond K, Cardellino R (2006a) Genetic diversity within and between European pig breeds using microsatellite markers. Anim Genet 37:189–198
19. SanCristobal M, Chevalet C, Peleman J, Heuven H, Brugmans B, Van Schriek M, Joosten B, Rattink AP, Harlizius B, Groenen MAM, Amigues Y, Boscher MY, Russell G, Law A, Davoli R, Russo V, Dèsautés C, Alderson L, Fimland E, Bagga M, Delgado JV, Vega-Pla JL, Martinez AM, Ramos M, Glodek P, Meyer JN,

Gandini G, Matassino D, Siggens K, Laval G, Archibald A, Milan D, Hammond K, Cardellino R, Haley C, Plastow G (2006b) Genetic diversity in European pigs utilizing amplified fragment length polymorphism markers. Anim Genet 37:232–238
20. Van Zeveren A, Peelman L, Van de Weghe A, Bouquet Y (1995) A genetic study of Belgian pig populations by means of seven microsatellites. J Anim Breed Genet 112:191–204
21. Sollero BP, Paiva SR, Faria DA, Guimarães EF, Castro STR, Egito AA, Albuquerque MSM, Piovezan U, Bertani GR, Da San Mariante A (2009) Genetic diversity of Brazilian pig breeds evidenced by microsatellite markers. Livest Sci 123:8–15
22. Souza CA, Ramayo Y, Megens HJ, Rodríguez MC, Loarca A, Caal E, Soto H, Melo M, Revidatti MA, De la Rosa SA, Shemereteva IN, Okumura N, Cho IC, Delgado JV, Paiva SR, Crooijmans RPMA, Schook LB, Groenen MAM, Ramos-Onsins SE, Pérez-Enciso M (2010) Porcine colonization of the Americas: a 60k SNP story. In: 9th WCGALP, Leipzig, Germany (Communication 0510)
23. Alves E, Barragán C, Fernández AI, Rodríguez C, Silió L (2006) Success rate of genetic clustering of domestic and wild pigs as a function of the number of markers. In: 8th WCGALP, Belo Horizonte, Brazil (Communication 33–24)
24. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
25. Flury C, Weigend S, Ding X, Täubert H, Simianer H (2007) Haplotype kinship for three populations of the Goettingen minipig. Genet Sel Evol 39:159–179
26. Petit RJ, El Mousadik A, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. Conserv Biol 12:844–855
27. Toro MA, Mäki-Tanila A (2007) Genomics reveals domestication history and facilitates breed development. In: Oldenbroek JK (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 75–102
28. Eding H, Crooijmans RPMA, Groenen MAM, Meuwissen THE (2002) Assessing the contribution of breeds to genetic diversity in conservation schemes. Genet Sel Evol 34:613–633
29. Fabuel E, Barragán C, Silió L, Rodríguez MC, Toro MA (2004) Analysis of genetic diversity and conservation priorities in Iberian pigs based on microsatellite markers. Heredity 93:104–113
30. Bennewitz J, Meuwissen THE (2005) A novel method for the estimation of the relative importance of breeds in order to conserve the total genetic variance. Genet Sel Evol 37:315–337
31. Ollivier L, Foulley JL (2005) Aggregate diversity: new approach combining within- and between-breed genetic diversity. Livest Prod Sci 95:247–254
32. Toro MA, Fernández J, Caballero A (2009) Molecular characterization of breeds and its use in conservation. Livest Sci 120:174–195
33. Ciobanu DC, Day AE, Nagy A, Wales R, Rothschild MF, Plastow GS (2001) Genetic variation in two conserved local Romanian pig breeds using type 1 DNA markers. Genet Sel Evol 33: 417–432
34. Iannuccelli N, Riquet J, Mercat MJ, Legros H, SanCristobal M, Lacoste A, Bidanel JP, Milan D (2006) Diversité génétique des populations porcines françaises dans les régions chromosomiques soumises à la sélection: projet DIVQTL. Les Actes du BRG 6:111–128
35. Bonin A, Nicole F, Pompanon F, Miaud C, Taberlet P (2007) Population adaptive index: a new method to help measure intraspecific genetic diversity and prioritize populations for conservation. Conserv Biol 21:697–708
36. Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, 4th edn. Longman, Harlow
37. Draganescu C, Ghita E, Nagy A (2008) Note on the genetic history of the Romanian Saddleback (Bazna) pig breed conservation nucleus. Archiva Zootechnica 11:65–69
38. Nomura T, Ieiri S, Yamashita J (2008) Effective size and inbreeding in closed breeding herds of pig. 1: Pedigree analysis. Jpn J Swine Sci 45:149–155
39. Dikić M, Salajpal K, Karolyi D (2009) Biological characteristics of Turopolje pig breed as factors in renewing and preservation of population. DAGENE annual meeting, Zagreb, Croatia, http://www.dagene.eu/annual zagreb 2009/Dikic et al_DAGENE 2009.pdf
40. Welsh CS, Blackburn HD, Schwab C (2009) Population status of major US swine breeds. J Anim Sci 87(Suppl 3):160
41. Tholen E, Staack J, Müller P, Ingwersen J (2010) Degree of endangerment of different German pig herdbook populations. In: 9th WCGALP, Leipzig, Germany (Communication 0264)
42. Uimari P, Sevón-Aimonen ML, Strandén I (2010) Pedigree analysis of Finnish Landrace and Yorkshire pig populations. In: 9th WCGALP, Leipzig, Germany (Communication 0090)
43. Villanueva B, Sawalha RM, Roughsedge T, Rius-Vilarrasa E, Woolliams JA (2010) Development of a genetic indicator of biodiversity for farm animals. Livest Sci 129:200–207
44. Wang J (2005) Estimation of effective population sizes from data on genetic markers. Philos Trans R Soc Lond B Biol Sci 360:1395–1409
45. Álvarez I, Royo LJ, Gutiérrez JP, Fernández I, Arranz JJ, Goyache F (2008) Relationship between genealogical and microsatellite information characterizing losses of genetic variability: empirical evidence from the rare Xalda sheep breed. Livest Sci 115:80–88
46. Tixier-Boichard M, Ayalew W, Jianlin H (2008) Inventory, characterization and monitoring. Anim Genet Res Info 42:29–44, Food and Agriculture Organization, Roma, Italy
47. Toro MA, Rodriganez J, Silio L, Rodriguez C (2000) Genealogical analysis of a closed herd of black hairless Iberian pigs. Conserv Biol 14:1843–1851
48. Aspi J, Roininen E, Ruokonen M, Kojola I, Vilà C (2006) Genetic diversity, population structure, effective population size and demographic history of the Finnish wolf population. Mol Ecol 15:1561–1576

49. Meuwissen THE (2007) Operation of conservation schemes. In: Oldenbroek JK (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 167–194
50. Ollivier L, Alderson L, Gandini GC, Foulley JL, Haley CS, Joosten R, Rattink AP, Harlizius B, Groenen MAM, Amigues Y, Boscher MY, Russell G, Law A, Davoli R, Russo V, Matassino D, Désautés C, Fimland E, Bagga M, Delgado JV, Vega-Pla JL, Martinez AM, Ramos AM, Glodek P, Meyer JN, Plastow GS, Siggens KW, Archibald AL, Milan D, SanCristobal M, Laval G, Hammond K, Cardellino R, Chevalet C (2005) An assessment of European pig diversity using molecular markers: partitioning of diversity among breeds. Conserv Genet 6:729–741
51. Simon DL, Buchenauer D (1993) Genetic diversity of European livestock breeds. EAAP publication 66, Wageningen Pers, Wageningen, Netherlands
52. Gandini GC, Ollivier L, Danell B, Distl O, Georgoudis A, Groeneveld E, Martyniuk E, Van Arendonk JAM, Woolliams JA (2004) Criteria to assess the degree of endangerment of livestock breeds in Europe. Livest Prod Sci 91:173–182
53. Lynch M, Conery J, Burger R (1995) Mutation accumulation and the extinction of small populations. Am Nat 146:489–518
54. Theodorou K, Couvet D (2006) On the expected relationship between inbreeding, fitness, and extinction. Genet Sel Evol 38:371–387
55. Bennewitz J, Meuwissen THE (2005) Estimation of extinction probabilities of five German cattle breeds by population viability analysis. J Dairy Sci 88:2949–2961
56. Tenesa A, Navarro P, Hayes BJ, Duffy DL, Clarke GM, Goddard ME, Visscher PM (2007) Recent human effective population size estimated from linkage disequilibrium. Genome Res 17(4):520–526
57. Hayes BJ, Visscher PM, McPartlan HC, Goddard ME (2003) Novel multilocus measure of linkage disequilibrium to estimate past effective population size. Genome Res 13:635–643
58. Amaral AJ, Megens HJ, Crooijmans RPMA, Heuven HCM, Groenen MAM (2008) Linkage disequilibrium decay and haplotype block structure in the pig. Genetics 179:569–579
59. Du FX, Clutter AC, Lohuis MM (2007) Characterizing linkage disequilibrium in pig populations. Int J Biol Sci 3:166–178
60. Harmegnies N, Farnir F, Davin F, Buys N, Georges M, Coppieters W (2006) Measuring the extent of linkage disequilibrium in commercial pig populations. Anim Genet 37:225–231
61. England PR, Cornuet JM, Berthier P, Tallmon DA, Luikart G (2006) Estimating effective population size from linkage disequilibrium: severe bias in small samples. Conserv Genet 7:303–308
62. Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 7:167–184
63. Schwartz MK, Tallmon DA, Luikart G (1998) Review of DNA-based census and effective population size estimators. Anim Conserv 1:293–299
64. Wang J (2009) A new method for estimating effective population sizes from a single sample of multilocus genotypes. Mol Ecol 18:2148–2164
65. Meuwissen THE, Goddard ME (2007) Multipoint identity-by-descent prediction using dense markers to map quantitative trait loci and estimate effective population size. Genetics 176:2551–2560
66. Vitalis R, Couvet D (2001) Estimation of effective population size and migration rate from one- and two-locus identity measures. Genetics 157:911–925
67. Abdallah JM, SanCristobal M, Chevalet C (2006) Estimation of effective size and migration rates in twelve European pig populations using non equilibrium model. In: 8th WCGALP, Belo Horizonte, Brazil (Communication 33–19)
68. Baas TJ, Goodwin RN, Christian LL, Johnson RK, Robison OW, Mabry JW, Clark K, Tokach M, Henry S, Berger PJ (2003) Design and standards for genetic evaluation of swine seedstock populations. J Anim Sci 81:2409–2418
69. Moeller SJ, Goodwin RN, Johnson RK, Mabry JW, Baas TJ, Robison OW (2004) The National Pork Producers Council maternal line national genetic evaluation program: a comparison of six maternal genetic lines for female productivity measures over four parities. J Anim Sci 82:41–53
70. Gibson JP, Ayalew W, Hanotte O (2007) Measures of diversity as inputs for decisions in conservation of livestock genetic resources. In: Jarvis DI, Padoch C, Cooper HD (eds) Managing biodiversity in agricultural ecosystems. Columbia University Press, New York, pp 117–140. ISBN 978-0231136488
71. Nichol ST, Arikawa J, Kawaoka Y (2000) Emerging viral diseases. Proc Natl Acad Sci USA 97:12411–12412
72. Pybus OG, Rambaut A (2009) Evolutionary analysis of the dynamics of viral infectious disease. Nat Rev Genet 10:540–550
73. Covey S, Merrill AR, Merrill RR (1994) First things first. Simon and Schuster, New York. ISBN 0-684-80203-1
74. Mendelsohn R (2003) The challenge of conserving indigenous domesticated animals. Ecol Econ 45:501–510
75. Ollivier L, Foulley JL (2009) Managing genetic diversity, fitness and adaptation. In: Van der Werf JHJ, Graser HU, Frankham R, Gondro C (eds) Adaptation and fitness in animal populations. Springer, Berlin, pp 201–228
76. Simianer H, Reist-Marti SB, Gibson J, Hanotte O, Rege JEO (2003) An approach to the optimal allocation of conservation funds to minimize loss of genetic diversity between livestock breeds. Ecol Econ 45:377–392
77. Bennewitz J, Eding H, Ruane J, Simianer H (2007) Selection of breeds for conservation. In: Oldenbroek JK (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 131–146
78. Barker JSF (2001) Conservation and management of genetic diversity: a domestic animal perspective. Can J For Res 31: 588–595
79. Rege JEO (1999a) Characterisation and conservation of animal genetic resources: What is it about? In: Rege JEO (ed)

Economic valuation of animal genetic resources. In: Proceedings of an FAO/ILRI workshop. International Livestock Research Institute, Nairobi, Kenya pp 22–24
80. Delanotte M (2007) Un avenir en rose pour une race qui a parfois broyé du noir. L'Union Agricole 21 December 2007. limousin.synagri.com/ca1/PJ.nsf/TECHPJPARCLEF/08102
81. Woolliams JA, Berg P, Mäki-Tanila A, Meuwissen THE, Fimland E (2005) Sustainable management of animal genetic resources. Nordic Gene Bank Farm Animals, Ås, Norway
82. Rathje TA (2000) Strategies to manage inbreeding accumulation in swine breeding company nucleus herds: some case studies. J Anim Sci 79(Suppl 1):1–8
83. Ollivier L, James JW (2004) Predicting the annual effective size of livestock populations. Genet Res 84:41–46
84. Woolliams JA (2007) Genetic contributions and inbreeding. In: Oldenbroek JK (ed) Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers, Wageningen, pp 147–165
85. Kinghorn BP, Banks R, Gondro C, Kremer VD, Meszaros SA, Newman S, Shepherd RK, Vagg RD, Van der Werf JHJ (2009) Strategies to exploit genetic variation while maintaining diversity. In: Van der Werf JHJ, Graser HU, Frankham R, Gondro C (eds) Adaptation and fitness in animal populations. Springer, Berlin, pp 191–200
86. Kremer VD, Newman SN, Kinghorn BP, Knap PW, Wilson ER (2009) Strategic management of pig genetics. In: EAAP, Barcelona, Spain (Communication 13–25)
87. Nimbkar C, Gibson J, Okeyo M, Boettcher P, Soelkner J (2007) Sustainable use and genetic improvement. In: Report of the scientific forum on animal genetic resources held during the international technical conference on animal genetic resources for food and agriculture, Interlaken, Switzerland. Food and Agriculture Organization, Rome, Italy, pp 49–64
88. Whittemore CT (2006) Development and improvement of pigs by genetic selection. In: Kyriazakis I, Whittemore CT (eds) Whittemore's science and practice of pig production, 3rd edn. Blackwell, Oxford, pp 184–262
89. Knap PW (1990) The herdbook society/breed association. In: 4th WCGALP, Edinburgh, UK, 15:439–442
90. Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M, De Haan C (2006) Livestock's long shadow: environmental issues and options. Food and Agriculture Organization, Rome
91. Brandjes PJ, De Wit J, Van der Meer HG, Van Keulen H (1995) Environmental impact of animal manure management. International Agricultural Centre, Wageningen
92. Dourmad JY, Sève B, Latimier P, Boisen S, Fernández J, Van der Peet-Schwering C, Jongbloed AW (1999a) Nitrogen consumption, utilisation and losses in pig production in France, the Netherlands and Denmark. Livest Prod Sci 58:261–264
93. Dourmad JY, Guingand N, Latimier P, Sève B (1999b) Nitrogen and phosphorus consumption, utilisation and losses in pig production: France. Livest Prod Sci 58:199–211
94. Bourdon D, Dourmad JY, Henry Y (1995) Réduction des rejets azotés chez les porcs en croissance par la mise en oeuvre de l'alimentation multiphase, associée a l'abaissement du taux azoté. Journées de la Recherche Porcine en France 27:269–278
95. De Lange CF, Gillis AM, Simpson GJ (2001) Influence of threonine intake on whole-body protein deposition and threonine utilization in growing pigs fed purified diets. J Anim Sci 79:3087–3095
96. Buraczewska L, Święch E, Le Bellego L (2006) Nitrogen retention and growth performance of 25 to 50 kg pigs fed diets of two protein levels and different ratios of digestible threonine to lysine. J Anim Feed Sci 15:25–36
97. Pomar C, Hauschild L, Zhang GH, Pomar J, Lovatto PA (2010) Precision feeding can significantly reduce feeding cost and nutrient excretion in growing animals. In: Sauvant D, Van Milgen J, Faverdin P, Friggens N (eds) Modelling nutrient digestion and utilisation in farm animals. Wageningen Academic Publishers, pp 327–334
98. Crocker AW, Robison OW (2002) Genetic and nutritional effects on swine excreta. J Anim Sci 80:2809–2816
99. De Verdal H, Narcy A, Le Bihan-Duval E, Chapuis H, Bastianelli D, Même N, Mignon-Grasteau S (2010) Selection for excretion traits in chicken. In: 9th WCGALP, Leipzig, Germany (Communication 0123)
100. Cassandro M, Cecchinato A, Battagin M, Penasa M (2010) Genetic parameters of methane production in Holstein Friesian cows. In: 9th WCGALP, Leipzig, Germany (Communication 0837)
101. Van der Peet-Schwering CMC, Jongbloed AW, Aarnink AJA (1999) Nitrogen and phosphorus consumption, utilisation and losses in pig production: the Netherlands. Livest Prod Sci 58:213–224
102. Jones H, Warkup C, Williams A, Audsley E (2008) The effect of genetic improvement on emissions from livestock systems. In: 59th EAAP, Vilnius, Lithuania (Contribution 05-6)
103. Knap PW (1999) Simulation of growth in pigs: evaluation of a model to relate thermoregulation to body protein and lipid content and deposition. Anim Sci 68:655–679
104. Knap PW, Rauw WM (2008) Selection for high productivity in pigs. In: Rauw WM (ed) Resource allocation theory applied to farm animal production. CABI, Wallingford, pp 288–301
105. NRC (1998) Nutrient requirements of swine, 10th revised edn. National Academy Press, Washington, DC
106. Morel PCH, Wood GR (2005) Optimisation of nutrient use to maximise profitability and minimise nitrogen excretion in pig meat production systems. Acta Horticulturae (ISHS) 674: 269–275
107. Knap PW (2009) Voluntary feed intake and pig breeding. In: Torrallardona D, Roura E (eds) Voluntary feed intake in pigs. Wageningen Academic Publishers, Wageningen, pp 11–33
108. Teizzi S (1999) External effects of agricultural production in Italy and environmental accounting. Environ Resour Econ 13:459–472

109. Hartmann M, Hediger W, Peter S (2008) Reducing nitrogen losses from agricultural systems – an integrated economic assessment. Schriften der Gesellschaft für Wirtschafts- und Sozialwissenschaften des Landbaus (Landwirtschaftsverlag, Münster, Germany) 43:335–344
110. Fontein PF, Thijssen GJ, Magnus JR, Dijk J (1994) On levies to reduce the nitrogen surplus: the case of Dutch pig farms. Environ Resour Econ 4:455–478
111. Paul CJM, Ball VE, Felthoven RG, Grube A, Nehring RF (2002) Effective costs and chemical use in United States agricultural production: using the environment as a "free" input. Am J Agr Econ 84:902–915
112. Key N, McBride W, Mosheim R (2008) Decomposition of total factor productivity change in the US hog industry. J Agr Appl Econ 40:137–149
113. Fontein PF, Thijssen GJ, Magnus JR, Dijk J (1999) Optimal taxation for the reduction of nitrogen surplus Dutch dairy farms, 1975–1989. In: Mahendrarajah S, Jakeman AJ, McAleer M (eds) Modelling change in integrated economic and environmental systems. Wiley, Chichester, pp 273–296
114. Price R, Thornton S, Nelson S (2007) The social cost of carbon and the shadow price of carbon: what they are, and how to use them in economic appraisal in the UK. Department for Environment, Food and Rural Affairs, London
115. Wall E, Simm G, Moran D (2010) Developing breeding schemes to assist mitigation of greenhouse gas emissions. Animal 4:366–376
116. Hoste R, Puister L (2009) Productiekosten van varkens: een internationale vergelijking. LEI Wageningen UR, Den Haag, Netherlands. Report 2008-082
117. SVC (1997) The welfare of intensively kept pigs. Scientific Veterinary Committee of the European Commission, Brussels, Belgium. Report XXIV/B3/ScVC/0005/1997
118. Fraser D (2005) Animal welfare and the intensification of animal production. An alternative interpretation. Food and Agriculture Organization, Rome
119. Grandin T, Deesing MJ (1998) Genetics and behavior during handling, restraint and herding. In: Grandin T (ed) Genetics and the behavior of domestic animals. Academic, San Diego, CA, pp 113–144
120. FAWC (2009) Farm animal welfare in Great Britain: past, present, future. Farm Animal welfare Council, London
121. Rauw WM, Kanis E, Noordhuizen-Stassen EN, Grommers FJ (1998) Undesirable side effects of selection for high production efficiency in farm animals: a review. Livest Prod Sci 56:15–33
122. Knap PW, Rauw WM (2009) Selection for high production in pigs. In: Rauw WM (ed) Resource allocation theory applied to farm animal production. CAB International, Wallingford, pp 210–229
123. Knap PW (2005) Breeding robust pigs. Aust J Exp Agric 45:763–774
124. Goddard M (2009) Fitness traits in animal breeding programs. In: Van der Werf JHJ, Graser HU, Frankham R, Gondro C (eds) Adaptation and fitness in animal populations. Springer, Berlin, pp 41–52
125. Gjedrem T (1972) A study on the definition of the aggregate genotype in a selection index. Acta Agric Scand 22:11–16
126. Knol EF, Duijvesteijn N, Leenhouwers JI, Merks JWM (2010) New phenotypes for new breeding goals in pigs. In: EAAP Heraklion, Greece (Communication 02-2)
127. Knap PW (2009a) Robustness. In: Rauw WM (ed) Resource allocation theory applied to farm animal production. CAB International, Wallingford, pp 288–301
128. Rydhmer L, Lundeheim N (2008) Breeding pigs for improved welfare. In: Faucitano L, Schaefer AL (eds) Welfare of pigs from birth to slaughter. Wageningen Academic Publishers, Wageningen, pp 243–270
129. Newman S, Wang L, Anderson J, Casey D (2010) Utilizing crossbred records to increase accuracy of breeding values in pigs. In: 9th WCGALP, Leipzig, Germany (Communication 0632)
130. Henryon M, Sørensen AC, Berg P, Nielsen B (2010) Breeding pigs for resistance to disease is difficult even with genomic selection. In: 9th WCGALP, Leipzig, Germany (Communication 0854)
131. Onteru SK, Fan B, Garrick DJ, Stalder KJ, Rothschild MF (2010) Whole-genome association analyses for sow lifetime production, reproduction and structural soundness traits using the PorcineSNP60 beadchip. In: 9th WCGALP, Leipzig, Germany (Communication 0273)
132. Hermesch S, Huisman AE, Luxford BG, Graser HU (2006) Analysis of genotype by feeding level interaction in pigs applying reaction norm models. In: 8th WCGALP, Belo Horizonte, Brazil (Contribution 06-03)
133. Knap PW, Su G (2008) Genotype by environment interaction for litter size in pigs as quantified by reaction norms analysis. Animal 2:1742–1747
134. Friggens N, Van der Waaij L (2008) Modelling of resource allocation patterns. In: Rauw WM (ed) Resource allocation theory applied to farm animal production. CAB International, Wallingford, pp 302–320
135. Hughes BO, Duncan IJH (1988) The notion of ethological 'need', models of motivation and animal welfare. Anim Behav 36:1696–1707
136. Damm BI, Lisborg L, Vestergaard KS, Vanicek J (2003) Nest-building, behavioural disturbances and heart rate in farrowing sows kept in crates and Schmid pens. Livest Prod Sci 80:175–187
137. Ruis MAW, Te Brake JHA, Engel B, Buist WG, Blokhuis HJ, Koolhaas JM (2001) Adaptation to social isolation Acute and long-term stress responses of growing gilts with different coping characteristics. Physiol Behav 73:541–551
138. Held S, Cooper JJ, Mendl MT (2009) Advances in the study of cognition, behavioural priorities and emotions. In: Marchant-Forde JN (ed) The welfare of pigs. Springer, Berlin, pp 47–94
139. Manteuffel G (2002) Central nervous regulation of the hypothalamic-pituitary-adrenal axis and its impact on fertility,

immunity, metabolism and animal welfare – a review. Archive für Tierzucht 45:575–595
140. Tuyttens F (2007) Stereotypies. In: Velarde A, Geers R (eds) On farm monitoring of pig welfare. WAP, Wageningen, pp 41–46
141. Wemelsfelder F (2007) Apathy. In: Velarde A, Geers R (eds) On farm monitoring of pig welfare. WAP, Wageningen, pp 47–52
142. Mason G, Bateson M (2009) Motivation and the organization of behaviour. In: Jensen P (ed) The ethology of domestic animals, 2nd edn. CABI, Wallingford, pp 38–56
143. Beattie VE, Walker N, Sneddon IA (1995) Effects of environmental enrichment on behaviour and productivity of growing pigs. Anim Welfare 4:207–220
144. Beattie VE, O'Connell NE, Moss BW (1999) The influence of environmental enrichment on behaviour, performance and meat quality of domestic pigs. In: BSAS conference, Scarborough (Communication 192)
145. Webster J (2006) Ideals and realities: what do we owe to farm animals? In: Turner J, D'Silva J (eds) Animals, ethics and trade. Earthscan, London, pp 149–158
146. Jensen P (2009) Behaviour genetics, evolution and domestication. In: Jensen P (ed) The ethology of domestic animals, 2nd edn. CABI, Wallingford, pp 10–24
147. Jensen P (2002) Behaviour of pigs. In: Jensen P (ed) The ethology of domestic animals, 1st edn. CABI, Wallingford, pp 159–172
148. Stolba A, Wood-Gush DGM (1989) The behaviour of pigs in a semi-natural environment. Anim Prod 48:419–425
149. Kruska DCT (2005) On the evolutionary significance of encephalization in some eutherian mammals: effects of adaptive radiation, domestication, and feralization. Brain Behav Evol 65:73–108
150. Bolhuis JE (2004) Personalities in pigs: individual characteristics and coping with environmental challenges. Ph.D. thesis, Wageningen University, The Netherlands
151. Carroll BJ (2009) The neuroendocrinology of mood disorders. In: Pfaff DW, Arnold AP, Fahrbach SE, Etgen AM, Rubin RT (eds) Hormones, brain and behavior, vol 5, 2nd edn. Academic, San Diego, CA, pp 2899–2926
152. Loijens LWS (2002) Stress, endogenous opioids and stereotypies in tethered pigs. Ph.D. thesis, Wageningen University, The Netherlands
153. Karman AG (2003) Neuroendocrine adaptation to stress in pigs. Ph.D. thesis, Wageningen University, The Netherlands
154. Veenema AH (2003) Coping style and stressor susceptibility: neuroendocrine and neurochemical studies with genetically selected mouse lines. Ph.D. thesis, University of Groningen, The Netherlands
155. Benus RF, Bohus B, Koolhaas JM, Van Oortmerssen GA (1991) Heritable variation for aggression as a reflection of individual coping strategies. Cell Mol Life Sci 47:1008–1019
156. De Kloet ER (2004) Hormones and the stressed brain. Ann NY Acad Sci 1018:1–15
157. Keeling L, Jensen P (2009) Abnormal behaviour, stress and welfare. In: Jensen P (ed) The ethology of domestic animals, 2nd edn. CABI, Wallingford, pp 85–101
158. Ramos A, Mormède P (1998) Stress and emotionality: a multidimensional and genetic approach. Neurosci Biobehav Rev 22:33–57
159. Van Oortmerssen GA, Bakker TC (1981) Artificial selection for short and long attack latencies in wild Mus musculus domesticus. Behav Genet 11:115–126
160. Cools AR, Brachten R, Heeren D, Willemen A, Ellenbroek B (1990) Search after neurobiological profile of individual-specific features of Wistar rats. Brain Res Bull 24:49–69
161. Touma C, Bunck M, Stein H, Zeh R, Landgraf R (2006) Mice selected for high or low stress reactivity: a new animal model for affective disorders. Front Neuroendocrinol 27:56
162. Morris R (2006) Stress and the hippocampus. In: Andersen P (ed) The hippocampus book. Oxford University Press, New York, pp 751–768
163. Poletto R, Steibel JP, Siegford JM, Zanella AJ (2005) Effects of early weaning and social isolation on the expression of glucocorticoid and mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase 1 and 2 mRNAs in the frontal cortex and hippocampus of piglets. Brain Res 1067:36–42
164. Kanitz E, Puppe B, Tuchscherer M, Heberer M, Viergutz T, Tuchscherer A (2009) A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain. Physiol Behav 98:176–185
165. Pottinger TG (2000) Genetic selection to reduce stress in animals. In: Moberg GP, Mench JA (eds) The biology of animal stress: basic principles and implications for animal welfare. CABI, Wallingford, pp 291–308
166. D'Eath RB, Conington J, Lawrence AB, Olsson IAS, Sandøe P (2010) Breeding for behavioural change in farm animals: practical, economic and ethical considerations. Anim Welfare 19(Suppl 1):17–27
167. Mormède P (2005) Molecular genetics of behaviour: research strategies and perspectives for animal production. Livest Prod Sci 93:15–21
168. Jensen P (1994) Fighting between unacquainted pigs. Effects of age and of individual reaction pattern. Appl Anim Behav Sci 41:37–52
169. Couret D, Otten W, Puppe B, Prunier A, Merlot E (2009) Behavioural, endocrine and immune responses to repeated social stress in pregnant gilts. Animal 3:118–127
170. Turner SP, Roehe R, D'Eath RB, Ison SH, Farish M, Jack MC, Lundeheim N, Rydhmer L, Lawrence AB (2009) Genetic validation of post-mixing skin injuries in pigs as an indicator of aggressiveness and the relationship with injuries under more stable social conditions. J Anim Sci 87:3076–3082
171. Murani E, D'Eath RB, Turner SP, Evans G, Foury A, Kurt E, Thölking L, Klont R, Ponsuksili S, Mormède P, Wimmers K (2009) Identification of genes involved in the genetic control of aggressiveness, stress responsiveness, pork quality and their interactions. In: EAAP Barcelona, Spain (Communication 26-7)

172. Terenina E, Bazovkina D, Rousseau S, Salin F, Monllor S, Kulikov A, Turner SP, D'Eath RB, Mormede P (2010) Association between aggressive behavior and candidate gene polymorphisms: study of the brain serotonergic system in pigs. In: 9th WCGALP, Leipzig, Germany (Communication 0864)
173. Pauly C, Ampuero S, Bee G (2010) Expected effects on carcass and pork quality when surgical castration is omitted: results of a meta-analysis study. In: EAAP Heraklion, Greece (Communication 17-6)
174. PigCas (2008) Attitudes, practices and state of the art regarding piglet castration in Europe. University of Newcastle, UK. http://w3.rennes.inra.fr/pigcas/Public reports/D3 3 Final report evaluation.pdf
175. Squires EJ (2006) Possibilities for selection against boar taint. Acta Vet Scand 48(Suppl 1):S8
176. Tajet H, Andresen Ø, Meuwissen THE (2006) Estimation of genetic parameters of boar taint; skatole and androstenone and their correlations with sexual maturation. Acta Vet Scand 48(Suppl 1):S9
177. Merks JWM, Bergsma R, Bloemhof S, Roelofs-Prins DT, Knol EF (2010) Quantitative genetic opportunities to ban castration. In: EAAP Heraklion, Greece (Communication 17-7)
178. Moe M, Lien S, Aasmundstad T, Meuwissen THE, Hansen MHS, Bendixen C, Grindflek E (2009) Association between SNPs within candidate genes and compounds related to boar taint and reproduction. BMC Genet 10:32
179. Schenkel FS, Squires EJ (2010) Reducing boar taint in pigs using SNP markers. In: 9th WCGALP, Leipzig, Germany (Communication 0674)
180. Breuer K, Sutcliffe MEM, Mercer JT, Rance KA, O'Connell NE, Sneddon IA, Edwards SA (2005) Heritability of clinical tail-biting and its relation to performance traits. Livest Prod Sci 93:87–94
181. Turner SP, Roehe R, Lawrence AB (2010) Social behaviour in pigs. In: 9th WCGALP, Leipzig, Germany (Communication 0060)
182. Taylor NR, Main DCJ, Mendl M, Edwards SA (2009) Tail-biting: a new perspective. Vet J 186(2):137–147
183. Bolhuis JE, Schouten WGP, Schrama JW, Wiegant VM (2006) Effects of rearing and housing environment on behaviour and performance of pigs with different coping characteristics. Appl Anim Behav Sci 101:68–85
184. Bracke MBM (2007) Vulva biting. In: Velarde A, Geers R (eds) On farm monitoring of pig welfare. Wageningen Academic Publishers, Wageningen, pp 65–70
185. Harris MJ, Li YZ, Gonyou HW (2003) Savaging behaviour in gilts and sows. Can J Anim Sci 83:819–821
186. Kjaer JB, Mench JA (2003) Behaviour problems associated with selection for increased production. In: Muir WM, Aggrey SE (eds) Poultry genetics, breeding and technology. CAB International, Wallingford, pp 67–82
187. Su G, Kjaer JB, Sørensen P (2005) Variance components and selection response for feather-pecking behavior in laying hens. Poult Sci 84:14–21
188. Labouriau R, Kjaer JB, Abreu GCG, Hedegaard J, Buitenhuis AJ (2009) Analysis of severe feather pecking behavior in a high feather pecking selection line. Poult Sci 88:2052–2062
189. Wysocki M, Stratz P, Preuss S, Bennewitz J (2010) Functional investigation of candidate genes affecting feather pecking in chickens. In: 9th WCGALP, Leipzig, Germany (Communication 0254)
190. Quilter CR, Gilbert CL, Oliver GL, Jafer O, Furlong RA, Blott SC, Wilson AE, Sargent CA, Mileham A, Affara NA (2008) Gene expression profiling in porcine maternal infanticide: a model for puerperal psychosis. Am J Med Genet B Neuropsychiatr Genet 147B:1126–1137
191. Ellen ED, Visscher J, Van Arendonk JAM, Bijma P (2008) Survival of laying hens: genetic parameters for direct and associative effects in three purebred layer lines. Poult Sci 87:233–239
192. Bergsma R, Kanis E, Knol EF, Bijma P (2008) The contribution of social effects to heritable variation in finishing traits of domestic pigs (Sus scrofa). Genetics 178:1559–1570
193. Chen CY, Kachman SD, Johnson RK, Newman S, Van Vleck LD (2008) Estimation of genetic parameters for average daily gain using models with competition effects. J Anim Sci 86:2525–2530
194. Muir WM, Craig JV (1998) Improving animal well-being through genetic selection. Poult Sci 77:1781–1788
195. Cheng H, Muir WM (2005) The effects of genetic selection for survivability and productivity on chicken physiological homeostasis. Worlds Poult Sci J 61:383–397
196. Cheng HW, Eicher SD, Chen Y, Singleton P, Muir WM (2001) Effect of genetic selection for group productivity and longevity on immunological and hematological parameters of chickens. Poultry Sci 80:1079–1086
197. Gunsett F (2005) Group selection in swine – a case study. National Swine Improvement Federation Conference, Ottawa, Canada. www.nsif.com/Conferences/2005/pdf/GroupSelectionSwine.pdf
198. Muir WM, Schinckel AP (2002). Incorporation of competitive effects in breeding pro-grams to improve productivity and animal well being. In: 7th WCGALP, Montpellier, France (Communication 14-07)
199. Bijma P (2009) Maintaining fitness by within breed selection. In: Van der Werf JHJ, Graser HU, Frankham R, Gondro C (eds) Adaptation and fitness in animal populations. Springer, Berlin, pp 103–124
200. Conington J, Gibbons J, Haskell MJ, Bünger L (2010) The use of breeding to improve animal welfare. In: 9th WCGALP, Leipzig, Germany (Communication 0057)
201. Faure JM (1980) To adapt the environment to the bird or the bird to the environment? In: Moss R (ed) The laying hen and its environment. Nijhoff, Den Haag, pp 19–42
202. Garnett T (2009) Livestock-related greenhouse gas emissions: impacts and options for policy makers. Environ Sci Policy 12:491–503

203. Hörning B (2008) Auswirkungen der Zucht auf das Verhalten von Nutztieren. Tierzuchtfonds für artgemässe Tierzucht, Kassel University Press, Kassel, Germany
204. Lee DHK (2006) Problems in the environmental adaptation of domestic animals. Ann NY Acad Sci 91:608–616
205. François D, Boissy A, Jacquiet P, Allain D, Bibé B, Rupp R, Moreno CR, Bodin L, Hazard D, Bouix J (2010) Genetics of adaptation traits for harsh environment in sheep. In: 9th WCGALP, Leipzig, Germany (Communication 0453)
206. Herre W, Röhrs M (1990) Haustiere – zoologisch gesehen, 2nd edn. Fischer Verlag, Stuttgart
207. Belyaev DK, Trut LN (1975) Some genetic and endocrine effects of selection for domestication in silver foxes. In: Fox MW (ed) The wild canids. Van Nostrand Reinhold, New York, pp 416–426
208. Reiss MJ, Straughan R (1996) Improving nature? The science and ethics of genetic engineering. Cambridge University Press, Cambridge
209. Webster J (2005) Ideals and realities: what do we owe to farm animals? In: From Darwin to Dawkins: the science and implications of animal sentience (DVD). Compassion in World Farming Trust, Petersfield
210. FAWC (2004) Report on the welfare implications of animal breeding and breeding technologies in commercial agriculture. Farm Animal Welfare Council, London
211. Appleby MC, Sandøe PT (2002) Philosophical debate on the nature of well-being: implications for animal welfare. Anim Welfare 11:283–294
212. Thompson PB (2010) Why using genetics to address welfare may not be a good idea. Poult Sci 89:814–821
213. Rollin BE (2003) Ethics and species integrity. Am J Bioeth 3:15–17
214. Gavrell Ortiz SE (2004) Beyond welfare: animal integrity, animal dignity, and genetic engineering. Ethics Environ 9:94–120
215. McInerney J (2004) Animal welfare, economics and policy. Defra, London. www.defra.gov.uk/evidence/economics/foodfarm/reports/documents/animalwelfare.pdf
216. Würbel H (2009) Ethology applied to animal ethics. Appl Anim Behav Sci 118:118–127
217. De Boer IJM, Cornelissen AMG (2002) A method using sustainability indicators to compare conventional and animal-friendly egg production systems. Poult Sci 81: 173–181
218. Cederberg C, Flysjö A (2004) Environmental assessment of future pig farming systems: quantifications of three scenarios from the FOOD21 synthesis work. Swedish Institute for Food and Biotechnology, Göteborg, Sweden, SIK-report 723. chaos.bibul.slu.se/sll/institutet_livsm_bioteknik/sik-rapport/SIK723/SIK723.pdf
219. Stern S, Sonesson U, Gunnarsson S, Kumm KI, Öborn I, Nybrant T (2005) Sustainable pig production in the future: development and evaluation of different scenarios. Swedish University of Agricultural Sciences, Uppsala, Sweden, Report FOOD21-5/2005. citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.121.7315&rep=rep1&type=pdf
220. Stott AW, Gunn GJ (2008) Use of a benefit function to assess the relative investment potential of alternative farm animal disease prevention strategies. Prev Vet Med 84:179–193
221. Lokhorst C, Groot Koerkamp PWG (2009) Precision livestock farming '09. Wageningen Academic Publishers, Wageningen
222. Krüger L (1961) Geschichtliche Entwicklung der Rassen in der europäischen Tierzucht. In: Hammond J, Johansson I, Haring F (eds) Handbuch der Tierzüchtung 3-1: Rassenkunde. Parey, Hamburg, pp 25–53
223. Mason IL (1969) A world dictionary of livestock breeds, types and varieties, 4th edn. CAB International, Wallingford
224. Porter V (1993) Pigs. A handbook to the breeds of the world. Helm Information, Mountfield, Robertsbridge
225. Falkenberg H, Hammer H (2007) Zur Geschichte und Kultur der Schweinezucht und -haltung. 3: Schweinezucht und -haltung in Deutschland von 1650 bis 1900. Züchtungskunde 79:92–110
226. Huisman A, Cherel P, Van Haandel B (2010) Linkage disequilibrium and signatures of selection on chromosome 1 in a commercial sire and dam line. In: 9th WCGALP, Leipzig, Germany (Communication 0840)

Plant Breeding Under a Changing Climate

M. Fernanda Dreccer[1], David Bonnett[2], Tanguy Lafarge[3,4]
[1]CSIRO Plant Industry Cooper Laboratory, Gatton, QLD, Australia
[2]CIMMYT Int, Mexico D.F, Mexico
[3]CIRAD, UMR AGAP, Montpellier, France
[4]IRRI, CESD, Los Baños, Philippines

Article Outline

Glossary

Phenotyping The activity of measuring the physiological, morphological, developmental, and chemical characteristics of plants.

Trait A measurable phenotypic character or attribute, for example, plant height.

Definition of the Subject

The next generation of crops, capable of being productive in an increasingly variable and changing climate, will rely on genetic interventions based on process understanding, selection of target traits in managed environments, and high-throughput phenotyping and genotyping more than ever before. This entry discusses examples from wheat and rice, recent advances in plant breeding for high yield potential environments, and also those where abiotic stress is a major limitation to productivity. The methodologies and lessons learnt are discussed in the context of breeding in the face of climate change.

Introduction

The effects of climate change on agricultural production and food security are already taking place, creating new challenges for plant breeders to act quickly. The consequences of climate change on agricultural systems across the globe will be heterogeneous [35]. The projections for 2050 indicate that the increase in temperature (1–3°C) and CO_2 together with rainfall changes may benefit crops in the mid- to high latitudes, as temperatures will be closer to optimal for growth and the growing season longer. Over the same period, a decline in agricultural productivity is projected for low-latitude agricultural systems due to detrimental thermal conditions and more frequent extreme weather–related events. In the longer term, if the effects of climate change are not counteracted, productivity could decline both in low and mid- and high latitudes, primarily due to detrimental impacts of high temperatures and water stress [35, 66]. Rising temperatures will lower production by limiting the length of the growing season, exerting direct negative effects on resource capture and processes underpinning growth and yield. Another consequence of rising global temperatures over the next few decades is likely to be the increase in evaporation and acceleration of the global hydrological cycle, which could potentially dry subtropical areas and increase precipitation at higher latitudes. Ongoing challenges to food security will result from these changes, as most developing countries are situated at low latitudes in regions that are already warm and semi-arid [66]. To illustrate this point, two thirds of the undernourished people in the world live in just seven countries (Bangladesh, China, the Democratic Republic of the Congo, Ethiopia, India, Indonesia, and Pakistan) and over 40% live in China and India alone [23].

While general trends are described above, changes can already be observed. In Australia, average temperatures have increased 0.9°C since 1950, with significant regional variation, while the frequency of hot days and nights has increased and that of cold days and nights has declined (www.climatechangeinaustralia.com.au). In parallel, since 1950, most of Eastern and Western Australia has experienced substantial rainfall decline,

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

while North-West Australia has become wetter. In this context, new crops and crop varieties represent a technical adaptation with the potential to be instrumental in at least reducing climate-related vulnerability at the farm level [33]. For Australia's wheat crops, it has been estimated that, in the absence of adaptive measures, a 1.5–2°C increase in temperature would cancel out the grain yield increase derived from a CO_2 doubling, assuming no change in varietal adaptation ([33] and references therein).

It is important to consider that plant breeding takes time. The objective of a plant breeding program is to create new genetic variation and select gene combinations to create genotypes with superior performance in the target population of environments (TPE) [16]. Combining a range of methods, from traditional plant breeding to molecular tools, it is estimated that it takes 7–12 years to release a wheat cultivar (David Bonnett, pers. comm.) and 5–10 years to release a rice cultivar [9]. Increased climate variability in terms of rainfall patterns and the trends in the evolution of major weather variables such as temperature will lead to longer-term changes in the TPE. Under increased weather variability, a higher genotype x environment interaction (GEI) is expected. An increase in GEI, observed through altered genotypic rankings, makes it harder for breeders to make sustained genetic gains, as already documented for drought [65]. The paradox is that at a time when farmers' needs for new varieties as an adaptation tool intensify, breeding progress may become slower.

In this context, three of the main challenges plant breeding faces in relation to adaptation to climate change are (1) identifying the new target population of environments (TPE), (2) translating this knowledge into practical selection methods to uncover new genetic variation, including large mass phenotyping of potential parental lines, progeny, and wild genetic resources, and (3) integrating complex genotypic information with the large volume of data from high-throughput phenotyping systems. This entry looks at some of the recent advances in plant breeding for high yield potential environments and also those where abiotic stress is a major limitation to productivity. The methodologies and lessons learnt could become useful when breeding in the face of climate change. Examples are given for rice and wheat, because of their important contribution in volume and value to the world economy [24].

Breeding in a Changing Environment

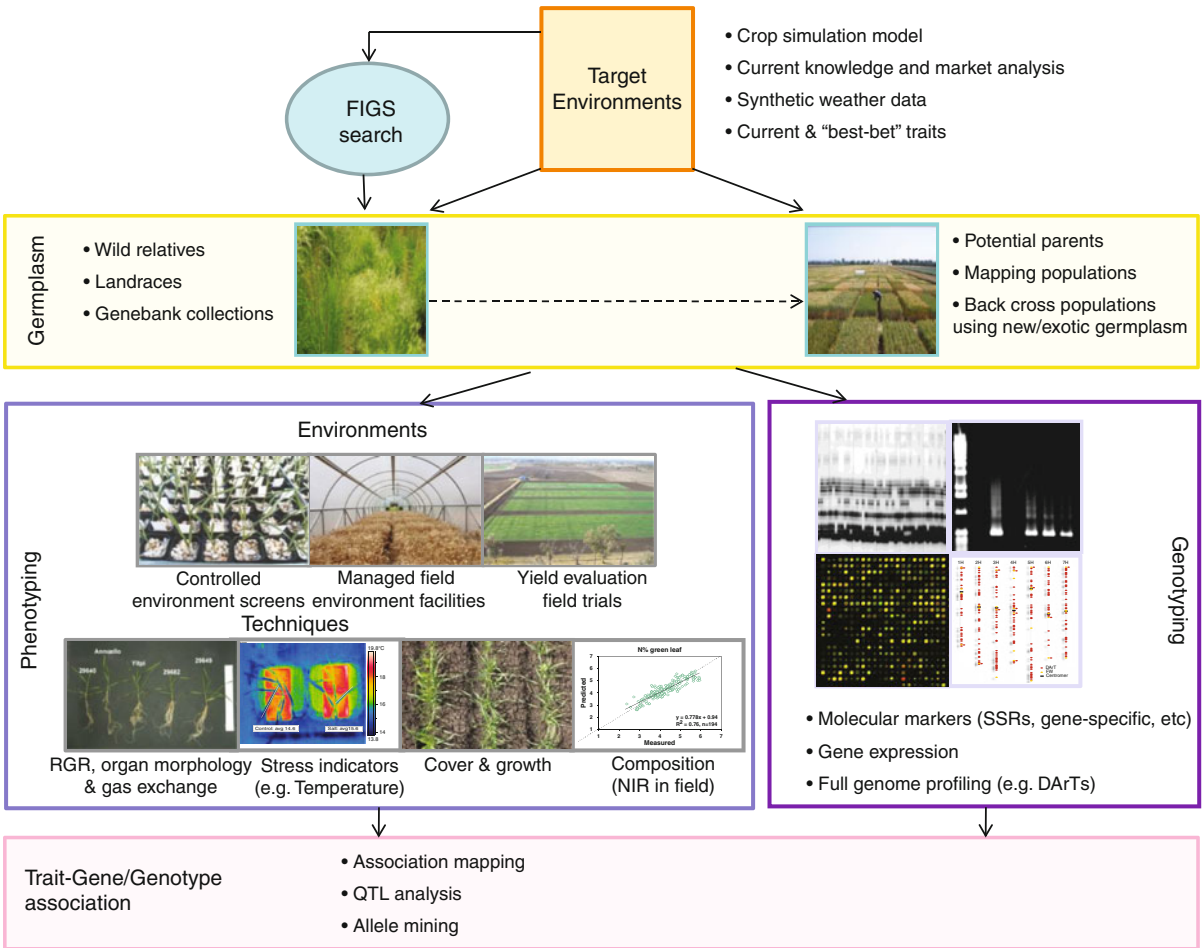
Yield is a complex trait underpinned by many different processes and, as such, highly influenced by environmental conditions. Breeding programs utilize multi-environment trials as a way of sampling the target population of environments (TPE). However, the conditions (weather, soil, agronomy) in those trials are not always a good representation of the TPE that the lines will grow in during their commercial life ([13] and references therein). This gap, between the "selection" TPE and the "commercial life TPE" may increase as a result of increased climate variability. Predicting which type of environments breeders will be targeting and their frequency of occurrence may become a key piece of information in designing the best targeting of selection schemes.

Some lessons can be learnt from experience in breeding for drought-prone environments that could be adopted more widely and potentially extended to selecting for adaptation to high temperatures. In drought-prone areas, an important proportion of the GEI has been attributed to the timing of drought stress with respect to the crop stage. Not surprisingly, different outcomes in yield progress can be expected when selection takes place under different drought patterns [13]. An attempt to describe "types of environment" for a particular region has been conducted for sorghum [13] and wheat [15] in Northern Australia utilizing historic weather data, soil characteristics, and current and virtual crop characteristics (e.g., sorghum: [14]) using the crop simulation model APSIM [38]. This could be expanded to a larger regional scale using synthetic weather data ("future climates") aiming at different outcomes. Although climate projections have their own uncertainty, there is consensus in some of the predicted global trends [32]. At a large scale, describing "types of environments" using synthetic weather data could be a tool to identify shifts in the cultivated area and regions that are likely to experience increased frequency of events that will lead to yield loss [42], for example, high temperature or drought during pollen meiosis in wheat [37] or high temperatures at anthesis in rice [36]. This information can be used to design the layout of multi-environment trials or create managed environment facilities that reliably "reproduce" these conditions, for example, irrigation in the desert (such

as CIMMYT's Obregon facility), rain-out shelters, temperature gradient tunnels, etc., that, depending on their scale, could be used for particular stages of the breeding program. Ultimately, this information could be used to set selection criteria prioritizing particular traits (e.g., [67]) and devise the appropriate tests and technologies to screen for them (Fig. 1).

A Combination of Breeding Approaches Needed to Advance Yield in a Changing Climate

A modern wheat variety carries an elite combination of alleles for productivity, grain quality, and resistance or tolerance to a suite of the most important biotic and abiotic stresses in the environments in which they are grown. In a breeding program targeting development of new varieties for production by farmers, it is usually necessary to make crosses between parents with a high co-ancestry and many attributes in common while differing for a small number of traits important to farmers and end users. Often they will be current varieties or advanced breeding lines. With this approach, the gains made in one breeding cycle are built on in subsequent cycles and progress is incremental. However, in spite of this relative conservatism, most crosses still fail to produce their desired outcome, and breeders



Plant Breeding Under a Changing Climate. Figure 1

Example of how to target traits to introduce in a breeding program for a changing climate. Defining target environments can help direct a FIGS search for exotic germplasm and set up specific populations. Phenotyping will be necessary both in the exotic germplasm and the breeding populations. Genotyping information combined with phenotypic data is the basis for trait–gene association

must make multiple crosses to spread risk. In drier environments subject to greater GEI, progress for yield is usually slower than in more favorable environments. In spite of these constraints, breeding programs have successfully improved water-limited yields over an extended period and new approaches must offer tangible benefits if they are to be adopted [46, 52, 75].

The already commonplace and increasing use of molecular markers in wheat breeding is a good example of the adoption of a technology that successfully supplements conventional approaches. Most markers implemented so far have been for genes of large effect associated with disease resistance, grain quality, or, more recently, the major genes controlling plant phenology. Few if any "yield," "drought tolerance" or "heat tolerance" quantitative trait loci (QTL) have been used in wheat breeding to date. Nonetheless, the major gene markers are useful in allowing more effective selection in earlier generations for a greater range of traits, while still leaving sufficient variation to allow selection of superior individuals for quantitative traits in later generations through conventional phenotypic selection (e.g., [77]). Removal of inferior individuals and alleles earlier in the breeding process means that fewer individuals entering the expensive later stages of yield and quality evaluation will be discarded because they lack some key major genes.

The identification of robust, yield-related QTL markers should allow early generation enrichment of the frequencies of these alleles in much the same way as for major genes. These QTL can be identified through a number of approaches. Initially, biparental mapping populations were used but these have two key problems. First, the population sizes and number of test environments required to accurately identify QTL for yield are large, even in the absence of GEI. Usually, suboptimal population sizes and numbers of test environments were used, leading to some QTL not being identified and the effects of others being overestimated [5]. Other problems with this approach are that only two parents are sampled per population and identified QTL are often population- or environment-specific (e.g., [60]). Further, parents commonly differed for major genes controlling height or phenology and these had by far the largest effects on the quantitative trait being measured making it difficult to identify previously unknown sources of variation in the face of further-reduced effective population sizes [54].

Association mapping and whole genome prediction models (e.g., based on full genome profiling techniques such as DArTs [39]) have been proposed as alternative methods to identify and combine useful variation for a range of traits [10, 30, 31, 43]. The advantages of this approach are that alleles from a greater range of parents can be examined which may have greater relevance to breeding populations if the set is appropriately formulated. In the context of a breeding program aiming to make progress for yield, the most appropriate lines are likely to be breeding lines, and the phenotyping will comprise the routine yield evaluation trials. A key advantage is that the resources allocated to phenotyping are likely to be much greater than is available to any stand-alone QTL mapping project and the associations identified are more likely to be relevant to breeding populations as they were developed in breeding populations. Given the likelihood that the effects of QTLs for complex traits will change over time through fixation of important regions and differing interactions with new alleles at other loci, a continual reassessment of the value of QTLs in breeding populations may be needed in parallel with their use in selection [57]. This requires integration of good multi-environment yield data and efficient whole genome fingerprinting techniques that can be applied to the large numbers of lines making up the yield trials of commercial breeding programs. This approach depends on large amounts of resource for phenotyping, genotyping, and information management that are currently not available within the public sector. Further, association mapping approaches may not give good clues to the underlying mechanisms responsible for the yield effects of QTLs, but given that conventional breeding has achieved yield gains despite ignorance of contributory mechanism(s), this need not be an impediment to their use. Given that there is not a perfect correlation between any one physiological parameter and yield and interrelationships between physiological characters and their effects on yield are often not well understood, widespread mapping and use of QTL for physiological characters are not likely in the public or private sectors in the near future.

A Niche for Indirect Selection for Yield Using Physiological Parameters

Given that all increases in yield must have a physiological basis, it should in theory be possible to identify and select for this variation. "Physiological" or "trait-based" breeding has a niche value as complementary to more conventional crossing and selection methods [63, 73]. For selection of physiological characters to be viable in a breeding program a demonstrated genetic correlation with yield is a prerequisite as is development of a cheap, high-throughput selection tool. The ultimate outcome of selection for yield-related physiological characters must be greater genetic gain per breeding cycle or per unit of investment. The fact that physiological measurements do not depend on knowledge of the number and location of QTLs segregating in any given population means they can be used across a greater number of populations. This also means they can be used to screen exotic germplasm, such as wild relatives, sources for variation that may be based on novel alleles and could be introgressed into breeding populations. High order or composite traits evaluated in the field have been particularly successful as targets of this approach, compared to traits evaluated at the cellular level, which are more prone to be subject to upscaling problems [72]. This is also of value given that agronomically important complex traits have not been particularly amenable to improvement using marker-assisted selection [31].

An analytic physiological approach is also likely to be useful to improve candidate traits for which genetic variation is not readily available, as could be the case in the response to high temperatures, and a targeted search and introduction strategy is needed. To illustrate the possibilities, an example of the use of physiological traits–based breeding to cope with limited water in wheat and improve yield under favorable conditions in rice is presented below.

Packaging Traits to Cope with Limited Water in Wheat and Links with Breeding for High Temperatures

A number of relationships between yield and physiological parameters have been identified in wheat and indirect selection methods for yield subsequently implemented. A good example resulted from the discovery of a positive relationship between irrigated yields and stomatal conductance in a historical series of CIMMYT wheats [25]. Subsequent research showed that selection for higher stomatal conductance could be used in indirect selection for increased yield in irrigated conditions [17]. Selection for high stomatal conductance using canopy temperature as a surrogate is now a routine procedure in the rainfed wheat program at CIMMYT (Manes, personal communication 2010).

Discovery of the relationship between C^{12}/C^{13} carbon isotope discrimination (CID) and yield under drought is another example that grew from postulation of a relationship based on theoretical considerations, subsequent identification of variation in wheat germplasm, demonstrating a relationship with yield, development of a selection tool and germplasm, and ultimately in release of improved varieties Drysdale and Rees [55, 56, 65].

Later genetic dissection identified QTL for CID in several mapping populations that had not been specifically developed for mapping CID and in which the parents were mainly commercial varieties [60]. Although the parents of these populations did not have the most extreme CID levels, the populations showed the genetic complexity of the trait, that diversity for CID alleles was present in current varieties and that it was possible to recover transgressive segregants for CID from these populations as extreme as any identified in previous germplasm surveys. Therefore, with knowledge of the relationship between CID and yield under drought, availability of an appropriate selection screen should allow breeders to indirectly select for higher yield in drought-prone environments. Although use of mass spectrometry to determine carbon isotope composition is a relatively expensive procedure and has not been applied routinely by commercially focused breeding programs, it was successfully applied in germplasm development efforts at CSIRO that led to the release of varieties Drysdale and Rees in collaboration with varietal breeding programs [55, 56]. Development of cheaper techniques to screen for the increased WUE that result in the CID

differences may be applicable on a more routine basis in breeding programs.

QTL mapping of water soluble carbohydrate (WSC) contents in stem [60] and coleoptile length [59] reveal similarly widespread and potentially useful variation for these traits in elite germplasm. In cereals, water soluble carbohydrates (WSC) stored in stems have been acknowledged as contributing to maintenance of grain filling rate when photosynthesis declines due to various stresses, for example, drought ([6, 47 81], heat stress [7], and possibly disease [8]. Increased coleoptiles length can be a useful option for systems utilizing moisture-seeking strategies, such as sowing in deep furrows. This is likely to be a useful combination of traits for climate change in Australia, with the projected temperature increase and the decrease in average annual and winter rainfall (fewer and drier sowing opportunities) in the southern areas of the wheatbelt toward 2030 [32]. Progress for these traits could be made by selecting variation already present in breeders' populations if an efficient selection screen were available.

As mentioned before some of the traits targeted for drought stress are potentially useful under heat stress, which is particularly the case for those related to transpirational cooling [54]. Increased root growth in a soil profile with water available at depth can increase the transpirational cooling of the crop, uncoupling it from air temperature and helping keep tissues in a "safer" temperature window. Epicuticular waxiness is another trait with a dual function, reducing heat load and transpiration. Waxiness can be scored visually in the field, but, despite the theoretical impact there have been no comprehensive studies of its impact in crops [64]. Flowering time has been exploited under terminal drought as a simple way of manipulating the water balance, early flowering leaving more water available to be used during grain filling. Early flowering can also be used to avoid high temperatures during grain filling. In both cases, advancing flowering can carry the penalty of lower biomass at flowering and increased probability of frost damage.

Some processes are directly affected by high temperatures, among them respiration, inflorescence fertility, and starch composition, and hence grain quality ([2, 4, 78, 82]). Night respiration and photorespiration are processes directly affected by temperature; however, the lack of an easy way to phenotype large number of lines and study its effects at the crop level makes it inaccessible as a target for breeders at this point in time. Susceptibility of pollen to high temperatures and traits contributing to heat tolerance (biochemical mechanisms) and avoidance (e.g., anther dehiscence early in the day) is a topic much researched in rice (see [78] and references therein). Pollen sterility induced by drought and genetic variation for it has been confirmed in wheat [37] and could also be potentially triggered by high temperatures. This is likely to be a trait to be screened for in controlled environment facilities or using molecular markers, given how unpredictable high temperatures can be at a particular crop stage in the field. For the purpose of marker development or QTL identification it will be important to "detangle" the phenotype appropriately as, for instance, reduced pollen sterility under high temperature could occur due to lower tissue temperature in a line that has high transpirational cooling due to higher stomatal conductance.

Physiological Traits to Raise Yield Potential in Rice: Different Targets for Temperate Versus Tropical Regions

As highlighted earlier, an increase in the length of the growing season as well as improved growing seasonal conditions are forecasted for high latitudes under climate change. This section illustrates the experience in rice in breeding for increased yield potential. Yield potential is defined as grain yield only limited by incoming radiation and temperature at a given site. Most of rice production is derived from tropical and subtropical areas, where it is grown with irrigation water from the monsoon [79]. The yield potential of rice in the tropics has been stable at 9–10 t ha^{-1} for the last 20 years [51]. However, the arable land for rice is continuously decreasing as a consequence of increasing urbanization, in parallel with the increase in the population of rice consumers. An increase in yield potential of rice of 10–15% is now necessary to cope with this raising demand [69]. Among the main limitations to

rice yield potential in the tropics are (1) the limited amount of incoming radiation (combination of short days and cloudiness), (2) high relative humidity underlying high resistance to transpiration, and (3) a trend toward a short crop cycle to allow the growth of two to three crops per year. Indeed, the highest rice yield potential today is slightly higher or similar to that of IR64, that is, comparable to introgression lines of IR64 background [29], a benchmark inbred line which was developed by IRRI breeders in the middle of the 1980s. Most of the increases in rice yield in the field have been achieved with hybrid rice [50]. In contrast, the main gain in crop productivity in the last 20 years has been observed undernutrient or water limitation [34]. Grain quality has also been a great focus of the last 20 years and has diversified significantly to meet the variable expectations of consumers from different regions [27].

The International Rice Research Institute was a pioneer in using physiological concepts in breeding for an ideotype, as illustrated by the so-called "new plant type" which reached limited success [51]. These guiding principles are still utilized in current efforts to improve yield potential in rice focusing in increasing biomass and harvest index as discussed below. One challenging option is to develop a rice plant with the C_4 photosynthesis pathway: the radiation use efficiency would be increased considerably and so the yield potential up to by 30–50% [44, 68, 80]. It is taking an integrated program involving molecular biologists, geneticists, biotechnologists, and physiologists working together for a considerable number of years. The search for genetic variation in different aspects of the C_4 pathway, such as leaf anatomical and cellular specialization and variation in mechanisms underlying the CO_2 compensation point is already presenting a considerable phenotyping challenge [83, 84]. Another option, still challenging but perhaps more realistic in a shorter timeframe, is to improve the current plant types for high yield. In the tropics, both improved biomass accumulation and partitioning underpin the superiority of hybrid rice versus rice elite inbred lines, with margins between 10% and 20% from wet to dry seasons [11, 40, 49, 50]. It is possible to assume that breeding programs for yield potential could gain much by incorporating traits relevant to hybrid rice superiority into improved inbred lines and lead to a substantial yield gain [41].

Work conducted at the International Rice Research Institute has extensively examined the basis for yield differences between hybrid rice and elite inbred lines of similar crop duration [11, 12, 40]. With an initial focus in the tropics, these authors confirmed, under a range of contrasting conditions, that superior biomass production in the succeeding phenological phases and improved partitioning play a significant role in the higher yields of the hybrids. Hybrids are characterized by (1) higher crop growth rate during each phenological phase leading to overall higher plant biomass at maturity, (2) earlier cessation of tiller production (associated with earlier biomass partitioning to culm and earlier accumulation of reserves) with similar tiller production rate, (3) larger pool of reserves in the culm at anthesis (estimated through a lower value of specific culm length, SCL), (4) larger remobilization of the accumulated reserves from the culm to the panicle during grain filling (associated with quicker grain filling and higher SCL at maturity), and (5) lighter unfilled spikelets indicating that grain filling was more efficient with less partially filled spikelets (associated with larger number of filled grains) [40, 41]. It is clear that it is worth looking for genetic variation in storage of soluble sugars in the stem as well as remobilization capacity, in line with results in wheat [81] and in view of identifying the driving force of the dynamics of soluble sugars. Bueno et al. [12] speculated that improved partitioning, more than source supply, is the key component driving crop performance of high-yielding genotypes in the tropics where variability in biomass accumulation among genotypes is poorly expressed due to low evaporative demand. The sink strength index, as an improved harvest index taking into account the culm vigor [12, 41], can be used as an integrated trait for screening genotypes with high or low partitioning efficiency. It cannot, however, be considered as a "foundation" trait for high yield potential, unlike the traits cited earlier, and seems rather to be the integrated expression at maturity, as sink size at anthesis, of the cumulated higher efficiency of more simple traits. Some other important considerations concern remobilizing assimilates from senescing to productive tillers, avoiding lodging and delaying root senescence during grain filling. Maintaining functional roots throughout grain filling, and maintaining nitrogen uptake, should help delaying leaf senescence; however,

leaf senescence has to be fast toward the end of grain filling to maximize remobilization.

Identification of Variation for Physiological Traits from Exotic Germplasm Sources

While the wheat example cited above indicates considerable variation for several yield-related physiological traits already existing in varieties and advanced breeding lines adapted to drier environments, for some traits, key genetic variation is lacking in existing breeding material. An example of this problem is the difficulty of recovering long coleoptile semidwarfs in wheats carrying the semidwarf alleles *Rht-B1b* and *Rht-D1b* [1, 76]. These alleles are virtually ubiquitous in modern semidwarf genes but have negative pleiotropic effects on coleoptile length. In order to develop semidwarf wheats with substantially longer coleoptiles the introduction of novel dwarfing genes that do not affect coleoptile length is necessary [22, 58]. For other traits such as root depth, likely to be related to yield under drought or heat stress, variation has been found in landraces and wild or synthetic hexaploid material that is greater or absent in existing wheat germplasm available to breeders [61, 62]. The introduction and exploitation of variation from synthetic hexaploid wheats in the CIMMYT wheat program is an example of the potential gains that can be made from exotic germplasm sources. These synthetic wheats were produced by re-synthesizing bread wheat from progenitor species the tetraploid durum wheat and the diploid wild grass *Triticum tauschii* [70, 71]. Derivatives of crosses between synthetic hexaploids and bread wheat now comprise around 30% of the breeding populations in CIMMYTs rainfed wheat program and the best lines have superior yield under drought stressed and more favorable environments than the best conventional wheats [18]. While this demonstrates the possibility of gains from exotic germplasm sources, a more targeted approach in which exotic sources are prescreened for traits related to yield under drought or high temperatures may produce even greater gains. A possible pathway of integration is shown in Fig. 1. In many cases novel yield-related variation in exotic germplasm sources would be difficult or impossible to identify simply by screening directly for yield because it is present in agronomically poor backgrounds. In such instances, screening for physiological traits likely to be related to yield may be a useful precursor to crossing to introduce new variation [62].

A number of other approaches may allow selection of better initial material even prior to screening for physiological traits. Molecular diversity studies have shown, for example, that genetic diversity of emmer wheat is greater than that in durum wheat [19] and studies of synthetic wheats produced by crossing emmers with *A. tauschii* have shown greater yield under drought stress in Mexico, Pakistan, and Eastern India than synthetics produced by durum wheat x *A. tauschii* crosses [71]. Given the distribution of emmer wheats in drought-prone Mediterranean environments the useful variation present in this material may have been predictable. Better predictions based on a greater array of climatic, soil, and location data as exemplified by the focused identification of germplasm strategy or FIGS (e.g., [20, 21]) should further improve the quality of germplasm selected to screen for variation in phenotypic traits (Fig. 1).

Robust and time-efficient phenotyping is also critical for trait-based selection of potential parents for crossing blocks and evaluation of the progeny, such as needed to underpin physiological breeding [63]. Noninvasive technologies, such as those based on spectral reflectance and thermal sensing have a role in the identification and selection of traits in a breeding context, by allowing several crop characteristics to be surveyed in a single measurement at the crop scale [45] (Fig. 1). For example, spectral reflectance has been used to simultaneously survey canopy cover, nitrogen, and water status of the crop [25, 48], while canopy temperature has been used as an indicator of leaf conductance and water use [85]. Potential for continuous developments for high throughput include introducing changes in platforms (wireless systems, unmanned aerial vehicles, etc.) to allow more frequent data capture and greater area coverage. While glasshouse or lab-based screens have been indicated as an option for some traits (e.g., [28, 74]), in most cases, there is a low correlation between this level of evaluation and field-based rankings. The interactions involved not only in upscaling to the crop/canopy level but with the changing environment itself get in the way (examples in [72]). Instead, field canopy scale measurements have more potential since selection can take place at the

crop level, which avoids scaling up issues (e.g., [62]). For instance, reflectance-based indices have proved very useful as indirect selection criteria to increase the efficiency of selection in wheat growing in a reasonably stable environment where the terminal drought is managed [3], while indicators of canopy cover and canopy temperature were a good proxy for performance or QTL detection in a hot environment [54].

Conclusions and Future Directions

Improved crop varieties will be a vital component of adaptation to climate change. Plant breeding will have to operate at a higher level of efficiency to make the necessary genetic progress to address current and projected food needs. In this context, the three main challenges plant breeding faces in relation to climate change are (1) identifying the new target population of environments, (2) translating this knowledge into practical selection methods to uncover new genetic variation, including mass phenotyping of potential parental lines, progeny, and wild genetic resources, and (3) linking genetic and phenotypic information. How to link and interpret the new and comprehensive information on genotypic characteristics and the large volume of data generated by high-throughput phenotyping platforms will be a critical step toward selecting the next generation of traits fit to less predictable environments.

Acknowledgments

The authors thank Lynne McIntyre (CSIRO) and Andrzej Kilian (DArTs Pty Ltd) for contributing illustrations on genotyping. FD acknowledges the financial support of the Department of Agriculture, Fisheries and Forestry, CSIRO and the Climate Adaptation Flagship.

Bibliography

Primary Literature

1. Allan RE (1989) Agronomic comparisons between Rht1 and Rht2 semi-dwarf genes in winter wheat. Crop Sci 29:1103–1108
2. Atkin OK, Tjoelker MG (2003) Thermal acclimation and the dynamic response of plant respiration to temperature. Trends Plant Sci 8:343–351
3. Babar MA, van Ginkel M, Reynolds MP, Prasad B, Klatt AR (2007) Heritability, correlated response, and indirect selection involving spectral reflectance indices and grain yield in wheat. Aust J Agric Res 58:432–442
4. Barnabas B, Jager K, Feher A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31:11–38
5. Beavis WD (1998) QTL analyses: power, precision, and accuracy. In: Patterson AH (ed) Molecular dissection of complex traits. CRC Press, Boca Raton, pp 145–162
6. Bidinger F, Musgrave RB, Fischer RA (1977) Contribution of stored pre-anthesis assimilates to grain yield in wheat and barley. Nature 270:431–433
7. Blum A, Sinmena B, Mayer J, Golan G, Shpiler L (1994) Stem reserve mobilisation supports wheat-grain filling under heat stress. Aust J Plant Physiol 21:771–781
8. Blum A (1998) Improving wheat grain filling under stress by stem reserve mobilisation. Euphytica 100:77–83
9. Brar D, Virk P (2010) How a modern rice variety is bred. Rice Today 9(Jan–March):11–12
10. Breseghello F, Sorrells ME (2006) Association mapping of kernel size and milling quality in wheat (Triticum aestivum L.) cultivars. Genetics 161(2):1165–1177
11. Bueno CS, Lafarge T (2009) Higher crop performance of rice hybrids than elite inbreds in the tropics. 1. Hybrids accumulate more biomass during each phenological phase. Field Crops Res 112:229–237
12. Bueno CS, Pasuquin E, Tubana B, Lafarge T (2010) Improving sink regulation, and searching for promising traits associated with hybrids, as a key avenue to increase potential of the rice crop in the tropics. Field Crops Res 118:199–207
13. Chapman SC (2008) Use of crop models to understand genotype by environment interactions for drought in real-world and simulated plant breeding trials. Euphytica 161:191–208
14. Chapman SC, Cooper M, Podlich D, Hammer GL (2003) Evaluating plant breeding strategies by simulating gene action and dryland environments. Agron J 95:99–113
15. Chenu K, Cooper M, Hammer GL, Mathews KL, Dreccer MF, Chapman SC (2011) Environment characterisation as an aid to wheat improvement – interpreting genotype-environment interaction by modelling water-deficit patterns in north eastern Australia. J Exp Bot 62:1743–1755
16. Comstock RE (1977) Quantitative genetics and the design of breeding programs. In: Proceedings of the international conference on quantitative genetics. Iowa State University Press, Ames, 16–21 Aug 1976, pp 705–718
17. Condon AG, Reynolds MP, Rebetzke GJ, Ginkel M van, Richards RA, Farquhar GD (2007) Using stomatal aperture-related traits to select for high yield potential in bread wheat. In**:** Wheat production in stressed environments. Proceedings of the 7th international wheat conference, Mar del Plata, 27 Nov–2 Dec 2005, pp 617–624
18. Dreccer MF, Chapman SC, Ogbonnaya FC, Borgognone MG, Trethowan RM (2008) Crop and environmental attributes underpinning genotype by environment interaction in synthetic bread wheats evaluated in Mexico and Australia. Aust J Agric Res 59:447–460

19. Dreisigacker S, Kishii M, Lage J, Warburton M, Ogbonnaya FC, van Ginkel M, Brettell R (2008) Use of synthetic hexaploid wheat to increase diversity for CIMMYT bread wheat improvement. Aust J Agric Res 59(5):413–420
20. El-Bouhssini M, Street K, Joubi A, Ibrahim Z, Rihawi F (2009) Sources of wheat resistance to Sunn pest, Eurygaster integriceps Puton, in Syria. Genet Resour Crop Evol 56:1065–1069
21. El-Bouhssini M, Street K, Amri A, Mackay M, Ogbonnaya FC, Omran A, Abdalla O, Baum M, Dabbous A, Rihawi F (2011) Sources of resistance in bread wheat to Russian wheat aphid (Diuraphis noxia) in Syria identified using the Focused Identification of Germplasm Strategy (FIGS). Plant Breeding 130:96–97
22. Ellis MH, Rebetzke GJ, Chandler P, Bonnett DG, Spielmeyer W, Richards RA (2004) The effect of different height reducing genes on early growth characteristics of wheat. Funct Plant Biol 31:583–589
23. FAO (2010) The state of food insecurity in the world. Chief publishing policy and support branch office of knowledge exchange, research and extension FAO, Rome, p57
24. FAO (2010) FAO Statistical yearbook 2010. Economic and social development department, FAO, Rome
25. Fischer RA, Rees D, Sayre KD, Lu Z-M, Condon AG, Saavedra AL (1998) Wheat yield progress associated with higher stomatal conductance and photosynthetic rate and cooler canopies. Crop Sci 38:1467–1475
26. Fitzgerald GJ, Rodriguez D, Christensen LK, Belford R, Sadras VO, Clarke TR (2006) Spectral and thermal sensing for nitrogen and water status in rainfed and irrigated wheat environments. Precision Agric 7:233–248
27. Fitzgerald MA, McCough SR, Hall RD (2009) Not just a grain of rice: the quest for quality. Trends Plant Sci 14:133–139
28. Gregory PJ, Bengough AG, Grinev D, Schmidt S, Thomas WTB, Wojciechowski T, Young IM (2009) Root phenomics of crops: opportunities and challenges. Funct Plant Biol 36:922–929
29. Guan YS, Serraj R, Liu SH, Xu JL, Ali J, Wang WS, Venus E, Zhu LH, Li ZK (2010) Simultaneously improving yield under drought stress and non-stress conditions: a case study of rice (*Oryza sativa* L.). J Exp Bot 61:4145–4156
30. Heffner EL, Sorrells ME, Jannink JL (2009) Genomic selection for crop improvement. Crop Sci 49:1–12
31. Heffner EL, Lorenz AJ, Jannink J-L, Sorrells ME (2010) Plant breeding with genomic selection: gain per unit time and cost. Crop Sci 50:1681–1690
32. Hennessy KJ, Whetton PH, Preston B et al (2010) Climate projections. In: Stokes C, Howden M (eds) Adapting agriculture to climate change-preparing Australian agriculture, forestry and fisheries for the future. CSIRO Publishing, Collingwood, pp 21–48
33. Howden SM, Gifford RG, Meinke H (2010) Grains. In: Stokes C, Howden M (eds) Adapting agriculture to climate change-preparing Australian agriculture, forestry and fisheries for the future. CSIRO Publishing, Collingwood, pp 21–48
34. Horie T, Shiraiwa T, Homma K, Katsura K, Maeda S, Yoshida H (2005) Can yields of low land rice resume the increases that they showed in the 1980s? Plant Prod Sci 8:259–274
35. IPCC (2007) Climate change 2007: impacts, adaptation, and vulnerability. Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, UK
36. Jagadish SVK, Craufurd PQ, Wheeler TR (2007) High temperature stress and spikelet fertility in rice (Oryza sativa L.). J Exp Bot 58:1627–1635
37. Ji XM, Shiran B, Wan JL, Lewis DC, Jenkins CLD, Condon AG, Richards RA, Dolferus R (2010) Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. Plant Cell Environ 33:926–942
38. Keating BA, Carberry PS, Hammer GL, Probert ME, Robertson MJ, Holzworth D, Huth NI, Hargreaves JNG, Meinke H, Hochman Z, McLean G, Verburg K, Snow V, Dimes JP, Silburn M, Wang E, Brown S, Bristow KL, Asseng S, Chapman S, McCown RL, Freebairn DM, Smith CJ (2003) An overview of APSIM, a model designed for farming systems simulation. Eur J Agron 18: 267–288
39. Jaccoud D, Peng K, Feinstein D, Kilian A (2001) Diversity arrays: a solid state technology for sequence information independent genotyping. Nucleic Acids Res 29:e25
40. Lafarge T, Bueno CS (2009) Higher crop performance of rice hybrids than elite inbreds in the tropics. 2. Does sink regulation, rather than sink size, play a major role? Field Crops Res 114:434–440
41. Lafarge T, Bueno C, Pasuquin E, Wiangsamut B (2009) Biomass accumulation and sink regulation in hybrid rice: consequences for breeding programs and crop management. In: Xie F, Hardy B (eds) Accelerating hybrid rice development. International Rice Research Institute, Los Baños, p 698. Invited oral presentation, international symposium on hybrid rice, Changsha, Hunan, 11–15 Sept 2008, pp 453–474
42. Li Y, Ye W, Wang M, Yan X (2009) Climate change and drought: a risk assessment of crop-yield impacts. Clim Res 39: 31–46
43. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829
44. Mitchell PL, Sheehy JE (2000) Performance of a potential C4 rice: overview from quantum yield to grain yield. Stud Plant Sci 7:145–163
45. Montes M, Melchinger AE, Reif JC (2007) Novel throughput phenotyping platforms in plant genetic studies. Trends Plant Sci 12:433–436
46. O'Brien L (1982) Victorian wheat yield trends, 1898–1977. J Aust Inst Agric Sci 48:162–168
47. Palta JA, Kobata T, Turner NC (1994) Carbon and nitrogen in wheat as influenced by postanthesis water deficits. Crop Sci 34:118–124

48. Peñuelas J, Fillela Y (1998) Visible and near-infrared reflectance techniques for diagnosing plant physiological status. Trends Plant Sci 3:151–156
49. Peng S, Cassman KG, Virmani SS, Sheehy J, Khush GC (1999) Yield potential trends of tropical rice since the release of IR8 and the challenge of increasing rice yield potential. Crop Sci 39:1552–1559
50. Peng S, Yang J, Laza MRC, Sanico A, Visperas RM, Son TT (2003) Physiological bases of heterosis and crop management strategies for hybrid rice in the tropics. In: Virmani SS, Mao CX, Hardy B (eds) Hybrid rice for food security, poverty alleviation, and environmental protection. Proceedings of the 4th international symposium on hybrid rice, 14–17 May 2002, Hanoi. IRRI, Los Baños, pp 153–170
51. Peng S, Kush GS, Virk P, Tang Q, Zhu Y (2008) Progress in ideotype breeding to increase rice wheat consortium. Field Crops Res 108:32–38
52. Perry MW, D'Antuono MF (1989) Yield improvement and associated characteristics of some Australian spring wheat cultivars introduced between 1860 and 1982. Aust J Agric Res 40:457–472
53. Pinter PJ, Hatfield JL, Schepers JS, Barnes EM, Moran MS, Daughtry CST, Upchurch DR (2003) Remote sensing for crop management. Photogram Eng Rem S 69:647–664
54. Pinto RS, Reynolds MP, Mathews KL, McIntyre CL, Olivares-Villegas JJ, Chapman SC (2010) Heat and drought adaptive QTL in a wheat population designed to minimize confounding agronomic effects. Theor Appl Genet 121:1001–1021
55. Plant Varieties Journal (2002) Official Journal of Plant Breeder's Rights Australia, 15(1):74
56. Plant Varieties Journal (2004) Official Journal of Plant Breeder's Rights Australia, 17(2):254–261
57. Podlich DW, Winkler CR, Cooper M (2004) Mapping as you go: an effective approach for marker-assisted selection of complex traits. Crop Sci 44:1560–1571
58. Rebetzke GJ, Richards RA, Fischer VM, Mickelson BJ (1999) Breeding long coleoptile, reduced height wheats. Euphytica 106:159–168
59. Rebetzke GJ, Ellis MH, Bonnett DG, Richards RA (2007) Molecular mapping of genes for coleoptile growth in bread wheat. Theor Appl Genet 114:1173–1183
60. Rebetzke GJ, van Herwaarden AF, Jenkins C, Weiss M, Lewis D, Ruuska S, Tabe L, Fettell N, Richards RA (2008) Quantitative trait loci for soluble stem carbohydrate production in wheat. Aust J Agric Res 59:891–905
61. Reynolds MP, Mujeeb-Kazi A, Sawkins M (2005) Prospects for utilising plant-adaptive mechanisms to improve wheat and other crops in drought & salinity-prone environments. Ann Appl Biol 146:239
62. Reynolds MP, Dreccer MF, Trethowan R (2007) Drought adaptive traits derived from wheat wild relatives and landraces. J Exp Bot 58:177–186
63. Reynolds MP, Manes Y, Izanloo A, Langridge P (2009) Phenotyping for physiological breeding and gene discovery in wheat. Ann Appl Biol 155:309–20
64. Reynolds MP, Hays D, Chapman S (2010) Breeding for adaptation to heat and drought stress. In: Reynolds MP (ed) Climate change and crop production. CAB International, Wallingford, pp 71–91
65. Richards RA, Rebetzke GJ, Condon AG, van Herwaarden AF (2002) Breeding opportunities for increasing the efficiency of water use and crop yield in temperate cereals. Crop Sci 42:111–121
66. Rosenzweig C (2009) Climate change and agriculture. In: Meyers RA (ed) Encyclopedia of complexity and systems science., pp 1071–1082. doi:10.1007/978-0-387-30440-3_70, Part 3
67. Semenov MA, Halford NG (2009) Identifying target traits and molecular mechanisms for wheat breeding under a changing climate. J Exp Bot 60:2791–2804
68. Sheehy JE, Ferrer AB, Mitchell PL, Elmido-Mabilangan A, Pablico P, Dionora MJA (2008) How the rice crop works and why it needs a new engine. In: Sheehy JE, Mitchell PL, Hardy B (eds) Charting new pathways to C4 rice. Proceedings of the international workshop, IRRI, Los Baños, pp 3–26
69. Swaminathan MS (2007) Science and shaping the future of rice. In: Aggarwal PK, Ladha JK, Singh RK, Devakumar C, Hardy B (eds) Science, technology, and trade for peace and prosperity. Proceedings of the 26th international rice research conference, 9–12 Oct 2006, New Delhi, pp 3–14
70. Trethowan RM, Reynolds MP, Sayre KD, Ortiz-Monasterio I (2005) Adapting wheat cultivars to resource conserving farming practices and human nutritional needs. Ann Appl Biol 146:404–413
71. Trethowan RM, Mujeeb-Kazi A (2008) Novel germplasm resources for improving environmental stress tolerance of hexaploid wheat. Crop Sci 48:1255–1265
72. Sinclair TR, Purcell LC, Sneller CH (2004) Crop transformation and the challenge to increase yield potential. Trends Plant Sci 9:70–75
73. Sinclair TR, Purcell LC (2005) Is a physiological perspective relevant in a 'genocentric' age? J Exp Bot 56:2777–2782
74. Sirault XRR, James RA, Furbank RTA (2009) A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. Funct Plant Biol 36:970–977
75. Vandeleur RK, Gill GS (2004) The impact of plant breeding on the grain yield and competitive ability of wheat in Australia. Aust J Agric Res 55:855–861
76. Whan BR (1976) The association between coleoptile length and culm length in semi-dwarf and standard wheats. J Aust Inst Agric Sci 42:194–196
77. Wang J, Chapman SC, Bonnett DG, Rebetzke GJ (2009) Simultaneous selection of major and minor genes: use of QTL to increase selection efficiency of coleoptile length of wheat (Triticum aestivum L.). Theor Appl Genet 119:65–74
78. Wassmann R, Jagadish SVK, Heuer S, Ismail A, Redona E, Serraj R, Singh RK, Howell G, Pathak H, Sumfleth K (2009) Climate change affecting rice production: the physiological and agronomic basis for possible adaptation strategies. Adv Agron 101:59–122
79. Wassmann R, Jagadish SVK, Sumfleth K, Pathak H, Howell G, Ismail A, Serraj R, Redona E, Singh RK, Heuer S (2009) Regional

vulnerability of climate change impacts on Asian rice production and scope for adaptation. Adv Agron 102:91–133
80. Yin X, Struik PC (2009) C3 and C4 photosynthesis models: an overview from the perspective of crop modelling. NJAS Wageningen J Life Sci 57:27–38
81. Dreccer MF, van Herwaarden AF, Chapman SC (2009) Grain number and grain weight in wheat lines contrasting for stems soluble carbohydrate concentration. Field Crops Res 112: 43–54
82. Wardlaw IF, Wrigley CW (1994) Heat tolerance in temperate cereals-an overview. Aust J Plant Phys 21:695–703
83. Hibberd JM, Sheehy JE, Langdale JA (2008) Using C-4 photosynthesis to increase the yield of rice - rationale and feasibility. Current opinion Plant Biol 11:228–231
84. Furbank RT, von Caemmerer S, Sheehy JE, Edwards G (2009) C(4) rice: a challnege for plant phenomics. Funct Plant Biol 36:970–977
85. Pinter PJ, Jr, Zipoli G, Reginato RJ, Jackson RD, Idso SB, Hohman JP (1990) Canopy temperature as an indicator of differential water use and yield performance among wheat cultivars. Agric Water Management 18:35–48

Plant Molecular Pharming, Industrial Enzymes

Saifullah Khan[1], Vidya Rajan[2], John Howard[2]
[1]Plant Biotechnology Section, International Center for Chemical and Biological Sciences, HEJ research Institute, University of Karachi, Karachi, Pakistan
[2]Applied Biotechnology Institute, San Luis Obispo, CA, USA

Article Outline

Glossary

Diafiltration Method of cross-flow filtration that separates filtrate from solids.

Industrial enzymes Proteins that are used in commercial applications where very specific catalysts are needed.

Pharmacognosy The study of medicines derived from natural sources.

Plant molecular pharming Production of pharmaceuticals or industrial enzymes from genetically engineered plants.

Sustainability Production to meet present needs without compromising the ability of future generations to meet their own needs.

Transgenic plants Plants genetically engineered by the introduction of foreign genes using recombinant DNA technology.

Definition of the Subject

Plants have been domesticated since around 10,000 years ago in the fertile Babylonian crescent [1] and husbandry and breeding techniques have been applied to increase yield and storage and to retard spoilage [2]. Plants have been used since time immemorial for their medicinal properties, and in ACE 78 Discorides first described 600 medicinal plants in De Materia Medica. Although the first synthetic drug, salicylic acid, entered the market in 1897, plants are still used for pharmacognosy – the preparation of drugs from natural sources [3]. The types of plants used for this purpose, however, are usually distinct from those for food, feed, or fiber. With the advent of molecular techniques, all plants now have the potential to serve as production vehicles for natural or engineered products that were previously limited to other hosts [4, 5]. Plant molecular pharming of industrial proteins refers to recombinant proteins used in industrial processes and produced in plants. Enormous quantities of a variety of enzymes go into the making of products such as paper, leather, detergents, pharmaceuticals, food, beverages, chemicals, and fabric, to name a few, and the economical production of these industrially important enzymes is crucial to commerce. This production must be balanced with the need for sustainability and environmental stewardship.

Sustainable production of industrial enzymes requires that resources are not over-exploited and that the environment is not polluted by wastes. The use of plants as "green" factories can meet both criteria. Plants are a renewable resource and thus generally are not over-exploited and wastes are biodegradable. The problems associated with fertilization runoff, spread of transgenic genes to non-target plants, and crop and land usage must be addressed to allay public concern about the use of transgenic plants. But, combined with modern farming and containment methods, transgenic plants have the potential to produce large quantities of target material safely and sustainably [6, 7].

Introduction

This article provides a review of plant-based production of industrial enzymes as a sustainable solution to increasing demand. The history of interest in sustainable development, industry and industrial enzymes will

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

set the background for a detailed analysis of how plant-based production systems compare in terms of sustainability to processes that currently exist. A compilation of potentially useful industrial enzymes produced in plants is given and a discussion of the economic, social, and environmental sustainability advantages provided by plant-based production systems is also provided.

Historical Background of Industrial Enzymes

In view of the importance of enzymes to industrial processes, a brief overview of the events leading to this relationship is provided below.

Development of Applications for Industrial Enzymes

Until mechanization and the industrial revolution, most people eked out a subsistence living using human and animal power for agriculture, production, and transport of people and goods. The industrial revolution eventually made possible tremendous increases in commerce, and consequent social upliftment through improved earnings, literacy, and working conditions. There were costs, however, in degradation of the environment through unregulated expansion of industries and consequent pollution. In 1956, the British Parliament enacted the world's first clean air act. Since then, governments have imposed restrictions on locations of industries and the disposal of pollutants and effluents. These measures have helped considerably to alleviate the impact of pollutants on the environment and health [8], but have been costly to the industries themselves. For example, costs imposed on the meat and meat by-products industries, which include industrial enzymes, are passed on to the consumer through increased prices [9] which is often negative for commerce. Thus, the pressure mounts to lower prices across the board.

An assessment of the true cost of product manufacturing is provided by Life Cycle Assessments (LCA) which measures, usually by mass loadings, the cradle-to-grave impact of every stage from resource procurement, inputs for manufacture, and outputs of wastes throughout the process [10]. Although it is often difficult to predict the LCA of a process, especially impacts on biodiversity and environment, key parameters affecting LCA are renewability of the raw material resource and energy inputs. Enzymes lower the activation energy of chemical reactions and are sourced from renewable resources, and thus should lower impact. A theoretical life cycle comparison between the production of biodiesel using inorganic and enzyme catalysis favored the latter as having lower environmental impact, lowered toxicity, and lowered greenhouse gas emissions, all attributed to lower steam heating requirements [11]. Production of large quantities of enzymes therefore comprises a key consideration for future industrial manufacturing processes.

Current Uses of Industrial Enzymes

Today, many industries use enzymes to manufacture a variety of goods (see Table 1) from food to paper to high-value pharmaceuticals. The utilization of industrial enzymes has now extended to almost all industries handling organic compounds. Enzymes are used in the production of detergents, reagents for the analysis of drugs or blood components, food or food additives, for fiber processing or pulp processing in the paper industry, and for environmental purification. The method of enzyme use also varies, for example, as an enzyme preparation, on the surface of an insoluble carrier in a bioreactor, or a biosensor with the enzyme integrated into an electrode. The annual world industrial enzyme market (excluding pharmaceuticals) is in billions of dollars and is composed largely of enzymes used as detergent ingredients and for food processing applications [12, 13]. Thus, enzymes constitute a key lubricant of commercial success in many fields.

Enzymes are formally classified and given an Enzyme Classification (EC) number by the International Union of Biochemistry and Molecular Biology (IUBMB) based on the reaction that they catalyze (http://www.chem.qmul.ac.uk/iubmb/). Each major category is further divided into subclasses and sub-subclasses. Several enzymes with different trivial or common names may share the same EC number based on the type of reaction that they catalyze (see Table 2). For example, lipases and amylases share the same major category, hydrolases, because they catalyze the breakdown of substrate by hydrolysis. However, enzymes of industrial importance are generally referred to by their trivial name, and trivial names will be used in this review. The source of industrial enzymes can be fungal, bacterial, animal, or plant and with the advent

Plant Molecular Pharming, Industrial Enzymes. Table 1 Enzymes used in various industrial segments and their applications (Reprinted from [13]. With permission from Elsevier)

Industry	Industrial enzyme	Industrial effect/application
Detergent laundry and dish wash	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase,	Starch liquefication and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition-fuel)
Food including dairy	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (in situ emulsifier)
	Phospholipase	Dough stability and conditioning (in situ emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestability-phosphorus release
	Xylanase	Digestibility
	β-glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment

Plant Molecular Pharming, Industrial Enzymes. Table 1 (Continued)

Industry	Industrial enzyme	Industrial effect/application
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oil	Lipase,	Transestrefication
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chial alcohol and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxlic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

of molecular techniques, many of the genes for these enzymes have been cloned and transformed into organisms that are easy and convenient to grow in an industrial setting. Optimizing gene expression and culture conditions can increase quantities of enzymes produced and consequently lower costs.

In medicine, the prohibitive cost of insulin isolated from human cadavers created a market for porcine insulin despite the negative side effects. Eli Lilly introduced recombinant insulin in 1982, which decreased the cost to a more manageable level through the use of higher-affinity analogs [14], thereby saving innumerable lives. This price decrease is attributed to the use of transgenic bacteria, improved production methods, and also to lower costs of high-purity protease used to cleave the insulin molecule to active form [15].

Another prominent example today is the use of enzymes in the bioconversion of grain to ethanol [16]. In this case the enzymes very efficiently break down starch to fermentable sugars that can then be used to make ethanol. In the near future the hope is that enzymes will also be used to convert cellulosic material into fermentable sugars as well.

The global industrial enzyme market increased from US $1 billion in 1995 to $1.5 billion in the 5-year period to 2000 with growth rates ranging from 2% to 25% annually [13]. The value of the market created by enzyme technology is much higher, at around $80–130 billion. In case studies by the Organization of Economic Cooperation and Development (OECD), the application of biotechnology has generally benefited by improved costs and sustainability, and

Plant Molecular Pharming, Industrial Enzymes. Table 2 Summary of classes of enzymatic reactions and industrial uses

Enzyme class	Reaction catalyzed	Example of industrial enzyme/use
Oxidoreductases	Oxidation or reduction of substrate	Biocatalysis/fine chemical synthesis
Transferases	Transfer of a group from one molecule to another molecule	Transglutaminases/fine chemical synthesis
Hydrolases	Bond cleavage while water is added	Proteases, esterases/food, beverage and paper pulp
Lyases	Non-hydrolytic cleavage of bond and remove group from their substrate	Pectate lyases/food, beverage
Isomerases	Conversion of one isomer to another	Glucose isomerase/food, beverage
Ligases	Joining of two molecules at the expense of chemical energy	Synthetases/fine chemical synthesis

operational costs lowered by between 9% and 90% [12]. It is projected that the enzyme-based biotechnological industries will continue to grow, fuelled by trends including the demand for chiral chemicals, cost savings, emerging technologies, and sustainable industrial development [12, 17]. Novel enzymatic activities can also be generated without prior knowledge of detailed mechanisms [18], providing further impetus for enzyme-based industries.

Enzyme-based industries have certain advantages compared to traditional chemical manufacturing industries. For example, the production of many specialty chemical compounds, especially pharmaceuticals, relies on the use of chirality, or handedness, since many bioactive compounds and receptors show chirality. Chemical processes generally generate a mixture of compounds that are right-handed and left-handed, and separating them is problematic. Enzymes can be rationally designed to produce specific chiral molecules [19–21]. The worldwide market for single enantiomer drugs exceeds $100 billion [22], making this search a worthwhile investment. In addition, enzymes being proteins are biodegradable and not harmful to the environment or difficult to dispose, unlike traditional chemicals which persist in the environment and are sometimes poisonous or bioaccumulate. For example, paper pulp was traditionally whitened using chlorine-based bleaches which are strongly oxidizing. New whiteners incorporate xylanases which are much less harmful than chlorine in the environment [23], and increase chlorine penetration, allowing less chlorine to be used [24, 25]. Many chemicals used in traditional industrial processes are strongly acidic or basic which may skew the pH of the effluents or cause damage to containers. Some enzymes do require extreme pH or temperatures for activity (e.g., some proteases and enzymes from thermophiles) but most industrial enzymes have moderate pH and temperature requirements, overcoming these problems.

Production of Industrial Enzymes

As the source of industrial enzymes varies, so do production procedures. Enzymes sourced from microbes, as well as transgenic enzymes produced in microbes, are usually grown in fermenters and can be extracted from the microbial cells. A preferred method is to secrete the enzyme into the culture media making extraction and purification much easier [26]. Microbial production utilizes microorganisms that have been modified and evaluated for safety and efficient production. Many have been used since historical times in the manufacture of fermented foods such as beer, cheese, soy sauce, and yogurt [13]. The first US patent granted for a transgenic microorganism with an industrial application was US Patent 4,259,444 following a Supreme Court ruling to Ananda Chakrabarty from General Electric Corporation for developing *Pseudomonas* strains harboring plasmids that could degrade aromatic hydrocarbons [27], and subsequently for mixed culture of *Arthrobacter* and *Pseudomonas* suitable "in the biological treatment of a contaminated

material including many persistent compounds of diverse chemical constitution" [28]. The ease of transformation of bacteria with plasmids and protection by patent law catalyzed the development of an industry of selected organisms producing proteins for various purposes.

Methods to optimize gene expression and enhance protein accumulation and purification continue to be developed. The industrial strains used to produce enzymes today are mostly proprietary and are selected for high production and accumulation levels. However, a disadvantage of bacteria is that proteins are not modified post-translationally and are often insoluble and accumulate into inclusion bodies. This can affect activity, especially when the protein is of eukaryotic origin, and methods to recover active proteins from this inactive conglomeration are often tedious [29].

Yeast is often the microbe of choice to express eukaryotic proteins, but hyperglycosylation of transgenic proteins has been observed. Fungal systems are relatively robust, but they have different metabolic pathways, post-translational processing, codon usage, and may form inclusion bodies [30, 31].

Following selection of an efficient microbial producer, the organism is grown in optimized conditions on media which may be solid or liquid. Most industrial enzymes are generally produced in 50–500 m^3 stirred fermenters. A major challenge with fermenters such as these is to maintain sterility, as contamination can cause loss of the entire batch. The integrity of the high producer must also be monitored to ensure that a lower-producing mutant does not outcompete the high-producing strain. But besides operational costs, capital costs can also be high, which makes this system quite expensive [32].

The same considerations apply to cultures of insect and mammalian cell cultures used to overcome the problems of eukaryotic protein expression seen in microorganisms such as glycosylation, folding, subunit association, γ-carboxylation, and cleavage [33]. For cell cultures, each production scale has to be optimized to the product and may vary from high-value, low-demand products being made in small multiple-unit reactors (flasks or roller bottles) and bulk products in large 10,000 L single-unit batch reactors to be cost-effective [34]. In fermenters, cultured mammalian cells are affected by shear forces and are also sensitive to growth conditions such as pH and temperature, metabolites, and dissolved oxygen, which may affect product quantity and quality [5]. Such variation does not permit streamlining or standardization, and makes animal cultures more expensive to operate.

Enzymes such as catalase from liver and rennet from stomach can also be isolated directly from animal tissues, often as byproducts of the meat industry. Transgenic animal sources have the advantages of appropriate modifications of proteins [33], but problems of scale and costs of production, maintenance, and waste disposal. A serious concern with the use of animals is the risk of contamination of end products, especially when they may be used for human therapeutics or consumption, with animal pathogens such as viruses, mycoplasmas, and prions. More recently, Bovine Spongiform Encephalopathy (BSE) or mad cow disease was shown to be transmitted by contamination by small amounts of infective prions in blood and other animal tissues [35]. This had led to fears of contracting this disease by using protein products derived from animals.

Although plants were one of the first sources to be used to produce industrial enzymes such as papain from papaya and β-amylase from barley, only recently have they been developed as recombinant protein production systems [5, 36]. There are several theoretical reasons why plants may be one of the best sources for the long-term supply of exogenous enzymes including: (1) plants represent the least expensive method to produce proteins in general, (2) they do not require the large amount of capital for production compared to microbial fermenters, (3) production can be scaled up or down without major changes in infrastructure, (4) almost any plant in theory can function as a production system, (5) proteins can be targeted to specific compartments allowing for increased accumulation in the desired tissue with little interference in other tissues to reduce potential toxicity to the cell, (6) plants have convenient storage, transport, and processing of component materials; and (7) plants have the potential to combine lines with different enzymes through crossing [37–39].

There are other advantages. Many pharmaceuticals targeted for use in animals are toxic to animal cells. Plants do not share the same receptors and are capable of accumulating such proteins. Since plants do not

form inclusion bodies, the proteins stay soluble and can be purified more easily. Plants can further be engineered to produce protein in specific tissue, allowing the other parts of the plant to be processed to offset the cost of production. Further, the protein is thereby precluded from interfering with metabolism in other parts of the plant. Proteins can also be targeted to subcellular compartments, further reducing the risk of toxicity, as well as increasing production levels. This potential to express different proteins in different locations allows flexibility in storage and purification options [5, 40–42], and will be discussed in more detail below.

One obvious advantage is that plant-produced biologics are free of animal source tissue, thereby eliminating the fear of transmitting animal pathogens. The lower trophic levels plants occupy as producers is advantageous because it indicates that the energy input into plant growth is lower, and therefore the LCA impact is lower compared with microbes and cell lines grown in fermenters and animals on farms. Plants grown in fields require little more than air, rain, soil, and nutrients. Microbes and animals utilize plant material for growth, and unless they can utilize plant resources at 100% efficiency, they can never be as energy proficient as plants themselves. The low cost of production, ability to post-translationally modify proteins and clear growing, handling, and processing knowhow make plants valuable for industrial enzyme production.

In addition to the advantages of plants listed above, plant systems are particularly well suited to inexpensively yield large amounts of a desired product in a relatively small area. For instance, the cost of transgenic seed for extraction of β-glucuronidase in 1998 was estimated to be only $0.20/kg [43], which was considerably less than bacterial cultures [44]. Moreover, because some plant tissues such as seeds can store proteins for years without loss of activity under ambient conditions, a ready supply of material can be manufactured into final form on an as-needed basis [45]. Propagation from stored seed, rapid scale-up, large volumes, and long-term storage are particularly advantageous for industrial enzymes. Low cost combined with the ability to use the raw material directly for industrial processes encourage development in this direction. These advantages have led to a recent increase in use of this technology for the production of new biologics.

While it seems unlikely that one production system could meet all potential needs for the diversity of products, plants do offer some clear theoretical advantages over other systems. A summary of characteristics of different production systems is shown in Table 3.

Production of Recombinant Proteins in Plants

There are a plethora of plants to choose from for heterologous protein production. The choice of the best plant type depends on how the characteristics of the final product complement the characteristics of the plant. Key factors include the ability of certain tissues to accumulate proteins, detrimental compounds such as toxins that may be produced in certain tissues that can co-purify with the protein products, the potential for the industrial crop to inadvertently mix with other food crops or weeds, the ease of purification of the protein from the plant tissue, and the potential to use the plant tissue directly eliminating the need to purify or extract the protein product. Table 4 lists some of the characteristics of different plant systems that can be used for protein production. While most plant systems can be used in theory, the associated cost can make this unsustainable for many industrial proteins. For high volume, the most cost-efficient system is with commodity grains. Grains provide the advantages of high protein content, feasibility for long-term storage, and the ease of downstream processing which give them great potential for future industrial protein production.

While plants are the least expensive source of biomass, they have not been developed to the extent of their microbial counterparts to accumulate proteins. The cost of producing the proteins is inversely related to the amount accumulated in the biomass so this has a direct bearing on the economics. In the past decades, most of the research on plants has focused on improving traditional uses of plants so there has not been much incentive to look at protein accumulation for use as a production vehicle for protein products. Currently high level of expression in plants is usually recognized at levels of 0.1% of the dry weight of the plant tissue. This leaves much room for improvement in the future.

Plant Molecular Pharming, Industrial Enzymes. Table 3 Features of different industrial enzyme production systems

Production system	Bacterial	Fungal	Animal cell lines	Transgenic animals	Plant
Speed of creating transgenic plants	Rapid	Rapid	Rapid	Slow	Moderate
Capital cost to produce raw ingredients (fermenters, chambers)	High	High	Very high	Low to moderate	Low to moderate
Consumables (media and resources)	Moderate	Moderate	High	Moderate	Low
Processing cost	Moderate	Low	Moderate	Moderate	Low
Production issues	Contamination, maintenance of high producers	Contamination	Contamination, animal pathogens	Animal pathogens	

Plant Molecular Pharming, Industrial Enzymes. Table 4 Characteristics of plant systems for the production of transgenic plants (Reprinted from [39]. With permission from Elsevier)

Crop	Advantages	Disadvantages
Wild species	Clearly distinguishable from crops	Low yield
		Outcross to native plants
		Little known about safety
Domesticated species	High yields	Potential to intermix with crops used for other purposes
	Infrastructure and experience exist	
Food	High margin of safety for human health products	Greater potential to intermix with food supply
Non-food	Less potential to intermix with food supply	Greater potential for toxic, antinutritional, or allergenic agents
Fresh tissue	Abundant biomass	Harvest/Transport/Storage
Seed or dry tissue	Harvest/Transport/Storage	
	High protein content	
Hydroponics, cell cultures	Limited exposure to environment	High cost
		Limited knowledge of product safety
Field grown	Low cost	Higher potential to intermix
	Infrastructure in place	
Modified food/feed grain designed for industrial applications	Clearly distinguished by color/shape	Not yet developed
	Non-transferable genetics	
	Low cost	
	Infrastructure and experience transferable from commodity crop	

Commodity plants currently used as a food, feed, or fiber source are being investigated as a production vehicle for industrial proteins. There has been public concern that use of food plants to produce industrial enzymes or pharmaceuticals may lead to inadvertent exposure to these products and cause safety concerns. Production of industrial or pharmaceutical compounds in organisms used in the food chain is far from new. In addition to the many native products isolated from animals, recombinant food organisms such as yeast or eggs play a major role in the production of pharmaceuticals and industrial proteins. There is also precedent in plants for species that produce both food and industrial products. Rapeseed is used primarily for the production of an industrial oil crop while canola seed which was derived from rapeseed, with subtle genetic differences, is used predominantly as a food crop. The key is to keep food and production streams separate [6] and failure to do so can create problems whether the organism is a traditional food or non-food source.

Current government regulations put plants on par with other production systems to prevent inadvertent products entering the food chain or harming the environment. While many of the industrial enzymes in use today are already in the food chain, these added precautions are necessary to limit exposure or can be used to protect against protein products that may not be in the food chain or have not undergone the rigorous or long-term testing needed to give confidence that there are no detrimental effects.

One concern often voiced by the public is that transgenic plants have the potential for dissemination of the transgene through pollen when grown in an open environment. The pollen may be ingested by non-target species, or hybridize with other plants. This situation has been recognized by regulatory agencies and there are strict controls on containment of transgenic plants and pollen. These include physical isolation and temporal delays as well as molecular containment strategies such as pollen and seed sterility and RNA interference have also been adopted to restrict dissemination [46–48]. These measures can lead to increased costs, but are necessary for safety and to allay public unease about transgenic crops.

The types of industrial proteins can include non-enzyme proteins and enzymes that have industrial use for food, feed, or pharmaceutical applications. This also includes proteins used in the making of pharmaceuticals [49], including plantibodies [50–52] and edible vaccines [53, 54]. This may also include pharmaceuticals such as therapeutics and vaccines but these will not be discussed in this contribution.

Like pharmaceutical products, some industrial proteins may require appropriate post-translational modification and folding to be active. Most higher plants can accommodate this in a manner very similar to that which occurs with animal cells with minor modifications. Since plants do not form inclusion bodies, and since many proteins normally harmful to animal cells do not affect plant cells, plants are increasingly and successfully being used for their production.

One of the main limitations in using plants today to produce industrial proteins is the demand that cost must be extremely low compared to pharmaceuticals. This requires that the expression level be high. There are various options in terms of plant type, tissue, and intracellular location, allowing for great potential. However, this versatility also causes uncertainty in the early stages of developing a plant expression system. In addition, the plant's ability to accumulate a particular hydrolytic or oxidative enzyme has the potential for interference with the plant's metabolism and cause damage to the plant long before protein accumulates. While this has been seen in a number of cases, there have also been various ways to overcome this problem by tissue and subcellular targeting. In addition the use of thermophilic [55] and pro-enzymes [56] or the requirement for cofactors lacking in the plant have all been used to increase protein accumulation [57–59]. A list of enzymes from various sources (bacterial, fungal, animal, and synthetic) produced in plants is given in Table 5. This table provides a snapshot of the accumulation levels of specific proteins in selected tissues and the problems that investigators may have encountered by expressing the protein in the tissue.

Options for Plant Transformation

The transformation technology used to express industrial enzymes can have a major impact on the accumulation of the recombinant protein. Therefore, it is important to select the type of transformation protocol that will best fit the application of the enzyme application. As the transformation process is discussed in

Plant Molecular Pharming, Industrial Enzymes. Table 5 List of industrial proteins produced in plants

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
α-Amylase	Starch degradation; food and beverages, biofuels, textiles, and paper industries	*Bacillus licheniformis*	Tobacco	0.3% total soluble protein (TSP) in leaf	Unaltered plant phenotype Secreted into intercellular space; extra complex sugar chains added; degradation products identical to native protein	[60]
α-Amylase		*Bacillus licheniformis*	*Nicotiana tabacum* SR1	0.4% TSP in seed	Constitutive expression; hydrolysis products identical to purified *B. licheniformis* α-amylase	[61] European Patent 044,9376
Thermostable α-Amylase		*Bacillus licheniformis*	*Vicia norbonnensis* L.	1 mU/mL seed supernatant	Seed specific USP promoter; accumulation in cotyledon protein bodies; post-translationally modified	[62]
α-Amylase OS103		*Oryza sativa* cDNA	*Nicotiana benthamina*	5% TSP in leaf	Viral infection causes mild chlorosisand stunting; moderate glycosylation of protein in plants	[63]
α-Amylase		*Bacillus licheniformis*	*Medicago sativa*	0.01% TSP in leaf	Unaltered phenotype	[64]
Bifunctional thermostable Amylopullula-nase (APU)	Pullulan and amylose degradation; detergent industry	*Thermoanaero-bacter ethanolicus* 39E (ATCC53033)	*Oryza sativa* L. cv Tainung 67	5.7% total soluble protein in seed	Unaltered phenotype; elevation in pullulanase correlates with decrease in amylose; amyloplast location; starch completely hydrolyzed upon heating	[65]
Aprotinin	Inhibitor of trypsin and proteases; medical and research uses	Optimized bovine aprotinin sequence	Maize	0.4% TSP in seed	Multiple copies in genome; ubiquitin promoter; protein accumulation in seed embryo; transgenic protein biochemically identical to native protein	[66]

Plant Molecular Pharming, Industrial Enzymes. Table 5 (Continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Aprotinin cDNA fusion with extension signal		Synthetic bovine	*Nicotiana benthamina*	7,100 trypsin inhibitory units/mg extract protein	Transient TMV virion transfection; product biochemically similar to native protein; large-scale production on 1.5 acres open field or 2,500 sq. ft. greenhouse yields 1 kg purified enzyme.	[67]
Arginine decarboxylase (adc) cDNA	Degradation of arginine; medical and research uses	*Datura stramonium*	Rice	2-fold increase in putrescine levels following stress removal	Under drought conditions, wild-type plants are severely affected, whereas transgenic plants have normal phenotype	[68]
Avidin	Irreversibly binds biotin; research uses	Chicken egg white	*Zea mays*	2.3% TSP in seed; 230 mg/kg seed	Partial to complete male sterility in high expressing plants; similar to native glycoprotein; stable during storage for over 3 months	[69]
Endochitinase (ech42) cDNA	Chitin degradation; research and agricultural uses	*Trichoderma atroviride*	Alfalfa	Up to 2,650-fold higher than control plants	Unaltered phenotype; high expression levels do not correlate with higher resistance to pathogen challenge	[70]
Endocellulase E1	Cellulose degradation; biofuels and paper industries	*Acidothermus cellulolyticus*	*Zea mays* L.	Higher levels in ER than mitochondria; max 2.0% total soluble protein	Targeted to ER and mitochondria; ER targeted E1-cellulase called “Spartan Corn 1”	[71]
Endo-1,4-βD-glucanase (E1 cellulase)	Cellulose degradation; biofuels and paper industries	*Acidothermus cellulolyticus*	*Zea mays* L.	6.1% (ER) and 5.6% (vacuole) TSP in seed	No apparent effect on growth; truncated catalytic domain accumulates in ER and vacuole; 16% TSP in single seed indicates high accumulation potential	[41]
Endoglucanase E1		*Acidothermus cellulolyticus*	Lemna minor 8627	0.24% TSP; 0.2 U/mg protein in fresh tissue	Unaltered phenotype	[72]

Endoglucanase E1		*Acidothermus cellulolyticus*	Tobacco	0.25% total soluble protein in leaf (apoplastic targeting)	Unaltered phenotype; stored seeds had 45% more activity after 1 year	[73]
Endoglucanase E1		*Acidothermus cellulolyticus*	Tobacco	Ammonia explosion (AFEX) treatment yielded 35% original activity	AFEX pretreatment is not a suitable method for releasing cellulase enzymes in transgenic plants	[74]
E1 endoglucanase		*Acidothermus cellulolyticus*	*Nicotiana tabacum*	1.35% TSP in leaf	Chloroplast targeting; normal growth and development; activity decreases with leaf age and upon dehydration	[75]
Cellobiohydrolase I (CBHI)	Cellulose degradation; biofuels and paper industries	*Trichoderma reesei*	*Zea mays* L.	3.2% (cell wall) and 4.1% (ER) TSP in seed	Holoenzyme in cell wall; single seed levels of 17.9% indicate high accumulation potential	[41]
Exo-cellobiohydrolase I (CBHI)	Cellulose degradation; biofuels and paper industry	*Trichoderma reesei*	Tobacco	0.11% TSP in leaf and 66.1 μmol/h/g total leaf protein activity; 0.082% TSP in callus and 83.6 μmol h/g total callus protein activity	Unaltered phenotype	[76]
Thermostable (1–3, 1–4) β-glucanase (codon adapted)		*Bacillus* spp.	*Hordeum vulgare* L. (barley)	40 ng enzyme/2 × 105 protoplasts for codon modified constructs compared to none for unmodified constructs	Biolistic transformation; codon usage important for expression; unaltered phenotype; germination induced expression of enzyme in grain	[77]
β (1–3, 1–4)-glucanase		*Bacillus* spp.	*Hordeum vulgare*	1.29 g/mg TS; 5.4% TSP in grain endosperm	Large variations in enzyme levels between transformants; levels stable for 3 years	[78]
Endo-1,4-β -glucanase (EGI, cellulase)		*Trichoderma reesei* egl1	*Hordeum vulgare* L. Kymppi and Golden Promise	0.025% TSP in seed	Plant morphology normal but reduced seed setting in transgenic plants	[79]
Endoglucanase holoenzyme (E1) and catalytic domain (E1cd)		*Acidothermus cellulolyticus*	*N. tabacum*	1.6% TSP in leaf	Unaltered phenotype; apoplast targeting of catalytic domain achieves 500-fold greater expression than cytosolic full length E1	[80]

Plant Molecular Pharming, Industrial Enzymes. Table 5 (Continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
1,4-β -D-endoglucanase (E1)		*Acidothermus cellulolyticus*	*Solanum tuberosum* L.	2.6% TSP in leaf	Unaltered phenotype; dual crop applications: leaf targeting allows tubers to be used for culinary applications	[81]
Thermostable endo-1,4-$\tilde{\beta}$-D-glucanase		*Acidothermus cellulolyticus*	*Nicotiana tabacum*	Not quantified	Targeting to chloroplast in vitro and in vivo	[82]
Modified endoglucanase cellulase (egl)		*Ruminococcus albus*	BY-2 tobacco suspension cells	0.1% TSP; 30-fold greater truncated form activity than endogenous cellulase	Unaltered phenotype; three forms (pre-form; mature form; truncated form); truncated form has highest expression	[83]
Hybrid (1,4) –β-glucanase (cel-hyb1)		*Neocallimastix patriciarum*	Barley grain	1.5% total grain protein	Endosperm targeted; codon optimization leads to 527-fold increase in expression levels; stable during post-harvest storage	[84]
Catalytic domain endo 1,4-β-D-glucanase (E1cd)		*Acidothermus cellulolyticus*	*Zea mays*	2.1% TSP in leaf and 0.845 nmol/μg/min activity; 2.08% TSP in root and 0.835 nmol/μg/min activity	Set seeds at maturity	[85]
Catalytic domain 1,4-β-endoglucanase E1		*Acidothermus cellulolyticus*	*Zea mays*	1.13% TSP	Unaltered phenotype; apoplast targeted; successful conversion of corn stover into glucose following AFEX pre-treatment	[86]
Thermostable catalytic domain endo-1,4-β-glucanase		*Acidothermus cellulolyticus*	*Oryza sativa* L. Japonica cv. Taipei 309	4.9% TSP	Unaltered phenotype; constitutive promoter; capable of hydrolyzing AFEX-treated stover	[87]
Truncated endoglucanase (t-egl)		*Ruminococcus albus*	Tobacco	0.5% TSP	Unaltered phenotype; cell disruption allows cell wall digestion to occur	[88]

Thermostable 1,4 β -D-endoglucanase catalytic domain		Acidothermus cellulolyticus	*Arabidopsis thaliana*	26% TSP in leaf	No abnormal phenotype; apoplast targeting; activity and immunochemically similar to native enzyme	[89]
Thermostable 1,4-β -D-Endoglucanase E2 and E3		*Thermomonospora fusca*	*M. sativa* L.	E2-0.1% TSP	Unaltered phenotype	[90]
			N. tabacum L.	E3 0.02% TSP		
			S. tuberosum L.			
Thermostable cellulases (Cel6A, Cel6B)		*Thermobifida fusca*	Tobacco	4% TSP	Homoplasmic, transplastomic plants using plastid-directed vector; not optimized	[91]
Recombinant hyperthermostable endoglucanase Cel5A		GenBank accession number At3g4890	Tobacco	5.2% TSP in leaf	Unaltered phenotype; chloroplast targeted; stable active enzymes	[92]
Chimeric chymosin (rennin)	Milk curd formation; dairy industry	Bovine	*Brassica napas*	0.5% (w/w) TSP	Seed targeted	[93] US Patent 7,390,936
Coumarate-3-hydroxylase (C3H)	Lignin modification; biofuels	*Medicago sativa*	*Medicago sativa* cv Regen SY	C3H levels 5% of wild type levels	No serious phenotypic impairment	[94]
Hyperthermo-philic α-glucosidase	Breakdown of maltose; brewing and research uses	*Sulfolobus solfataricus*	*Nicotiana tabacum*	0.04% TSP in leaf	Enzyme levels increase at maturity; unaltered phenotype	[95]
			cv. Xanthi			
Glycosyl hydrolase β-glycosidase	Degradation of glycosidic bonds; biofuels and research uses	*Sulfolobus solfataricus*	Tobacco	0.15% TSP in leaf	Enzyme levels increase at maturity; unaltered phenotype	[95]
ADP-glucose pyrophosphory-lase modified (Sh2r6hs)	Starch synthesis; agricultural and research uses	*Zea mays*	*Triticum aestivum*	5-fold greater protein accumulation	Modified large subunits permit greater stability and yield; endosperm specific promoter	[96]

Plant Molecular Pharming, Industrial Enzymes. Table 5 (Continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
ADP-glucose pyrophosphorylase modified large subunit (Shrunken 2 gene Sh2r6hs)		*Zea mays* L.	*Triticum aestivum* L.	91% more activity in the presence of 10 mM Pi	Transgenic wheat plants produced 38% more seed weight; 31% higher biomass; transgene stable after five generations	[97]
Human placental β-glucosidase (GCase)	Degradation of glycosidic bonds; biofuels and research uses	Human placenta	Tobacco	750 U/kg seed	Seed viability totally impaired above 500 U/kg; taken up by human fibroblasts; free from immunogenic xylose and fucose	[98]
β-glucuronidase (GUS)	Degradation of glucuronic acid residues; research uses	*E. coli*	*Zea mays*	0.7% TSP in seed	Functionally equivalent to native protein; dispersed in cytoplasm; single integration in transformant with highest expression	[99]
β-glucuronidase (GUS) with αAmy8 regulatory and signal sequence		αAmy8 sequences from rice	Rice, tobacco, and potato suspension cells	40% total secreted proteins	Fusion to β-glucuronidase (GUS); inducible by sugar; tunicamycin causes ER accumulation	[100]
Laccase I	Lignin degradation; biofuels, wood, and paper industry	*Trametes versicolor*	*Zea mays*	0.55% TSP 4 ng/mg dry weight (T2) to 70 ng/mg dry weight (T6)	Variable expression levels; breeding and selection increased levels 20-fold in five generations; embryo-preferred promoter with cell wall targeting supports highest expression; germplasm background affects germination frequency	[110]
Laccase		*Trametes versicolor*	*Zea mays*	0.20% of dried, defatted corn germ	Contains both water soluble and immobilized laccase; some laccase is inactive apoenzyme form	[58]

Laccase riceMaL and ricePcyL cDNA		*Melanocarpus albomyces* *Pycanoporus cinnabarinus*	Rice	13 ppm (riceMaL) 39 ppm (ricePycL)	Endosperm targeted; seed production was normal; recombinant protein is biochemically similar to native proteins, but had lower kinetic parameters	[101]
Lipase	Lipid breakdown; dairy, food, biofuels, and detergent uses	Recombinant dog gastric lipase	Tobacco	5% (vacuolar retention signal) and 7% (secretion signal) of acid extractable protein	Active glycosylated protein with similar properties to native protein; specific activity dependent on subcellular compartment; Normal leaf morphology	[102]
Lipase (rDGL)		Recombinant dog gastric lipase	Maize seed endosperm	Specific activities of 3 U/mg protein(grinding); 3.7 U/mg protein (defatting) and 9 U/mg protein (dry milling)	Comparison of relative efficiency of grinding, defatting, and dry milling of seed prior to selective extraction	[103]
Lipase		Dog gastric lipase	Tobacco	360 U/mg protein	Impact of subcellular targeting on glycosylation; transient expression system	[104]
Manganese-dependent lignin peroxidase (MnP)	Lignin degradation; biofuels, wood, and paper industry	*Phanerochaete chrysosporium*	*Medicago sativa* L.	0.5% TSP in leaf	Reduction in dry matter and height related to expression levels; yellow foliage; MnP expression segregates in sexual progeny	[64]
Manganese peroxidase (MnP)	Lignin degradation; biofuels, wood, and paper industry	*Phanerochaete chrysosporium*	Maize	15% TSP in seed 3% TSP in leaf	Cell wall targeting yields full length MnP; cytoplasmic targeting produces truncated products; seed-targeted promoter has higher expression levels and improved plant health outcomes over constitutive promoter	[59]
Anionic peroxidase cDNA	Lignin structural modification; biofuels, wood, and paper industry	cDNA clone for the primary isoenzyme	*N. tabacum*; *N. sylvestris*	>10× higher peroxidase activity compared to wild type	CMV35S promoter; chronic severe wilting through loss of turgor in leaves initiated at the time of flowering	[105]

Plant Molecular Pharming, Industrial Enzymes. Table 5 (Continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Phytase phyA2	Phytic acid breakdown; animal feed uses	*Aspergillus niger*	Maize	2,200 U/kg of seed	Embryo-specific globulin-1 promoter; different glycosylation pattern; stable over four generations; normal transgenic seed germination	[106]
Phytase		*Schwanniomyces occidentalis*	Rice	4.6–10.6 U/g fresh weight in leaves	Stable in silage for 12 weeks	[107]
Rationally designed phytase		*Aspergillus fumigatus*	*Triticum aestivum* L. (wheat)	4,777 FTU/kg seed flour	Vacuole accumulation despite apoplast targeting; unaltered phenotype	[108]
Secretory phytase (PHY)		Synthetic gene	Potato	40% more phosphate in transgenic plants	Trichoblast-specific promoter; healthy plants, but with altered leaf shape	[109]
Chimeric phytase ex::phyA		*Aspergillus niger*	*Nicotiana tabacum*	3.7-fold more phytase secretion and 52% higher P accumulation in transgenic plants	Presence of soil phytate essential	[110]
Phytase phyA		*Aspergillus niger*	*Triticum aestivum* L.	4-fold increase in plants with constructs with a-amylase signal peptide; 56% increase in plants with constructs without signal peptide; Phytase activity 3,000 FTU/kg	Endosperm, but not embryo accumulation; gene stability over three generations	[111]
Phytase gene		*Aspergillus fumigatus*	Rice Japonica var Taipei 309	130-fold increase in grain phytase level	Unchanged phenotype; coexpressed in endosperm with Phaseolus vulgaris ferritin gene and overexpressed endogenous cysteine-rich metallothionein	[112]

Phytase MtPHY1		*Medicago truncatula*	Arabidopsis	12.3- to 16.2-fold higher levels in root apoplast	Dry weight of transgenic plant upto 4.0 times higher than control and P content up to 5.5-fold higher; root-specific and constitutive promoters used	[113]
Phytase		*Aspergillus niger*	Tobacco	1% TSP in seed	Transgenic seed added directly to chicken feed shows improved nutritional quality	[114]
Phytase GmPhy		*Glycine max* L. Merr.	Soybean tissue culture	2–3 fold higher than controls	Novel phytase similar to purple acid phosphatases	[115]
Phytase cDNA		*Aspergillus niger*	*Nicotiana tabacum*	26% dry weight of leaves 14.4% TSP in leaf	Constitutive expression with secretion signal from tobacco pathogen-related protein S; differences in glycosylation compared with native protein	[116]
Phytase phyA		*Aspergillus niger*	*Brassica napus* (Canola)	600 U/g of multi-copy T1seed; 103 U/g in single copy line	Unchanged morphology; seed-specific CruA promoter used; gene-dosage related expression; stable over three generations; no correlation between high expression and seed germination	[117]
Phytase phyA		*Aspergillus niger*	Soybean cell cultures	920 pKat M/g total soluble protein	Secretion and glycosylation may be necessary for activity; transgenic protein smaller than native protein, but has similar biochemical profile	[118]
Phytase SrPf6 (*S. ruminatum*) appA (*E. coli*)		*Selenomonas ruminantium* *Escherichia coli*	*Oryza sativa* L. cv. Tainung 67.	0.6 U/mg (appA); 1.4 U/mg (SrPf6) of TSP in seed; up to 60 times activity of control	No adverse effects; germination-inducible αAmy8 promoter and αAmy8 signal peptide; multiple copy number	[119]
Phytase cDNA		*Aspergillus niger*	*Medicago sativa*	2.0% TSP	Un-glycosylated, but stable	[120]
Transglutaminase (rTGp)	Formation of peptide bonds; food industry	Rat prostate	*Oryza sativa*	0.15 U/mg h leaf	Ca^{2+} dependent enzyme	[121]

Plant Molecular Pharming, Industrial Enzymes. Table 5 (Continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Trypsin	Protein hydrolysis; medical and research uses	Bovine pancreas	Zea mays	0.025% seed dry weight	Equivalent to native enzyme. levels suitable for commercial production; produced as zymogen	[56] US Patent 6,087, 558
Xylanase B xynB	Hemicellulose degradation; biofuels, wood, and paper industry	*Streptomyces olivaceoviridis*	Solanum tuberosum L.	5% TSP in leaf	Stable gene expression for several generations	[122]
Xylanase A xynA thermostable catalytic domain xynA1		*Clostridum thermocellum*	Rice	Not quantified	Normal plant phenotype; stable expression in seed and straw; activity in desiccated seed	[123]
Xylanase xynC-oleosin fusion		*Neocallimastix patriciarum*	Canola	2,000 U/kg seed (oil body of seed)	Fusion protein retains optimal temperature, Km, and specificity, but has reduced pH sensitivity	[124]
Xylanase Modified xynC		*Neocallimastix patriciarum*	Barley	0.004% dry weight of seed endosperm	GluB-1 promoter better than Hor2-4 promoter; protein stable during seed maturation, desiccation, and storage; 40% low fertility in one line;	[125]
Xylanase XYLII		*Trichoderma reesei*	*Arabidopsis*	1.2% (cytosol); 3.0% (chloroplast); 1.7% (peroxisome); 4.8% (chloroplast+peroxisome) total soluble protein	Unaltered phenotype; levels highest at flowering; dual targeting to chloroplasts and peroxisomes causes much higher levels than either compartment alone, although RNA levels are similar	[126]

Xylanase xynII		*Trichoderma reesei*	*Arabidopsis*	3.2% TSP in leaf	Chloroplast expression exhibit normal growth, but cytosolic accumulation affected transgenic plant growth	[127]
Xylanase Thermostable, truncated xyn2		*Clostridum thermocellum*	Tobacco	4.1% TSP in leaf	Unaltered phenotype; proteinase II signal peptide used; enzyme enrichment following heat treatment	[128]
Xylanase Thermostable, truncated xyn2		*Clostridum thermocellum*	*Nicotiana tabacum* L. cv Wisconsin	Not quantitated	Clearance zone develops at 3 h	[42]
xylanse (XYLD-A) and β(1-3, 1-4) glucanase (XYLD-C)		*Ruminococcus flavefaciens*	Tobacco	170 μM/min/m^2 xylanase and 2,000 μM/min/m2 glucanase in leaves	Unaltered phenotype; separate constructs; apoplast targeting; glucanase accumulated higher protein levels than xylanase	[129]
1-deoxy-D-xylulose-5-phosphate synthase DXS gene cDNA (CLA1)	Lignin structure modification; biofuels, wood, and paper industry	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Higher or lower levels than wild type	Normal morphology; variable levels of isoprenoid biosynthetic pathway products correlated with increased or decreased enzyme level	[130]

detail elsewhere, only a brief synopsis of how it impacts industrial enzymes is summarized here.

Both stably transformed and transiently expressing plants have been used to express proteins [131]. In stable transformation, the foreign DNA can be targeted to the cytoplasm or a number of different intracellular locations such as the nucleus [132] or into plastid genomes, usually the chloroplast [133]. Mitochondrial targeting is not as well established and has not been pursued in this context. Organelle transformation provides the advantages of high copy number and the transgenes are not passed on by the pollen [6]. This method however is not yet applicable to many types of plants. Stably transformed plants are time consuming to characterize and generate, but once produced, they can be stored as seed. This allows for a ready source upon demand. Transiently expressing foreign DNA can be inserted into somatic tissue with the purpose of short-term expression using viral vectors delivered using Agrobacterium or biolistics [134–136]. Transient expression is useful when a protein needs to be expressed at short notice.

Selection of Plant Species for Transformation

One consideration for industrial protein production is the type of plant used as the production vehicle. The options include: plants grown for their vegetative tissue for large volumes of biomass; plants harvested for grain for their enriched protein and facile storage characteristics; well-established cultivated crops where much is known about growing and processing; wild species that have little use today, making them distinct; food crops that have Generally Regarded as Safe (GRAS) status and pose no safety threat to the crop itself or host protein but have the potential for intermixing with food crops; or a non-food crop with decreased concerns about intermixing with food crops but with greater potential to have compounds that are detrimental or untested with regard to human safety (see Table 3).

Food crops are known to be safe when consumed and they have well-established procedures for growth, harvest, and storage. In cases where the final product may include some or all of the plant tissue as well as the recombinant protein, this has a significant advantage and direct applicability in the case of many industrial enzymes that are used in the food and feed industry.

For example, maize (*Zea mays*; corn) is well accepted as a safe product (GRAS), and is widely used in food, feed, and industrial applications today [137]. The production cost of maize is very low, and the infrastructure can handle large or small acreages for industrial or pharmaceutical products. Storage and transport of seed, and protein purification from flour are compatible and flexible with current practices without special handling. There are no known agents in maize that generally interfere with protein purification. Finally, the grain can be processed with little or no heat inactivation steps without affecting the protein's properties [4, 138, 139].

In addition, maize has an added advantage in that the kernels can be mechanically separated to yield a germ fraction with enriched protein and an endosperm fraction with enriched carbohydrate [137]. This facilitates use of the carbohydrate fraction for industrial applications such as ethanol production [140] or feed. In this way not only is the cost of the raw material reduced but the waste products are conveniently handled as well.

A disadvantage of maize is the fear of inadvertent mixing with the food supply. Intermixing potential can be handled by management practices but will need to gain the public's confidence. While there may be claims that no food source can ever be used to produce pharmaceutical products, it is common knowledge that both eggs and yeast are used to make pharmaceuticals. Not only is there no public outcry of intermixing in these instances, but their perception is that these production systems are distinct from the systems used to make food. This is the perception that food crops must earn as well. Maize as well as other plant systems must build an infrastructure dedicated to industrial protein production, which is as distinct from food production as edible eggs are from vaccine production. Furthermore, this must also be perceived by the public to reduce fears.

One advantage of non-food crops for industrial protein production is that they are less likely to be mistaken for food crops and therefore unlikely to be inadvertently mixed with the food supply. The disadvantage is that non-food crops must be assessed for toxins, allergens, or anti-nutritional agents that may accompany the recombinant protein. For industrial proteins where little purification is performed to keep

the cost down this can be a considerable problem if the protein precuts are to be used in food or feed applications.

Another question that is relevant to selecting a crop for recombinant industrial protein production is whether a cultivated species or wild species is preferred. Cultivated species show "domestication syndrome" and have several advantages for humans than that their wild relatives exhibit through husbandry and selection over thousands of years [141]. Although non-cultivated species have not undergone selection for higher yields or been subject to agronomic practices in past centuries, there may be yield advantages in crossing domesticated plants with wild relatives, with increase in yields of up to 50% in tomato fruit [142]. Finally, the impact of relatively unknown wild species on product safety is unknown. Determination of the degree of this impact would undoubtedly require extensive effort and time, which may be limiting factors for industry.

A further choice is whether to use an open-pollinated or a self-pollinating plant. Self-pollinating plants have the advantage of a lowered risk that pollen will unintentionally transfer onto other plants of the same species. Controlled pollen shed of open-pollinated crops can be used to help alleviate this concern by either physical or genetic means to prevent out-crossing onto weedy species or related food or feed crops [46–48]. The likelihood of gene transfer from engineered to wild plants depends on sexual compatibility, flowering time, and pollen transfer distance between the engineered and wild varieties [143]. This choice is made based on the growing location and the product required.

With a variety of species to select from and the wide variety of products that are possible, it is highly unlikely that any one system will work best for each of these steps and therefore, it is important to select the plant system that best suits the product. Since it is impractical to have thousands of different production systems, it is preferable to adapt a given system to the needs of various products.

Fortunately, some common features exist that will apply to most products enabling a few systems to accommodate most products. The key features include a potential for low cost of goods, maintenance of protein integrity, flexibility with regard to time and temperature for harvest, and maintenance of product safety and environmental safety [144, 145]. Production systems are discussed below as to how they relate to the overall efficiency of the system as well as to the regulatory aspects.

Selection of Plant Growth System

Transgenic plants can be grown in the traditional manner in an open field or in a contained chamber such as a greenhouse [146]. Row-grown transgenic crops are subject to USDA regulations on buffer zones to limit pollen spread and minimize the risk of the intermixing of food crops with "pharma" crops. If the protein source is green tissue, the plants may be harvested before flowering to limit pollen spread. This is also the case when transient transfection is used to express proteins in leaf and other green tissue. When row plants are required to flower and set seed for protein production, such as is the case for grain-targeted proteins, adequate precautions must be taken to avoid pollen transfer. Row plants are capable of higher product accumulation since they are capable of growth to higher biomass [38].

There are strict regulations and permit requirements imposed by APHIS, the branch of USDA with responsibility for animal- and plant-related services (www.aphis.usda.gov). A practical solution proposed for field growth is to dedicate areas to growth of transgenic plants, or to grow transgenic plants in locations within processing areas, such as cellulose-producing plants in a bioethanol production area [140]. This way the material is grown where it is utilized, cutting transportation costs. Alternatively, the transgenic plant can be isolated in greenhouses in soil or in liquid media. This solution is more expensive, but has the advantage of physical isolation of the transgenic plant from the environment.

The potential for inadvertent transfer of the transgene to non-target plants is considerably reduced by containment in a chamber, but production is necessarily limited by cost considerations. Within a chamber, plants can be grown in soil or in liquid growth media where exudates containing the protein of interest may be continuously produced and has similar advantages to plant cell cultures where proteins are secreted into the culture medium [147]. Scalability may also be an

issue. Exudates from roots also facilitate production when secreted into fluid growth medium [40], but chambers for contained growth in liquid medium can be capital intensive and require sterility and media-related expenses. On the other hand, secretion of transgenic proteins into guttation fluid provides a convenient, but labor-intensive method of recovery of secreted proteins. Proof-of-concept was shown with three transgenic proteins in tobacco, [42]. Using this "phyllosecretion" technology, Komaryntsky and colleagues engineered three proteins from different genetic backgrounds (bacterial xylanase, jellyfish green fluorescent protein and human alkaline phosphatase) into *N. tabacum*. The proteins were fused to endoplasmic reticulum signal peptides targeted to leaf apoplast and released into guttation fluid in plants maintained in high humidity conditions. Guttation fluid has lower overall protein content than apoplast fluid, and is also released throughout the plant's life. In addition to continuous production, the process is non-destructive of plants. Levels of up to 1.1 μg/g of leaf dry weight per day, comprising up to 3% TSP, were recovered from the guttation fluid using simplified downstream processing. Although tobacco is not an ideal plant for guttation fluid production, other plants such as tomato and some grasses are highly susceptible to the production of large quantities of guttation fluid and may provide alternative targets for phyllosecretion.

Optimization of Heterologous Protein Production

Because industrial enzymes require a very low cost of production the most critical factor determining the system of choice is the level of accumulation that occurs in the selected tissue. The optimization of expression of heterologous proteins in plants has been studied for various purposes: to improve nutritional value, insect resistance, salt and drought tolerance, and product quality. These other applications however are not nearly as demanding for the level of expression required as for industrial enzymes. Key steps to increase heterologous protein expression are the choice of plant, tissue location, various manipulations of the promoter, codon usage, and compartmentalization of the protein.

After selecting the type of plant, a determination has to be made about which target tissue is best to express the protein. Location of expression is guided by many factors such as the nature of the protein, whether the protein is to be used directly or purified, and accumulation levels desired. Often, strong constitutive promoters such as the CMV 35S promoter are used, and promoter analysis by site-specific mutations has allowed delineation of sequences that modulate expression in tissue-specific locations of this promoter [148]. *Cis*-acting elements in a green-tissue-specific rice promoter P_{D540} acting as activators or suppressors of activity were identified, thereby facilitating control of protein expression in different tissues [149]. Manipulations such as the use of the embryo-specific maize globulin-1 promoter also allow accumulation of proteins in specific locations within tissues [150]. Expression of the heterologous protein can also be controlled by the use of inducible promoters, and a search of patents reveals a plethora of such inducible promoters. A list of promoters in cereals is compared in [151]. Other promoter permutations including the use of inducible promoters, stacking transcriptional units, synthetic bi-directional promoters, global regulatory sequences to recruit transcription factors, and other such approaches have been studied to enhance transcription and are reviewed elsewhere [152].

Further, heterologous gene sequences should be optimized for the plant type and location. For example, chloroplast codon usage is similar to that of prokaryotes, whereas nuclear codon usage varies from plant to plant. Also, RNA silencing is a feature of many plants, and some viruses produce suppressors of silencing, and heterologous protein sequences are often not expressed at high levels because of RNA silencing [49]. This silencing can be turned off by the expression of the heterologous gene together with a suppressor of silencing [153, 154].

In addition to the choice of tissue location, highest expression levels can be obtained when the protein is directed to specific subcellular compartments and especially so if the protein is an enzyme that would interfere with normal cellular activities [38, 41, 152, 155]. Subcellular targeting is critical for accumulation and protein integrity of hydrolytic enzymes. Cell wall targeting allowed expression of full-length manganese peroxidases, but cytoplasmic targeting resulted in truncation of the peptide [41, 59]. In addition, a seed-preferred promoter allowed high accumulation without negative effects on plant health [59]. Interestingly, targeting xylanase to two subcellular locations – the

chloroplast and the peroxisome – accumulated 240% more enzyme than the chloroplast and 160% more than the peroxisome alone. Highest levels also accumulated during flowering time [126]. These are empirical observations, and such permutations may benefit studies of high-level expression of heterologous proteins. Medrano et al. have developed a transient expression system using *Nicotiana* to assess construct efficiency [156], which may be helpful.

Finally, decisions on the expression of a heterologous protein as a fusion or as a free protein depend upon target location and levels of expression desired. For example, fusions to oleosins accumulate in oil bodies in *Brassica napus* seeds [157], and fusions to proteins previously shown to stably accumulate in plant cells may have the effect of stabilizing heterologous proteins as well [158]. Fusions to the C-terminus of ubiquitin also have a stabilizing effect resulting in 10-fold increased levels [159]. Proteins accumulated at high levels may be subjected to cellular protease activity, thus using cell lines with lower protease levels may help stabilize the heterologous protein. The expression of some proteins, such as hydrolytic enzymes, can be detrimental to the cell. The strategy for the protease trypsin was to express it as a zymogen, allowing sufficiently high levels for commercial production, marketed as TrypZean [56].

Product Recovery from Transgenic Plants

The single most important consideration for recovery is whether the protein produced in plants can be used in crude form in the plant extract, or has to be purified prior to use. Obviously, crude extracts are considerably less demanding to make, but the end use of the protein determines the level of purity required. Purification of transgenic proteins is an expensive prospect, regardless of the system, accounting for about 94% of the production cost in the case of maize [44]. Decisions on recovery should form an integral part of assessing production options based on levels of expression, costs, and sustainability. These decisions are based on the nature of protein to be expressed, and include transient or stable expression, type of plant, targeting, and modifications required.

A variety of elegant solutions have been used to overcome the problems of purification, but vary in costs and efficiency. For example, roots have been used to secrete proteins into the growth medium, and guttation liquid produced by plants also provide a useful medium for secretion-based isolation of the transgenic protein, as the growth or secreted liquid can be recovered and filtered for protein recovery [42, 49]. However, they are considerably more labor and energy intensive due to growth in liquid medium as discussed above, and recovery varies based on loss due to dilution factor and protein stability in an extracellular environment [160].

When whole plants are used as production systems, the recovery process depends on the tissue being used for expression. Transient expression in roots or leaves generally requires processing of fresh wet tissue. If stable expression is used, the material may be wet or relatively dry depending on the tissue used. Proteins extracted in fresh tissue are generally unstable and have to be recovered immediately, whereas seed-expressed proteins have shelf-life as long as 3 years [45]. Grain can be subjected to either wet or dry milling followed by separation steps. These include fractionation to obtain enrichment of germ or endosperm in the case of seed, or subcellular compartments targeted for protein accumulation, protein precipitation, adsorption, chromatography, and diafiltration [44]. Methods have been developed to simplify recovery such as post-harvest protein expression using stress-inducible promoters [45, 161] and oleosin-partitioned proteins that can be recovered easily following oil-water separations [124, 162, 163]. A detailed comparison of the economics, processing, and regulatory constraints associated with the most common plant production systems is provided in [145].

In the ideal case, commodity plants that are used for industrial applications would also express the transgenic protein. These transgenic proteins can then be used directly in the industrial process without purification assuming the protein can survive the processing steps. In this best-case scenario, no other inputs are needed to produce, purify, or process the protein leading to lower cost and less detrimental impact on the environment.

Case Study: Economics of Cellulases Produced from Transgenic Plants With the current state of technology for biomass conversion, the overwhelming enzyme

requirement is for cellulases: endo-cellulase, exo-cellulase, and glucosidase [164]. The specific activity of most cellulases is quite low [155, 165] and much effort has focused on increasing their activity levels. However, even with improved enzymes and improved methods of production, the amount of cellulase required to deconstruct the volumes of biomass necessary for 30% replacement of gasoline are in the millions of tons. It has been estimated that 36 billion gallons can require as much as 3.6 million metric tons of cellulase per year [166]. This is an unprecedented challenge in terms of the amount of enzymes and the extreme low cost that is required.

The bioprocessing challenge for ethanol is how to deliver these extremely large volumes to a saccharification facility at an unprecedented low cost. This represents potentially the single greatest application of industrial enzymes.

Currently, cellulases are produced by fungal and bacterial systems, and are a costly component of ethanol production [167]. Plants offer the potential for a production system that can meet the low cost and high volumes required. To do this however, the level of expression needs to be extremely high and the choice of tissue needs careful consideration. There have been many attempts to express cellulase in many types of plants and these have been reviewed elsewhere [37]. Protein and tissue stability, tissue fractionation, protein extraction and formulation, storage, as well as transportation add to this cost and must be considered.

To achieve targeted production cost targets for biomass enzymes the following considerations must be employed: (1) eliminate transportation cost by integrating enzyme processing into biomass conversion facility; (2) minimize fractionation/extraction cost of transgenic material; and (3) reduce the contribution of transgenic plant material to enzyme production cost by capturing plant biomass value through byproduct credits or cellulose. It has been suggested [166] that in order to keep the enzyme cost down, plant production systems that accumulate cellulase in the normally unused or low-value portion of the plants can be competitive when the other parts of the plant are harvested for their traditional use. The obvious example is when the leaves of crops are used to produce the cellulase and the grain is used for food, feed, or other industrial applications. Using the grain for industrial applications is less of a regulatory burden than if the grain is to be used for food or feed. However, according to the US Food and Drug Administration Statement of Policy (http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Biotechnology/ucm096095.htm) this is still an option, "If plants (or materials derived from plants) used to make nonfood chemicals are also intended to be used for food, producers should consult with FDA to determine whether the non-food chemical would be a food additive requiring an authorizing regulation prior to marketing for food use." These guidelines may change and may vary between countries; therefore, current regulatory practices must be consulted and followed in each case.

A case has been proposed for a fully integrated and synergistic system of ethanol production using maize. Maize grain today is the major source of ethanol production in the USA [168]. Using the stover to make cellulosic ethanol has been proposed as a convenient, economical, and sustainable way to make cellulosic ethanol alongside grain ethanol allowing for lower transportation costs and synergy in the ethanol facilities [170].

Taking this approach one step further, it has been suggested that the leaves themselves can be used to generate the required enzymes [155]. This has great potential if the enzymes can survive in the processing steps and can reach the target levels.

Another option has been proposed using the germ fraction of the grain [140]. In this case the enzymes could be made in the germ which is separated in the dry milling prior to using the endosperm for grain ethanol. The acreage required to grow crops to produce this amount of enzymes has been modeled previously considering the proximity limitations of the lignocellulose biomass to the ethanol facility to avoid large transportation cost. This study demonstrated that if cellulase levels were 0.1% of the dry weight of the seed (1% of germ dry weight), this was more than sufficient to keep the acreage of the cellulase crop less than the acreage needed to supply the lignocellulose biomass. Additional models suggest that when expression levels reach 4% of the dry weight of tissue (0.4% weight of the grain) it may be possible to add the germ tissue without any fractionation [166]. Direct delivery of plant tissue can be the system of choice by eliminating

extraction and purification costs. In addition to the cost of production, this approach allows the entire plant to better utilized without additional acreage or input for growing the plants. There is also no additional stress on the environment due to making or processing the enzymes and this has the potential to meet the current volumes of cellulase projected at a cost below current targets.

The main reason why this is not in use today is that cellulase has not enjoyed the levels of expression required for the models above. However, there is every reason to believe that expression levels will continue to improve in plants as it is a relatively young science compared to microbial production. In maize specifically it has been reported that levels as high as 16% of the total soluble protein were observed [41] and more recently levels of >1% of dry weight in the germ fraction has been achieved [169].

Regulation of Growth and Use of Transgenic Plants

Assuming that one can create plants having industrial enzymes with the characteristics and cost benefits that are desirable, there is still the need to grow these on a commercial scale. The process of growing transgenic plants is highly regulated including those grown for research purposes. However, for research purposes, cost is not a primary concern and the environmental impact is usually minimal due to the small acreage. However, this changes dramatically during scale-up for commercialization. Assuming a yield of 1–10 kg industrial enzyme per acre would require 100–1,000 acres for a relatively low volume of industrial enzymes but this can increase 10- to 1,000-fold for larger volume enzymes. Therefore, the potential for certain individual enzymes to be greater than 100,000 acres creates regulatory scenarios that are much more complex than those addressed in a research environment.

Regulatory systems are a social issue and vary in different countries but they all address human and animal safety as well as environmental implications. They must also address perhaps the most controversial issue, public acceptance. The technical aspects of regulatory concerns are discussed below with the understanding that every country will have its own interpretation and standards for what is acceptable.

Product Safety

The first concern for any product is the inherent safety of the active ingredient. Having a production system different from the native host does not usually change the inherent properties of the enzyme itself. Factors such as exposure to humans, dosage, toxicity, allergenicity, and whether or not the proteins are already a part of the food chain are considered. It is not the intent of this entry to review the regulatory process in detail but rather to point out the difference when using plants as the production vehicle as opposed to other sources. Therefore, the focus is not on the inherent safety of the enzyme but what safety factors arise when produced in plants and what additional challenges are presented with plant production.

For products that have already undergone regulatory approval, it is critical to show, utilizing empirical data, that there is equivalency with the plant-produced process. In many cases plants can produce functionally and chemically equivalent proteins to those made in the native hosts. However, there may be exceptions which in turn can lead to difference in protein structure or function. These differences may be subtle such as a small signal peptide intended to target the protein to the vacuole that is not cleaved and instead retained in the final protein sequence, or carbohydrate structures could be added where none existed before. These types of changes may have no impact on function and may be acceptable in commercial products.

In contrast to that above, some changes may result in proteins that have altered functions or altered safety profiles. As an example, many industrial enzymes are bacterial in origin and therefore are not normally glycosylated proteins. If these bacterial proteins happen to have a glycosylation site that a eukaryote can recognize then that can create a challenge. The possibility exists that the enzyme will be glycosylated when expressed in eukaryotes and potentially lead to a change in function. Fortunately there is little evidence to suggest that glycosylation itself will change enzyme function unless the carbohydrate is added to a key amino acid either in the binding site or catalytic site. An example of this is the bacterial enzyme β-glucuronidase (GUS). GUS protein loses activity due to secretion-specific N-glycosylation of a key amino acid in the protein [171]. Contrary to this

observation, other proteins can be glycosylated such as the bacterial protein organophosphate hydrolase with no significant effect on its biochemical activity [57].

Glycosylation patterns in plants can also be different from those observed in other eukaryotes. Plant glycoproteins contain the same mannose backbone structure found in animal glycoproteins but the additional sugars added to the backbone are usually less complex in plants than animals. Another difference is in β−1-4 linkage versus β−1-6 linkage and the appearance of xylose. In one case the animal protein trypsin was produced in plants and showed evidence of O-glycosylation whereas it is not detected from the native porcine or bovine source [56]. Despite these changes no functional difference in catalytic activity has been observed.

In addition to catalytic activity, there is also the concern that the enzyme may be altered in a way that affects its activity in the desired application. There is little evidence of this for industrial enzymes but there are examples for where carbohydrate structures on proteins can affect their pharmacological properties. In the case of certain pharmaceutical proteins, sialic acid is a terminal sugar on the glycoprotein and leads to a longer half-life in the blood [172]. Since plants do not add sialic acid this leads to an alteration in clearance time in the blood [173]. On the other hand, the plant carbohydrate sequence for antibodies is also critical in vivo but the altered plant sequence appears to work as well as the animal carbohydrate sequence [174]. This demonstrates the need to test the industrial enzymes in the desired application when the composition of matter is different than the native source.

Demonstrating functional equivalency is still not enough from a regulatory standpoint. The addition of carbohydrates has been implicated in a number of studies for allergenicity. This raises the theoretical question of whether plant glycosylation can lead to allergenicity. On the surface this seems highly unlikely since plant proteins are eaten routinely, and plant glycoproteins would seem inherently safe. Therefore, just adding a plant carbohydrate does not make the protein allergenic. On the other hand, there have been reports showing that the carbohydrate sequence of pollen glycoproteins [175] is responsible for an allergenic reaction. Therefore, while generalizing that plant carbohydrates are allergenic is misleading, it is important not only to check whether there is glycosylation but aslo to find out how this may differ from the native source. While there are no current examples where the addition of a plant-specific glycosylation to a transgenic protein has lead to an increase in allergenicity there is still a theoretical concern.

Safety of Host Proteins

While inherent activity is a functionality concern, the addition of extraneous host material is also a regulatory issue. Since most industrial enzymes cannot be purified because of cost restraints, the host material must also be shown to be safe in the final product. For this reason production in plants that already have a proven history of safe use is a great advantage. Certain crops that are known to produce toxins, allergens, antinutritionals, or carcinogens present additional difficulties as hosts. Crops already known to have GRAS (generally regarded as safe) status will be at an advantage because of inherent safety of the crop. In the best case a GRAS enzyme can be produced in a GRAS host greatly reducing the regulatory burden [176].

Environmental Safety

The environmental impact of protein production from a regulatory standpoint can be a concern based on the additional acreage. The consequence of additional inputs, displacement of food crops, and the lack of containment leading to inadvertent exposure to animals and humans must be addressed [177].

Dedicated cropland for the sole purpose of producing industrial enzymes will in most cases be insignificant compared to the acreage already under development for current uses, if produced in tandem with commodity crops. Since expression levels must be high to keep costs low for industrial products, this translates into only a very small percentage (∼1%) of the acreage for even large-volume products. The exception to this scenario is if the by products can be utilized for other, value-added purposes, such as for biomass or feed. In such cases, there is no additional impact either in acreage or inputs.

Of greater concern is the issue of containment and inadvertent exposure. Regulations to evaluate containment of transgenic plants are similar in concept to

those for other hosts, but specifics are very different. One of the areas historically concerning the production process of industrial enzymes is the occurrence of allergenic reactions developed by workers in the production facility. This has led the industry to safer forms of controlling small-particle exposure during microbial production. The concern for plant is that pollen from production fields can act as a carrier of the protein and lead to exposure similar to that observed for microbial production. For this reason, it is suggested that expression of the protein be specific to tissues other than pollen, thereby eliminating this concern.

Field production has the potential to affect wildlife where the crop is grown. Unlike many applied pesticides, transgenic plants are generally non-toxic to wildlife. This may reduce the environmental threat as opposed to many chemicals. The specific protein produced still may have some unwanted effects on wildlife and this aspect needs to be addressed, particularly for endangered species that may be present in the production field. Products that require larger acreage will create more concerns.

The final evaluation and perhaps the most controversial is the potential impact for the industrial enzyme for inadvertent exposure into the food chain. This may occur for several theoretical reasons, including mislabeling of seed, spills during transport, or pollen dissemination into food crops or wild relatives. Pollen dispersion has received the most attention as the other possibilities of inadvertent exposure most likely because the other potential sources are similar to that of other host production systems. To control the flow of pollen several different methods can be employed. These range from male sterility systems and physical isolation of the crops from compatible agricultural crops or weeds. In addition, systems have been proposed for growing transgenic crops within an industrial crop zone thereby further reducing the possibility of inadvertent mixing [140].

The underlying assumption for the production of transgenic proteins produced in plants is that they must be kept at all times isolated from food crops. This is essential for proteins that have the potential for detrimental effects on the population. However, many industrial proteins are used in food processing or derived from material already in the food chain. In these cases the transgenic proteins have the potential to be deregulated. After demonstrating that there is no danger to human safety the strictest of containment conditions may be dropped although some containment may still be desirable, either from a regulatory standpoint or from a commercial necessity.

Plants as Sustainable Sources of Industrial Enzymes

Plants have the potential to provide a sustainable source of industrial enzymes. Most plants can be transformed stably or used to express transiently heterologous proteins at levels that can be used for industrial production. The key sustainable feature of plants is that they are a renewable resource. They do not require intensive efforts for growth and maintenance of sterility, although some may require containment. Even so, the infrastructure and capital investment for growing plants is considerably less than that for fermentation of microbes. In addition, plant waste can be disposed of without intensive treatment, unlike effluents from fermenters. Energy resources for plant growth are also lower than those for maintenance of temperature and sterility of fermenters. In addition, formulations of large volumes of media for culture represent a large input of water, often a limiting resource. Cooling of large fermentation chambers is also energy intensive. These considerations are less severe with plants. Although plants do need regular and sustained watering, they can be grown in traditionally non-irrigated areas or in irrigated land where the input can be spread out using efficient drip hoses and watering procedures over a long period of time, alleviating the need for vast quantities of sterile water at short notice. The water does not need to be sterile, which reduces the energy burden and thereby the LCA.

The projection of 2–25% annual growth of enzyme requirement indicates a massive increase in enzyme production if supply is to keep pace with demand. Large volumes of enzymes imply that a number of fermenters have to be constructed to produce microbial- or cell-culture-based enzymes, or a shift of paradigms to a more efficient supply. Plants can provide the large volumes of enzymes needed with relatively low capital investment, and may represent the only really low-cost option for providing the immense requirements of industrial enzymes anticipated with projected

growth. Depending on the level of purification required, the enzymes can either be used without purification (such as for the production of biofuels) or undergo processing (such as for pharmaceuticals). This decreased processing obviously lowers environmental impact of the procedure. Tissue from plants that are not target for protein accumulation can be processed or disposed of such as to provide a financial buffer to the industrial enzyme production aspect.

The key to using plants for the production of industrial proteins is the increasing expression of transgenic proteins to levels commensurate with economic recovery of protein. A search of the Sigma Aldrich Chemical Company for transgenic-plant-derived products showed that proteins expressed in rice (avidin, lactoferrin, lysozyme), corn (avidin, trypsin), and tobacco (tissue factor proteinase inhibitor II, bovine aprotinin) are commercially marketed. Cell Sciences (www.cellsciences.com) produces over 25 cytokines and growth factors from barley endosperm, especially marketable as they are animal, bacterial, and viral-free. These are fine chemicals produced with high purity, but evidently are also commercially viable, providing a proof of potential. Increasing accumulation levels in plant tissue is an important issue for commercial success.

Storage of enzymes is also energy consuming when produced from microbial fermentation. The protein is usually lyophilized for storage; an energy-intensive process requiring freezing and desiccation simultaneously. Green tissues from plants have to be processed immediately, frozen or dried for protein stability. However, proteins expressed in seeds have been shown to maintain stability, even at room temperature, for at least 3 years without noticeable degradation [45]. This decreases the LCA and increases sustainability, as well as facilitating rapid response to spikes of increased demand.

Plant-based production also results in less waste. The unused portions of the plant body can be funneled into other uses, such as ethanol from biomass, and there is little waste from the recovery process compared to effluents from fermenters, lower waste disposal requirements, and lower net production of greenhouse gases, which is better for environmental sustainability and society.

Future Directions

Plants have historically contributed to industrial processes from dyes and tannins for fabric and leather to drugs for healthcare and pharmaceuticals. The first generation of plant-derived recombinant proteins is now commercially available and the prospects of more products is in the pipeline with many groups working on expressing high levels of laccases, cellulases, plantibodies, and pharmaceutically important proteins. In some cases, enzymes can be directly delivered in the plants, such as cellulolytic enzymes expressed in plants to improve their degradation for production of bioethanol. Alternatively, enzymes can be expressed at high levels and isolated for industrial processes. In order to sustain either process, plants should accumulate proteins in sufficient quantities. Protein targeting to improve expression levels is a topic of major interest and additional studies on inducible expression are being pursued to enhance utility.

Currently, there is a major push to find and utilize renewable energy more efficiently. Plant starch and sugars are major sources of bioethanol, but their production from otherwise discarded lignocellulosic material hold out great promise for fuel production, enabling the real possibility of national fuel self-sufficiency. Plants are a renewable resource, and lower the LCA of processes since energy input into their production is substantially lower than other production systems. In addition, most products of plants can find use elsewhere for feed, silage, or biomass for renewable fuel production. The benefits of large-scale increases with little effort, lower costs, and potential to offset costs with downstream use of waste solely accrue to plants, making this a technology worth investing in.

Bibliography

1. Brown T, Jones M, Powell W, Allaby R (2009) The complex origins of domesticated crops in the fertile crescent. Trends Ecol Evol 24:103–109
2. Hallauer A (2007) History, contribution, and future of quantitative genetics in plant breeding: lessons from maize. Crop Sci 47:S4–S19
3. Raskin I, Ribnicky D, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno D, Ripoll C, Yakoby N (2002) Plants and human health in the twenty-first century. Trends Biotechnol 20:522–531
4. Giddings G (2001) Transgenic plants as protein factories. Curr Opin Biotechnol 12:450–454

5. Giddings G, Allison G, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 18:1151–1155
6. Murphy D (2007) Improving containment strategies in biopharming. Plant Biotechnol J 5:555–569
7. Chapotin S, Wolt J (2007) Genetically modified crops for the bioeconomy: meeting public and regulatory expectations. Transgenic Res 16:675–688
8. Hansell A, Knorr-Held L, Best N, Schmid V, Aylin P (2003) Copd mortality trends 1950–1999 in England and Wales – did the 1956 clean air act make a detectable difference? Epidemiology 14:S55
9. Hazilla M, Kopp R (1990) Social cost of environmental quality regulations: a general equilibrium analysis. J Polit Econ 98:853–873
10. Owens J (1997) Life-cycle assessment: Constraints on moving from inventory to impact assessment. J Ind Ecol 1:37–49
11. Harding K, Dennis J, Von Blottnitz H, Harrison S (2008) A life-cycle comparison between inorganic and biological catalysis for the production of biodiesel. J Cleaner Prod 16:1368–1378
12. van Beilen JB, Li Z (2002) Enzyme technology: an overview. Curr Opin Biotechnol 13:338–344
13. Kirk O, Borchert T, Fuglsang C (2002) Industrial enzyme applications. Curr Opin Biotechnol 13:345–351
14. Vajo Z, Fawcett J, Duckworth W (2001) Recombinant DNA technology in the treatment of diabetes: insulin analogs. Endocr Rev 22:706–717
15. Swartz J (2001) Advances in *Escherichia coli* production of therapeutic proteins. Curr Opin Biotechnol 12:195–201
16. Haki G, Rakshit S (2003) Developments in industrially important thermostable enzymes: a review. Bioresour Technol 89:17–34
17. Hatti-Kaul R, Törnvall U, Gustafsson L, Börjesson P (2007) Industrial biotechnology for the production of bio-based chemicals – a cradle-to-grave perspective. Trends Biotechnol 25:119–124
18. Seelig B, Szostak J (2007) Selection and evolution of enzymes from a partially randomized non-catalytic scaffold. Nature 448:828–831
19. Jaeger K, Eggert T (2004) Enantioselective biocatalysis optimized by directed evolution. Curr Opin Biotechnol 15:305–313
20. Bornscheuer U, Pohl M (2001) Improved biocatalysts by directed evolution and rational protein design. Curr Opin Chem Biol 5:137–143
21. Cherry J, Fidantsef A (2003) Directed evolution of industrial enzymes: an update. Curr Opin Biotechnol 14:438–443
22. Jaeger K, Eggert T, Eipper A, Reetz M (2001) Directed evolution and the creation of enantioselective biocatalysts. Appl Microbiol Biotechnol 55:519–530
23. Louwrier A (1998) Industrial products-the return to carbohydrate-based industries. Biotechnol Appl Biochem 27:1–8
24. Zhao J, Li X, Qu Y (2006) Application of enzymes in producing bleached pulp from wheat straw. Bioresour Technol 97:1470–1476
25. Dyer T, Ragauskas A (2002) Developments in bleaching technology focus on reducing capital, operating costs. In: IPST Technical Paper Series Number 929 (Paper, P. a., Ed.)
26. Headon D, Walsh G (1994) The industrial production of enzymes. Biotechnol Adv 12:635–646
27. Chakrabarty A (1981) Microorganisms having multiple compatible degradative energy-generating plasmids and preparation thereof. US Patent No 4,259,444, Google Patents (Office, U. P., Ed.), General Electric Company, United States
28. Chakrabarty A, Kellogg S (1985) Bacteria capable of dissimilation of environmentally persistent chemical compounds. US Patent No 4,535,061. University of Illinois Foundation, United States
29. Sahdev S, Khattar S, Saini K (2008) Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. Mol Cell Biochem 307:249–264
30. Twyman R, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10:185–218
31. Dominguez A, Ferminan E, Sanchez M, Gonzalez F, Perez-Campo F, Garcia S, Herrero A, San Vicente A, Cabello J, Prado M (1998) Non-conventional yeasts as hosts for heterologous protein production. Int Microbiol 1:131–142
32. McAloon A, Taylor F, Yee W, Ibsen K, Wooley R (2000) Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks. National Renewable Energy Laboratory Report, NREL, Fort Collins, CO
33. Houdebine L (2009) Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infect Dis 32:107–121
34. Kretzmer G (2002) Industrial processes with animal cells. Appl Microbiol Biotechnol 59:135–142
35. Prusiner S, Scott M, DeArmond S, Cohen F (1998) Prion protein biology. Cell 93:337–348
36. Ragauskas A, Williams C, Davison B, Britovsek G, Cairney J, Eckert C, Frederick W Jr, Hallett J, Leak D, Liotta C (2006) The path forward for biofuels and biomaterials. Science 311:484
37. Sainz M (2009) Commercial cellulosic ethanol: the role of plant-expressed enzymes. In Vitro Cell Dev Biol Plant 45:314–329
38. Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578
39. Howard JA, Hood E (2005) Bioindustrial and biopharmaceutical products produced in plants. Adv Agron 85:91–124
40. Borisjuk N, Borisjuk L, Logendra S, Petersen F, Gleba Y, Raskin I (1999) Production of recombinant proteins in plant root exudates. Nat Biotechnol 17:466–469
41. Hood E, Love R, Lane J, Bray J, Clough R, Pappu K, Drees C, Hood K, Yoon S, Ahmad A (2007) Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. Plant Biotechnol J 5:709–719
42. Komarnytsky S, Borisjuk N, Borisjuk L, Alam M, Raskin I (2000) Production of recombinant proteins in tobacco guttation fluid. Plant Physiol 124:927–934

43. Evangelista R, Kusnadi A, Howard J, Nikolov Z (1998) Process and Economic evaluation of the extraction and purification of recombinant β-glucuronidase from transgenic corn. Biotechnol Prog 14:607–614
44. Doran P (2000) Foreign protein production in plant tissue cultures. Curr Opin Biotechnol 11:199–204
45. Fischer R, Stoger E, Schillberg S, Christou P, Twyman R (2004) Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 7:152–158
46. Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20:581–586
47. Lee D, Natesan E (2006) Evaluating genetic containment strategies for transgenic plants. Trends Biotechnol 24:109–114
48. Lin C, Fang J, Xu X, Zhao T, Cheng J, Tu J, Ye G, Shen Z (2008) A built-in strategy for containment of transgenic plants: creation of selectively terminable transgenic rice. PLoS ONE 3:e1818
49. Lienard D, Sourrouille C, Gomord V, Faye L (2007) Pharming and transgenic plants. Biotechnol Annu Rev 13:115–147
50. Daniell H, Streatfield S, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219–226
51. Stoger E, Sack M, Fischer R, Christou P (2002) Plantibodies: applications, advantages and bottlenecks. Curr Opin Biotechnol 13:161–166
52. Orzáez D, Granell A, Blázquez M (2009) Manufacturing antibodies in the plant cell. Biotechnol J 4:1712–1724
53. Streatfield S, Howard J (2003) Plant production systems for vaccines. Expert Rev Vaccin 2:763–775
54. Lamphear B, Jilka J, Kesl L, Welter M, Howard J, Streatfield S (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine 22:2420–2424
55. Borkhardt B, Harholt J, Ulvskov P, Ahring B, Jørgensen B, Brinch-Pedersen H (2010) Autohydrolysis of plant xylans by apoplastic expression of thermophilic bacterial endo-xylanases. Plant Biotechnol J 8:363–374
56. Woodard S, Mayor J, Bailey M, Barker D, Love R, Lane J, Delaney D, McComas-Wagner J, Mallubhotla H, Hood E (2003) Maize (*Zea mays*)-derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. Biotechnol Appl Biochem 38:123–130
57. Pinkerton T, Howard J, Wild J (2008) Genetically engineered resistance to organophosphate herbicides provides a new scoreable and selectable marker system for transgenic plants. Mol Breed 21:27–36
58. Bailey M, Woodard S, Callaway E, Beifuss K, Magallanes-Lundback M, Lane J, Horn M, Mallubhotla H, Delaney D, Ward M (2004) Improved recovery of active recombinant laccase from maize seed. Appl Microbiol Biotechnol 63:390–397
59. Clough RC, Pappu K, Thompson K, Beifuss K, Lane J, Delaney DE, Harkey R, Drees C, Howard JA, Hood EE (2006) Manganese peroxidase from the white-rot fungus *Phanerochaete chrysosporium* is enzymatically active and accumulates to high levels in transgenic maize seed. Plant Biotechnol J 4:53–62
60. Pen J, Molendijk L, Quax W, Sijmons P, van Ooyen A, van den Elzen P, Rietveld K, Hoekema A (1992) Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction. Nat Biotechnol 10:292–296
61. Pen J, Hoekema A, Sijmons P, Van Ooyen A, Rietveld K, Verwoerd T, Quax W (1991) Production of enzymes in seeds and their use. Gist-Brocades N.V. (Wateringseweg 1 P.O. Box 1, MA Delft, NL-2600, NL) Mogen International N.V. (Einsteinweg 97, CB Leiden, NL-2333, NL) European Patent
62. Czihal A, Conrad B, Buchner P, Brevis R, Farouk A, Manteuffel R, Adler K, Wobus U, Hofemeister J, Bäumlein H (1999) Gene farming in plants: expression of a heatstable *Bacillus* amylase in transgenic legume seeds. J Plant Physiol 155:183–189
63. Kumagai M, Donson J, Della-Cioppa G, Grill L (2000) Rapid, high-level expression of glycosylated rice-amylase in transfected plants by an RNA viral vector. Gene 245:169–174
64. Austin S, Bingham E, Mathews D, Shahan M, Will J, Burgess R (1995) Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. Euphytica 85:381–393
65. Chiang C, Yeh F, Huang L, Tseng T, Chung M, Wang C, Lur H, Shaw J, Yu S (2005) Expression of a bi-functional and thermostable amylopullulanase in transgenic rice seeds leads to autohydrolysis and altered composition of starch. Mol Breed 15:125–143
66. Zhong G, Peterson D, Delaney D, Bailey M, Witcher D, Register Iii J, Bond D, Li C, Marshall L, Kulisek E (1999) Commercial production of aprotinin in transgenic maize seeds. Mol Breed 5:345–356
67. Pogue GP, Vojdani F, Palmer KE, Hiatt E, Hume S, Phelps J, Long L, Bohorova N, Kim D, Pauly M, Velasco J, Whaley K, Zeitlin L, Garger SJ, White E, Bai Y, Haydon H, Bratcher B (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. Plant Biotechnol J 8:1–17
68. Capell T, Bassie L, Christou P (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proc Natl Acad Sci USA 101:9909–9914
69. Hood E, Witcher D, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306
70. Samac D, Tesfaye M, Dornbusch M, Saruul P, Temple S (2004) A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*). Transgenic Res 13:349–361
71. Mei C, Park S, Sabzikar R, Ransom C, Qi C, Sticklen M (2009) Green tissue-specific production of a microbial endo-cellulase in maize *Zea mays* L. endoplasmic-reticulum and mitochondria converts cellulose into fermentable sugars. J Chem Technol Biotechnol 84:689–695

72. Sun Y, Cheng J, Himmel M, Skory C, Adney W, Thomas S, Tisserat B, Nishimura Y, Yamamoto Y (2007) Expression and characterization of *Acidothermus cellulolyticus* E1 endoglucanase in transgenic duckweed *Lemna minor* 8627. Bioresour Technol 98:2866–2872
73. Dai Z, Hooker B, Quesenberry R, Thomas S (2005) Optimization of *Acidothermus cellulolyticus* endoglucanase (E1) production in transgenic tobacco plants by transcriptional, post-transcription and post-translational modification. Transgenic Res 14:627–643
74. Teymouri F, Alizadeh H, Laureano-Pérez L, Dale B, Sticklen M (2004) Effects of ammonia fiber explosion treatment on activity of endoglucanase from Acidothermus cellulolyticus in transgenic plant. Appl Biochem Biotechnol 116:1183–1191
75. Dai Z, Hooker B, Anderson D, Thomas S (2000) Expression of *Acidothermus cellulolyticus* endoglucanase E1 in transgenic tobacco: biochemical characteristics and physiological effects. Transgenic Res 9:43–54
76. Dai Z, Hooker B, Quesenberry R, Gao J (1999) Expression of *Trichoderma reesei* exo-cellobiohydrolase I in transgenic tobacco leaves and calli. Appl Biochem Biotechnol 79:689–699
77. Jensen L, Olsen O, Kops O, Wolf N, Thomsen K, Von Wettstein D (1996) Transgenic barley expressing a protein-engineered, thermostable (1,1,4)-beta-glucanase during germination. Proc Natl Acad Sci USA 93:3487–3491
78. Horvath H, Jensen L, Wong O, Kohl E, Ullrich S, Cochran J, Kannangara C, Von Wettstein D (2001) Stability of transgene expression, field performance and recombination breeding of transformed barley lines. Theor Appl Genet 102:1–11
79. Nuutila A, Ritala A, Skadsen R, Mannonen L, Kauppinen V (1999) Expression of fungal thermotolerant endo-1,4-beta-glucanase in transgenic barley seeds during germination. Plant Mol Biol 41:777–783
80. Ziegelhoffer T, Raasch J, Austin-Phillips S (2001) Dramatic effects of truncation and sub-cellular targeting on the accumulation of recombinant microbial cellulase in tobacco. Mol Breed 8:147–158
81. Dai Z, Hooker B, Anderson D, Thomas S (2000) Improved plant-based production of E1 endoglucanase using potato: expression optimization and tissue targeting. Mol Breed 6:277–285
82. Jin R, Richter S, Zhong R, Lamppa G (2003) Expression and import of an active cellulase from a thermophilic bacterium into the chloroplast both in vitro and in vivo. Plant Mol Biol 51:493–507
83. Kawazu T, Ohta T, Ito K, Shibata M, Kimura T, Sakka K, Ohmiya K (1996) Expression of a *Ruminococcus albus* cellulase gene in tobacco suspension cells. J Ferment Bioeng 82:205–209
84. Xue G, Patel M, Johnson J, Smyth D, Vickers C (2003) Selectable marker-free transgenic barley producing a high level of cellulase (1, 4-ß-glucanase) in developing grains. Plant Cell Rep 21:1088–1094
85. Biswas G, Ransom C, Sticklen M (2006) Expression of biologically active *Acidothermus cellulolyticus* endoglucanase in transgenic maize plants. Plant Sci 171:617–623
86. Ransom C, Balan V, Biswas G, Dale B, Crockett E, Sticklen M (2007) Heterologous *Acidothermus cellulolyticus* 1, 4-beta-endoglucanase E1 produced within the corn biomass converts corn stover into glucose. Appl Biochem Biotechnol 137:207–219
87. Oraby H, Venkatesh B, Dale B, Ahmad R, Ransom C, Oehmke J, Sticklen M (2007) Enhanced conversion of plant biomass into glucose using transgenic rice-produced endoglucanase for cellulosic ethanol. Transgenic Res 16:739–749
88. Kawazu T, Sun J, Shibata M, Kimura T, Sakka K, Ohmiya K (1999) Expression of a bacterial endoglucanase gene in tobacco increases digestibility of its cell wall fibers. J Biosci Bioeng 88:421–425
89. Ziegler M, Thomas S, Danna K (2000) Accumulation of a thermostable endo-1, 4- -D-glucanase in the apoplast of *Arabidopsis thaliana* leaves. Mol Breed 6:37–46
90. Ziegelhoffer T, Will J, Austin-Phillips S (1999) Expression of bacterial cellulase genes in transgenic alfalfa (*Medicago sativa* L.), potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.). Mol Breed 5:309–318
91. Yu L, Gray B, Rutzke C, Walker L, Wilson D, Hanson M (2007) Expression of thermostable microbial cellulases in the chloroplasts of nicotine-free tobacco. J Biotechnol 131:362–369
92. Kim S, Lee D, Choi I, Ahn S, Kim Y, Bae H (2010) *Arabidopsis thaliana* Rubisco small subunit transit peptide increases the accumulation of *Thermotoga maritima* endoglucanase Cel5A in chloroplasts of transgenic tobacco plants. Transgenic Res 19(3):489–497
93. Rooijen G, Glenn K, Shen, Y, Boothe J (2008) Commercial production of chymosin in plants, United States Patent Application 20080184394
94. Ralph J, Akiyama T, Kim H, Lu F, Schatz P, Marita J, Ralph S, Reddy M, Chen F, Dixon R (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. J Biol Chem 281:8843–8853
95. Montalvo-Rodriguez R, Haseltine C, Huess-LaRossa K, Clemente T, Soto J, Staswick P, Blum P (2000) Autohydrolysis of plant polysaccharides using transgenic hyperthermophilic enzymes. Biotechnol Bioeng 70:151–159
96. Meyer F, Smidansky E, Beecher B, Greene T, Giroux M (2004) The maize Sh2r6hs ADP-glucose pyrophosphorylase (AGP) large subunit confers enhanced AGP properties in transgenic wheat (*Triticum aestivum*). Plant Sci 167:899–911
97. Smidansky E, Clancy M, Meyer F, Lanning S, Blake N, Talbert L, Giroux M (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc Natl Acad Sci U S A 99:1724–1729
98. Reggi S, Marchetti S, Patti T, Amicis F, Cariati R, Bembi B, Fogher C (2005) Recombinant human acid β-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. Plant Mol Biol 57:101–113
99. Witcher D, Hood E, Peterson D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh R (1998) Commercial production of β-glucuronidase (GUS): a model system for the production of proteins in plants. Mol Breed 4:301–312

100. Chan M, Chao Y, Yu S (1994) Novel gene expression system for plant cells based on induction of alpha-amylase promoter by carbohydrate starvation. J Biol Chem 269: 17635–17641
101. de Wilde C, Uzan E, Zhou Z, Kruus K, Andberg M, Buchert J, Record E, Asther M, Lomascolo A (2008) Transgenic rice as a novel production system for *Melanocarpus* and *Pycnoporus* laccases. Transgenic Res 17:515–527
102. Gruber V, Berna P, Arnaud T, Bournat P, Clément C, Mison D, Olagnier B, Philippe L, Theisen M, Baudino S (2001) Large-scale production of a therapeutic protein in transgenic tobacco plants: effect of subcellular targeting on quality of a recombinant dog gastric lipase. Mol Breed 7:329–340
103. Zhong Q, Gu Z, Glatz C (2006) Extraction of recombinant dog gastric lipase from transgenic corn seed. J Agric Food Chem 54:8086–8092
104. Mokrzycki-Issartel N, Bouchon B, Farrer S, Berland P, Laparra H, Madelmont J, Theisen M (2003) A transient tobacco expression system coupled to MALDI-TOF-MS allows validation of the impact of differential targeting on structure and activity of a recombinant therapeutic glycoprotein produced in plants. FEBS Lett 552:170–176
105. Lagrimini L, Bradford S, Rothstein S (1990) Peroxidase-induced wilting in transgenic tobacco plants. Plant Cell 2:7–18, Online
106. Chen R, Xue G, Chen P, Yao B, Yang W, Ma Q, Fan Y, Zhao Z, Tarczynski M, Shi J (2008) Transgenic maize plants expressing a fungal phytase gene. Transgenic Res 17:633–643
107. Hamada A, Yamaguchi K, Harada M, Horiguchi K, Takahashi T, Honda H (2006) Recombinant, rice-produced yeast phytase shows the ability to hydrolyze phytate derived from seed-based feed, and extreme stability during ensilage treatment. Biosci Biotechnol Biochem 70:1524–1527
108. Brinch-Pedersen H, Hatzack F, Stoger E, Arcalis E, Pontopidan K, Holm P (2006) Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): deposition pattern, thermostability, and phytate hydrolysis. J Agric Food Chem 54:4624–4632
109. Zimmermann P, Zardi G, Lehmann M, Zeder C, Amrhein N, Frossard E, Bucher M (2003) Engineering the root-soil interface via targeted expression of a synthetic phytase gene in trichoblasts. Plant Biotechnol J 1:353–360
110. George T, Simpson R, Hadobas P, Richardson A (2005) Expression of a fungal phytase gene in *Nicotiana tabacum* improves phosphorus nutrition of plants grown in amended soils. Plant Biotechnol J 3:129–140
111. Brinch-Pedersen H, Olesen A, Rasmussen S, Holm P (2000) Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. Mol Breed 6:195–206
112. Lucca P, Hurrell R, Potrykus I (2001) Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theor Appl Genet 102:392–397
113. Xiao K, Harrison M, Wang Z (2005) Transgenic expression of a novel M. truncatula phytase gene results in improved acquisition of organic phosphorus by Arabidopsis. Planta 222:27–36
114. Pen J, Verwoerd T, van Paridon P, Beudeker R, van den Elzen P, Geerse K, van der Klis J, Versteegh H, van Ooyen A, Hoekema A (1993) Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. Nat Biotechnol 11:811–814
115. Hegeman C, Grabau E (2001) A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. Plant Physiol 126:1598–1608
116. Verwoerd T, Van Paridon P, Van Ooyen A, Van Lent J, Hoekema A, Pen J (1995) Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. Plant Physiol 109:1199–1205
117. Ponstein A, Bade J, Verwoerd T, Molendijk L, Storms J, Beudeker R, Pen J (2002) Stable expression of phytase (phyA) in canola (*Brassica napus*) seeds: towards a commercial product. Mol Breed 10:31–44
118. Li J, Hegeman C, Hanlon R, Lacy G, Denbow D, Grabau E (1997) Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol 114:1103–1111
119. Hong C, Cheng K, Tseng T, Wang C, Liu L, Yu S (2004) Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. Transgenic Res 13:29–39
120. Austin-Phillips S, Koegel R, Straub R, Cook M (1999) Animal feed compositions containing phytase derived from transgenic alfalfa and methods of use thereof. US Patent No 5,900,525, Wisconsin Alumni Research Foundation, Madison, Wisconsin, USA
121. Claparols M, Bassie L, Miro B, Del Duca S, Rodriguez-Montesinos J, Christou P, Serafini-Fracassini D, Capell T (2004) Transgenic rice as a vehicle for the production of the industrial enzyme transglutaminase. Transgenic Res 13:195–199
122. Yang P, Wang Y, Bai Y, Meng K, Luo H, Yuan T, Fan Y, Yao B (2007) Expression of xylanase with high specific activity from *Streptomyces olivaceoviridis* A1 in transgenic potato plants (*Solanum tuberosum* L.). Biotechnol Lett 29:659–667
123. Kimura T, Mizutani T, Tanaka T, Koyama T, Sakka K, Ohmiya K (2003) Molecular breeding of transgenic rice expressing a xylanase domain of the xynA gene from *Clostridium thermocellum*. Appl Microbiol Biotechnol 62:374–379
124. Liu J, Selinger L, Cheng K, Beauchemin K, Moloney M (1997) Plant seed oil-bodies as an immobilization matrix for a recombinant xylanase from the rumen fungus *Neocallimastix patriciarum*. Mol Breed 3:463–470
125. Patel M, Johnson J, Brettell R, Jacobsen J, Xue G (2000) Transgenic barley expressing a fungal xylanase gene in the endosperm of the developing grains. Mol Breed 6:113–124
126. Hyunjong B, Lee D, Hwang I (2006) Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells. J Exp Bot 57:161–169

127. Bae H, Kim H, Kim Y (2008) Production of a recombinant xylanase in plants and its potential for pulp biobleaching applications. Bioresour Technol 99:3513–3519
128. Herbers K, Wilke I, Sonnewald U (1995) A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco has no detrimental effects and is easily purified. Biotechnology 13:63–66
129. Herbers K, Flint H, Sonnewald U (1996) Apoplastic expression of the xylanase and (1–3, 1–4) glucanase domains of the xyn D gene from *Ruminococcus flavefaciens* leads to functional polypeptides in transgenic tobacco plants. Mol Breed 2:81–87
130. Estevez J, Cantero A, Reindl A, Reichler S, Leon P (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem 276:22901–22909
131. Vain P (2007) Thirty years of plant transformation technology development. Plant Biotechnol J 5:221–229
132. Tzfira T, Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol 17:147–154
133. Daniell H, Cohill P, Kumar S, Dufourmantel N, Dubald M (2007) Chloroplast genetic engineering. In: Daniell H, Chase C (eds) Molecular biology and biotechnology of plant organelles. Springer, Dordrecht
134. Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 23:718–723
135. Sainsbury F, Lomonossoff G (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol 148:1212–1218
136. Ueki S, Lacroix B, Krichevsky A, Lazarowitz S, Citovsky V (2008) Functional transient genetic transformation of *Arabidopsis* leaves by biolistic bombardment. Nat Protoc 4:71–77
137. Watson S (1988) Corn marketing, processing, and utilization. US 9,011,308 (NAL/USDA, A. U., Ed.)
138. Hood E, Kusnadi A, Nikolov Z, Howard J (1999) Molecular farming of industrial proteins from transgenic maize. In: Sahidi F, Kolodziejczyk P, Whitaker J, Munguia A, Fuller G (eds) Chemicals via higher plant bioengineering. Kluwer Academic/Plenum Publishers, New York, pp 127–148
139. Ramessar K, Sabalza M, Capell T, Christou P (2008) Maize plants: an ideal production platform for effective and safe molecular pharming. Plant Sci 174:409–419
140. Howard J, Hood E (2007) Methods for growing nonfood products in transgenic plants. Crop Sci 47:1255
141. Gepts P (2002) A comparison between crop domestication, classical plant breeding, and genetic engineering. Crop Sci 42:1780–1790
142. Gur A, Zamir D (2004) Unused natural variation can lift yield barriers in plant breeding. PLoS Biol 2:1610–1615
143. Dale P (1992) Spread of engineered genes to wild relatives. Plant Physiol 100:13
144. Delaney B, Astwood J, Cunny H, Conn R, Herouet-Guicheney C, MacIntosh S, Meyer L, Privalle L, Gao Y, Mattsson J (2008) Evaluation of protein safety in the context of agricultural biotechnology. Food Chem Toxicol 46:S71–S97
145. Nikolov Z, Hammes D (2002) Production of recombinant proteins from transgenic crops. In: Hood EE, Howard JA (eds) Plants as factories for protein production. Kluwer Academic Publishers, Dordrecht, pp 159–174
146. Horn M, Woodard S, Howard J (2004) Plant molecular farming: systems and products. Plant Cell Rep 22:711–720
147. Hellwig S, Drossard J, Twyman R, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422
148. Lam E, Benfey P, Gilmartin P, Fang R, Chua N (1989) Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. Proc Natl Acad Sci U S A 86:7890–7894
149. Cai M, Wei J, Li X, Xu C, Wang S (2007) A rice promoter containing both novel positive and negative cis-elements for regulation of green tissue-specific gene expression in transgenic plants. Plant Biotechnol J 5:664–674
150. Belanger F, Kriz A (1991) Molecular basis for allelic polymorphism of the maize Globulin-1 gene. Genetics 129:863–872
151. Jones H, Sparks C (2009) Promoter sequences for defining transgene expression. In: Jones H, Shewry P (eds) Transgenic wheat, barley and oats: production and characterization protocols. Humana, Totowa
152. Streatfield S (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15
153. Vance V, Vaucheret H (2001) RNA silencing in plants – defense and counterdefense. Science 292:2277–2280
154. Baulcombe D (2003) Overcoming and exploiting RNA silencing. In: Vasil IK (ed) 10th IAPTC&B congress. Kluwer Academic Publishers, Orlando, pp 49–58
155. Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 9:433–443
156. Medrano G, Reidy M, Liu J, Ayala J, Dolan M, Cramer C (2009) Rapid system for evaluating bioproduction capacity of complex pharmaceutical proteins in plants. Meth Mol Biol 483:51–67
157. Van Rooijen G, Moloney M (1995) Structural requirements of oleosin domains for subcellular targeting to the oil body. Plant Physiol 109:1353–1361
158. Rigano M, Alvarez M, Pinkhasov J, Jin Y, Sala F, Arntzen C, Walmsley A (2004) Production of a fusion protein consisting of the enterotoxigenic *Escherichia coli* heat-labile toxin B subunit and a tuberculosis antigen in *Arabidopsis thaliana*. Plant Cell Rep 22:502–508
159. Garbarino J, Oosumi T, Belknap W (1995) Isolation of a polyubiquitin promoter and its expression in transgenic potato plants. Plant Physiol 109:1371–1378
160. Doran P (2006) Foreign protein degradation and instability in plants and plant tissue cultures. Trends Biotechnol 24:426–432

161. Kim K, Kwon S, Lee H, Hur Y, Bang J, Kwak S (2003) A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. Plant Mol Biol 51:831–838
162. Cramer C, Boothe J, Oishi K (1999) Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr Top Microbiol Immunol 240:95–118
163. Moloney M (2003) Oleosin partitioning technology for production of recombinant proteins in oil seeds. In: Vinci V, Parekh S (eds) Handbook of industrial cell culture: mammalian, microbial, and plant cells. Humana Press, Totowa
164. Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. Adv Biochem Eng Biotechnol 108:95–120
165. Jorgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels, Bioprod Biorefin 1:119–134, be
166. Howard JA, Nikolov Z, Hood EE (2011) Enzyme production systems for biomass conversion. In: Hood EE, Nelson P, Powell R (eds) The Plant Biomass Conversion. John Wiley & Sons Inc.
167. Hamelinck C, Hooijdonk G, Faaij A (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long-term. Biomass Bioenergy 28:384–410
168. Solomon B, Barnes J, Halvorsen K (2007) Grain and cellulosic ethanol: history, economics, and energy policy. Biomass Bioenergy 31:416–425
169. Howard JA, Hood EE (unpublished)
170. Sheehan J, Aden A, Paustian K, Killian K, Brenner J, Walsh M, Nelson R (2003) Energy and environmental aspects of using corn stover for fuel ethanol. J Ind Ecol 7:117–146
171. Groover AT, Fontana JR, Arroyo JM, Yordan C, McCombie WR, Martienssen RA (2003) Secretion trap tagging of secreted and membrane-spanning proteins using Arabidopsis gene traps. Plant Physiol 132:698–708
172. Cohen O, Kronman C, Velan B, Shafferman A (2004) Amino acid domains control the circulatory residence time of primate acetylcholinesterases in rhesus macaques (*Macaca mulatta*). Biochem J 378:117–128
173. Huang J, Sutliff T, Wu L, Nandi S, Benge K, Terashima M, Ralston A, Drohan W, Huang N, Rodriguez R (2008) Expression and purification of functional human α1-antitrypsin from cultured plant cells. Biotechnol Prog 17:126–133
174. Ma J, Hein M (1995) Plant antibodies for immunotherapy. Plant Physiol 109:341–346
175. Garcia-Casado G, Sanchez-Monge R, Chrispeels M, Armentia A, Salcedo G, Gomez L (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. Glycobiology 6:471–477
176. Cockburn A (2002) Assuring the safety of genetically modified (GM) foods: the importance of an holistic, integrative approach. J Biotechnol 98:79–106
177. Peterson R, Arntzen C (2004) On risk and plant-based biopharmaceuticals. Trends Biotechnol 22:64–66

Plant Molecular Pharming, Pharmaceuticals for Human Health

Andreas Schiermeyer, Stefan Schillberg
Department of Plant Biotechnology, Fraunhofer IME, Aachen, Germany

Article Outline

Glossary

Agrobacterium tumefaciens Gram negative phytopathogenic soil bacterium belonging to the family Rhizobiaceae. *A. tumefaciens* naturally infects a variety of dicotyledonous plant species and induces the formation of tumors (galls) by transferring genes located within the T-DNA.

Biopharmaceuticals Drugs, produced using modern biotechnological methods such as recombinant DNA technology, comprising proteins and/or nucleic acids for therapeutic or in vivo diagnostic purposes.

Epigenetic effects Changes caused in gene expression patterns that are not due to changes in the nucleotide sequence of the DNA but due to nucleotide modifications by methylation or RNA-directed mechanisms.

Glycosylation The co- or posttranslational addition of carbohydrate moieties to polypeptides. The carbohydrates may be either N-linked (at asparagine or arginine side chains) or O-linked (at serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side chains).

Immunoglobulin (Ig) Major component of the adaptive immune system with specific antibody activity. Immunoglobulins are produced by lymphocytes and consist of four polypeptide chains: two identical heavy and two identical light chains. Immunoglobulin G (IgG) is the principal immunoglobulin of the plasma with a molecular weight of 150 kDa. Immunoglobulin A (IgA) is a dimeric 400 kDa molecule secreted by mucosal surfaces. Beside the heavy and the light chains an IgA molecule contains a joining chain and the secretory component. Immunoglobulin M (IgM) is produced early during the immune response. Secreted IgM molecules have a star-shaped pentameric structure.

Molecular farming (also pharming) The production of pharmaceutical or technical proteins in plants or animals.

Monoclonal antibody An immunoglobulin secreted by a single clone of antibody producing cells.

Plastid Plant organelle bound by a double membrane containing its own circular genome. Several types of plastids are known that originate from a common precursor the proplastid. The most prominent form is the chloroplast found in the green tissues.

Posttranslational modification Any modification that occurs once a polypeptide has been synthesized, for example, proteolytic processing, glycosylation, methylation, phosphorylation, and prenylation.

Suspension culture Technique for the cultivation of plant cells or tissues in liquid culture medium under aseptic conditions using shake flasks or fermentation vessels.

T-DNA Transfer DNA. Natural T-DNA is located on large tumor inducing (Ti) or hairy root inducing (Ri) plasmids, although for gene transfer to plants it has been moved onto a smaller, more convenient vector. The T-DNA is transferred to the plant cell with the help of a range of virulence factors, and once in the nucleus it may be stably integrated into the plant genome.

Transformation Transfer of genetic information into a cell by biological (*A. tumefaciens*) or physical means (e.g., gene gun).

Transgene A segment of DNA that is introduced into the genome of a host cell by transformation, and

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

which integrates into the genome so that it is inherited like any other gene.

Transient expression The temporary expression of a transgene within a host cell without stable integration into the host genome.

Vaccine Preparation of immunogenic material that stimulates active immunity in the recipient without causing disease. Vaccines can be based on killed or attenuated microorganisms or isolated components of the disease causing agent (subunit vaccines).

Viral vector Genetic elements derived from viral genomes for the transient expression of transgenes in a host cell. Viral vectors have the ability to replicate autonomously in the host cell and might be able to infect distant cells (depending on the degree of engineering).

Zinc-finger nucleases Chimeric proteins consisting of a zinc-finger domain conferring sequence specific DNA binding and a nuclease domain for the introduction of a double-strand break at the target site.

Definition of the Subject and Its Importance

The demand for therapeutic proteins has increased in recent years and modern biotechnological methods have, until recently, ensured the production of safe and effective biopharmaceuticals to meet this demand. Various production platforms are currently used in the pharmaceutical industry, most based on the fermentation of engineered pro- and eukaryotic microorganisms, insect cells, or mammalian cells. However, the growth of the market for biopharmaceuticals is predicted to outpace production capacity using these platforms in the next decade, so alternatives are necessary. Intact plants and plant cell cultures are suitable production systems for a wide range of therapeutic proteins and could help to fulfill the need for increased production capacity. The production of pharmaceutical proteins in plants began with a monoclonal antibody expressed in transgenic tobacco plants more than 20 years ago. Since then many different plant species have been genetically engineered to produce valuable pharmaceutical proteins using a variety of transformation methods. Major progress has been achieved in transformation and expression technology, the downstream processing of transgenic plant material and the adaptation of regulatory procedures to encompass the new production platforms, allowing the first plant-made pharmaceuticals to begin clinical trials.

Introduction

Plant cells synthesize a vast array of secondary metabolites, many of which are already used as pharmaceuticals. Recombinant DNA technology combined with techniques for plant transformation and the regeneration of transgenic plants have allowed the pharmaceutical exploitation of plants to be extended to include the production of biopharmaceuticals such as subunit vaccines, antibodies, growth factors, cytokines, enzymes, and blood factors. In many cases, these products need to be purified from plant material and formulated in the same way as conventional biopharmaceuticals. However, many plants are "generally regarded as safe" for both topical and oral administration, so they are particularly suitable for the production of vaccines that can be delivered via the oral route or antibodies applied as topical microbicides, especially where such products are required on a large scale. This reflects the fact that plants can be grown inexpensively on an agricultural scale and that plant-derived pharmaceuticals for topical/oral administration would require only minimal processing. This could potentially bring the costs of production and distribution down to levels suitable for deployment in developing countries with limited financial resources and a poor medical infrastructure. This contribution describes the technologies that facilitate biopharmaceutical production in plants and plant cell cultures either through transient expression or stable transformation. It also discusses issues relating to posttranslational modification, extraction and purification, and regulatory compliance, focusing on those plant-derived pharmaceutical products that have advanced the furthest in the clinic. A compilation of selected technical achievements in plant molecular farming is provided in Table 1.

Plant Transformation

Pharmaceutical proteins can be produced in plants or plant cells either through transient expression or stable transformation. In the first case, the DNA encoding the protein is delivered into plant cells by *Agrobacterium tumefaciens* or a viral vector (or a combination of the two) but there is no integration of this DNA into the

Plant Molecular Pharming, Pharmaceuticals for Human Health. Table 1 Selected research achievements in the field of plant molecular farming between 1989 and 2008

Year	Achievement	Reference
1989	Full-size antibody (mouse IgG) expression in tobacco	[1]
1990	First human protein (HSA) produced in tobacco and potato	[2]
1992	First vaccine candidate (HBsAg) expressed in tobacco	[3]
1995	First secretory antibody (sIgA) produced in tobacco	[4]
1995	Plant seed oilbodies as vehicles for protein production and purification	[5]
1996	Expression of a protein-based polymer in tobacco	[6]
1998	First clinical trial with a vaccine candidate produced in transgenic potato	[7]
1999	Transient expression of an antibody by *Agrobacterium* vacuum infiltration	[8]
1999	N-glycan analysis of a plant-produced antibody	[9]
2000	Human growth hormone produced in tobacco chloroplasts	[10]
2001	N-glycan modification by expression of a human β-1,4 galactosyltransferase	[11]
2004	Knockout mutants of moss lacking plant-specific glycosylation	[12]
2004	Generation of *Arabidopsis* plants lacking plant-specific glycosylation	[13]
2005	*Agrobacterium*-mediated delivery of viral replicons	[14]
2006	Approval of a plant-made vaccine for veterinary medicine	[15]
2007	Production of glucocerebrosidase with terminal mannose residues	[16]
2008	Clinical phase I trial with plant-produced anti-idiotype vaccines	[17]
2008	Engineering of a CMP-sialic acid pathway in plants	[18]
2008	Phase III clinical trial with plant-made glucocerebrosidase	[19]

plant genome and the protein is synthesized for a few hours or days. In the second case, DNA delivered either by *A. tumefaciens* or a physical process such as particle bombardment integrates into the plant genome and becomes a permanent locus, allowing long-term production of the recombinant protein and the transmission of the trait to subsequent generations.

Each method has advantages and disadvantages that need to be evaluated on a case by case basis for each pharmaceutical protein, depending on its intended use and the production scale. The production of an immunoglobulin via stable integration into the nuclear genome was first reported in 1989 when Hiatt and colleagues produced a monoclonal IgG-class antibody in tobacco leaves [1]. They introduced the coding sequences for the gamma heavy chain and kappa light chain of the immunoglobulin into independent tobacco lines and then crossed plants from each line to stack the transgenes in a single plant, which was able to produce the full antibody. The same strategy was used to produce a chimeric secretory (sIgA/G) antibody, although in this case four transgenes were required (encoding the kappa light chain, the chimeric alpha/gamma heavy chain, the joining chain, and the secretory component) and four lines were bred over two generations to generate the final production crop [4]. Later on the assembly of a chimeric secretory sIgA/G antibody could be achieved by simultaneous transformation of all four components in rice plants [20]. These examples clearly show how plant cells can produce even the most complex proteins and modify and assemble them into functional oligomeric structures (two different cell types are required in mammals to produce secretory IgA antibodies). Stable transformation of the nuclear

genome enables the combination of several independent expression cassettes into a single transgenic line and also allows the introduction of a transgene from a laboratory cultivar into other varieties of the same species, to yield a germplasm that is particularly suited for a certain purpose. The latter strategy has been used to breed dent and sweet corn varieties that produce the HIV-specific antibody 2G12. The expression cassettes were initially introduced into a laboratory maize cultivar with little agronomic value and low yield [21]. Using conventional breeding transgenes can be transferred to a germplasm that is either inaccessible for direct transformation or that is particularly suited for the cultivation under certain climate conditions.

A drawback of stable nuclear transformation is the time needed to identify and establish a germplasm with the desired properties. A large number of primary transformants often need to be screened to identify plants showing high-level transgene expression. These lines then need to be analyzed at the molecular level to determine the number and arrangement of the transgenes. For breeding purposes single-copy integration events or multicopy single locus events with a regular transgene arrangement are preferred [22]. In contrast, multiple transgene copies with a complex integration pattern are likely to suffer from both transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) [23]. The transgenic plants must also be analyzed for unwanted pleiotropic effects that could be caused by the transgene itself or by the changes that are brought about by its integration, since transgene integration following both *Agrobacterium*-mediated transformation and particle bombardment is a random process. Precise transgene integration at a predefined locus can be achieved by homologous recombination (gene targeting) but this has not been possible for most plant species in the past due to its very low efficiency. A notable exception is the moss *Physcomitrella patens*, where transformation by homologous recombination is a straightforward and robust process [24]. In higher plants, efficient homologous recombination has become possible only recently with the use of zinc-finger endonucleases. These are engineered endonucleases containing zinc-finger motifs that bind precise DNA sequences and introduce double-strand breaks at the target site. This in turn stimulates DNA repair in the host, thereby facilitating homologous recombination. This has enabled the precise engineering of transgenic plants, although there have been no reports thus far concerning applications in molecular farming [25–28].

Another disadvantage of nuclear transgenic plants is that the target protein often accumulates at low levels, making them commercially unfeasible. This has been addressed by the use of plastid transformation, where the transgene is integrated in the circular chloroplast or chromoplast genome, typically by particle bombardment. Unlike nuclear transformation, homologous recombination is an efficient method for transgene integration into the plastid genome, allowing precise gene targeting. Every plastid contains several copies of the genome, and each plant cell contains many plastids [29]; therefore, it is possible in principle to generate plant cells containing several thousand transgene copies (and these are not subject to silencing because the TGS and PTGS mechanisms are not present in the plastid). To ensure the transgene is present in every copy of the plastid genome (the homoplasmic state), the primary transformants must undergo multiple rounds of selection and regeneration. This is generally achieved using the marker gene aminoglycoside 3″-adenylyltransferase, which confers resistance to the antibiotic spectinomycin [30]. The high transgene copy number and absence of silencing allows the accumulation of some target proteins to levels exceeding 10% of the total soluble protein (TSP) in the cell. Furthermore, since plastids are evolutionarily derived from bacteria, it is possible to express multiple genes as operons, producing polycistronic mRNA [31].

Many biopharmaceuticals are complex molecules that require several posttranslational processing steps to achieve a functional state. Plastids are equipped to form disulfide bridges, as demonstrated for the production of the human growth hormone somatotropin [10], and they can also assemble oligomers as demonstrated for the production of cholera toxin B-subunit (CTB), which assembled into functional GM1 ganglioside-binding pentamers [32]. Human serum albumin, which requires posttranslational removal of the N-terminal methionine residue, has also been produced successfully in plastids [33]. The enzyme methionine aminopeptidase cleaves off the initiating N-formylmethionine in plastids depending on the

subsequent amino acid sequence context, and this must be considered when dealing with proteins that need to have intact N-termini. Another elegant approach for the production of proteins with a defined N-terminus is the expression of the target protein as an N-terminal ubiquitin fusion protein. Endogenous ubiquitin-specific proteases then remove the ubiquitin moiety precisely, a strategy that has enabled the production of native somatotropin that carries an N-terminal phenylalanine residue [10].

Plastid transformation technique was limited to tobacco for many years but has recently expanded to incorporate certain crop species such as lettuce and tomato [34, 35]. Plastid transformation in crop plants opens new possibilities in the area of oral vaccines, where antigens are produced in the edible parts of plants and delivered via the oral route. A further advantage of plastid transformation is the biosafety aspect, since chloroplasts are inherited maternally in most species and are therefore not present in pollen [36]. However, there are also some limitations. Many biopharmaceuticals need to undergo co- and post-translational glycosylation in order to fold properly or in order to remain functional and stable, but this process does not occur in plastids. Certain target proteins also appear to be unstable or toxic when expressed in plastids, for example, the rotavirus coat protein VP6 and HIV p24 antigen undergo rapid degradation in the chloroplasts of older tobacco leaves, with significant accumulation only possible in the youngest leaves. A codon-optimized HIV p24 construct allowed homogenous expression but all the leaves turned yellow, and rearrangements were observed within the plastid DNA [37].

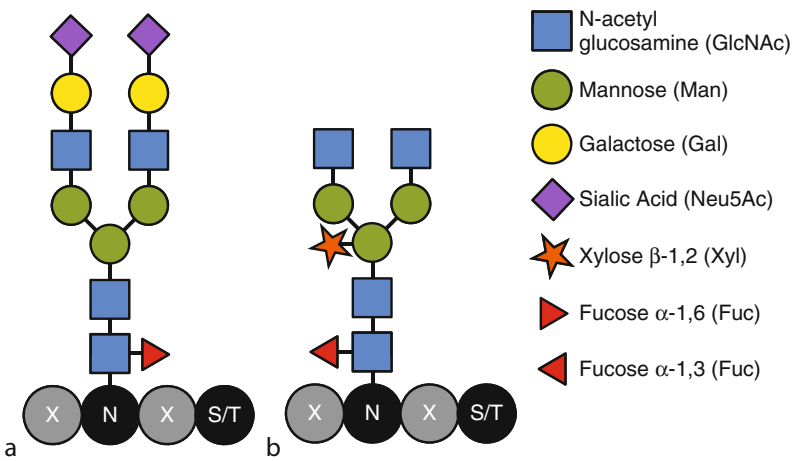
Transient expression allows more rapid production than stable transformation (nuclear or plastid). DNA encoding the pharmaceutical proteins is either included within a T-DNA cassette carried by an *A. tumefaciens* strain delivered into leaf tissue by vacuum infiltration [38, 39], or inserted into a viral vector that is used to infect the plant [40, 41]. Transient expression can be used for the rapid testing of expression constructs for subsequent stable transformation procedures or can be scaled up for use as production system in its own right. Most of the viral vectors are based on RNA viruses such as *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), and *Cowpea mosaic virus* (CPMV). These vectors have been used both to produce intact proteins and to produce chimeric virus particles that display peptide antigens on their surface. In such peptide display vectors, the target peptide is fused to the coat protein, and because each particle has many copies of the coat protein (and hence the antigen), the particles are strongly immunogenic and can be used without additional adjuvants to provoke an immune response. The versatility of this approach has been demonstrated with an experimental rabies vaccine that induced a protective immune response in mice. Furthermore human volunteers who ingested spinach leaves infected with the recombinant virus mounted a humoral immune response [42]. Conventional viral vectors have a limited capacity, and larger transgenes tend to be truncated or eliminated altogether as the virus spreads. This has been addressed by developing a series of deconstructed viral vectors in which the coat protein gene is deleted to provide space for the transgene. In order to deliver these vectors to a maximum number of plant cells the entire recombinant virus genome is incorporated as a DNA copy into a T-DNA cassette and delivered by *A. tumefaciens* via vacuum infiltration [43]. Two similar systems have been developed, one described as the launch vector system [44] and the other as the magnifection system [14]. They both exploit the ability of *A. tumefaciens* to infect a large range of plants, thereby extending the host range of the natural virus and using the efficient viral replication system to amplify the coding sequence of the target protein. In a proof of concept experiment, the accumulation of green fluorescent protein (GFP) peaked at 4 g kg^{-1} fresh weight in *Nicotiana benthamiana* plants transformed by magnifection [14]. The platform has been refined for the production of oligomeric proteins such as antibodies. Full-size IgG1 immunoglobulins have been produced successfully at levels of up to 0.5 g kg^{-1} fresh weight, by introducing the light and heavy chain coding sequences into two independent noninterfering vectors based on TMV and PVX, respectively [45].

Posttranslational Modification

Approximately 30% of all approved biopharmaceuticals contain N-linked glycans, so N-glycosylation is the most important posttranslational modification that needs to be

taken into account when manufacturing recombinant biopharmaceuticals in plants. The mechanism of N-glycosylation is conserved between plants and mammals, beginning in the endoplasmic reticulum (ER) with the transfer of an oligosaccharide precursor molecule onto asparagine residues within the sequence motif N-X-S/T (where X is any amino acid except proline). The precursor molecule is subsequently trimmed to yield a structure known as a high-mannose type glycan. The protein then passes into the Golgi apparatus where additional glycan modifications take place.

The final complex type glycan structures differ between plants and mammals (Fig. 1), in that plant glycoproteins contain core β1,2-xylose and α1,3-fucose residues whereas mammalian glycoproteins contain β1,4-galactose and terminal N-acetyl-neuraminic acid (sialic acid) residues [46]. Plant-specific glycosylation patterns have been found to induce an immune response upon injection in some mammalian species [47–49]. To prevent these immune responses several strategies have been explored to avoid the addition of plant-specific sugar residues to the glycan structure. One approach is the attachment of a C-terminal H/KDEL amino acid sequence motif to retain the target protein within the ER, thereby preventing exposure to the Golgi apparatus and the attachment of β1,2-xylose and α1,3-fucose residues [50]. This strategy has been applied successfully in the production of a chimeric mouse/human IgG1 antibody against human chorionic gonadotropin [51]. An alternative strategy is the knockout or knockdown of the endogenous plant β1,2-xylosyltransferase and α1,3-fucosyltransferase genes, which has been achieved in the moss *P. patens* by homologous recombination [12]. The double knockout mutant was used to express human erythropoietin devoid of plant-specific glycan structures [52]. In the model plant *Arabidopsis thaliana*, the β1,2-xylosyltransferase and α1,3-fucosyltransferase genes have been knocked out by T-DNA insertional mutagenesis [13]. This plant line has been used to produce the anti-HIV antibody 2G12 with a humanized glycan structure [53]. In the duckweed *Lemna minor*, the human anti-CD30 antibody MDX-060 was produced without plant glycans by co-introducing inverted repeat transgenes matching the sequences of the β1,2-xylosyltransferase and α1,3-fucosyltransferase genes, so that they were silenced by RNA interference (RNAi) [54]. This antibody also demonstrated stronger antibody-dependent cell-mediated cytotoxicity (ADCC) compared to its counterpart produced in Chinese hamster ovary (CHO) cells, reflecting the tenfold higher affinity of the plant-derived antibody for the human Fcγ receptor [54]. The same phenomenon has been demonstrated for other antibodies produced in the duckweed and moss systems [55, 56].



Plant Molecular Pharming, Pharmaceuticals for Human Health. Figure 1
Prototypes of protein N-glycosylation patterns from mammalian (**a**) and plant (**b**) origin. We are grateful to Holger Spiegel (Fraunhofer IME, Aachen, Germany) for the preparation of the graphics

To further humanize the glycan structure of biopharmaceuticals produced in plants, the coding sequence for human β1,4-galactosyltransferase has been introduced into tobacco [57, 58] and alfalfa plants [59]. The recombinant antibodies produced in these modified host plants not only possessed glycans with terminal galactose residues but they also contained fewer core β1,2-xylose and α1,3-fucose residues. Many human glycoproteins possess terminal sialic acid residues that play a pivotal role in serum stability. For example, erythropoietin usually has a half-life of 5 h in rat serum, but enzymatically trimming off the terminal sialic acid residues reduces the half-life to <2 min [60]. There is consequently an ongoing effort to introduce the CMP-sialic acid biosynthesis pathway into plants and thus far four of the enzymes (α2,6-sialyltransferase, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, N-acetylneuraminic acid phosphate synthase and CMP-N-acetylneuraminic acid synthetase) have been expressed successfully in *A. thaliana* [18, 61]. Recently, the components of the complete pathway have been transiently expressed in *N. benthamiana* and the co-expressed 2G12 mAb has been shown to become sialylated [62].

There is much less information available about the significance of O-glycosylation in pharmaceutical proteins produced in plants. Endogenous plant proteins tend to undergo O-glycosylation at clustered proline residues that are first converted into hydroxyproline, for example, those found in the extension family of hydroxyproline-rich glycoproteins (HRGPs). Such proline clusters are also present in the hinge region of IgA1 antibodies, so a recombinant IgA1 antibody expressed in maize similarly underwent hydroxylation followed by O-glycosylation [63]. An artificial O-glycosylation motif consisting of 20 tandem repeats of the dipeptide serine/proline has been fused to the C-terminus of human interferon α2b (IFNα2-$(SO)_{20}$) expressed in tobacco suspension cells. The fusion protein accumulated in the culture supernatant at levels two orders of magnitude higher than native IFNα2b, reflecting its more efficient secretion and its greater resistance toward proteolysis [64]. The antiviral activity of the fusion protein was comparable to that of native interferon but its higher molecular weight (75 kDa vs. 19.2 kDa) delayed renal clearance therefore increasing its serum half-life in mice by 13-fold [64].

The unwanted processing of recombinant proteins by endogenous plant proteases is a major obstacle in the field of molecular farming because the overall yield of the recombinant protein is reduced and the resulting protein fragments need to be removed during downstream processing. Proteolytic degradation has been observed irrespective of the subcellular localization of the target protein but the extracellular compartments (apoplast and culture medium) appear to be particularly rich in proteolytic enzymes [65–68]. This has been addressed using a number of strategies, including the co-expression of protease inhibitors [69–71] and the co-secretion of unrelated proteins that might act as bait for the proteases [72]. Although the proteases responsible for recombinant protein degradation have yet to be identified, there are indications that certain classes of proteases are involved (e.g., aspartic proteases, metalloproteases, and serine proteases) [66, 68]. Once the proteases responsible for recombinant protein degradation are known, knockout and knockdown strategies can be employed to reduce their abundance. However, proteases play a significant role in many aspects of plant development, stress responses, and pathogen defense, so their elimination may only be suitable for cell and tissue cultures that are grown under sterile and controlled conditions in the absence of pathogens.

Downstream Processing

Most biopharmaceuticals are formulated as a purified product so the majority of biopharmaceuticals produced in plants must be extracted from plant tissue and then purified and formulated in the same way as conventional biopharmaceutical products. Regardless of the upstream production platform, downstream processing can account for up to 80% of the total manufacturing costs for a biopharmaceutical protein [73], but the first downstream processing steps are largely determined by the specific production host [74]. If the target protein is produced in plant suspension cells and secreted into the culture medium, the purification process can begin directly after the cells have been removed by filtration or centrifugation. If the protein is produced in an intact plant and/or if it accumulates inside the plant cell, it must be released by mechanical disruption in the presence of an appropriate extraction buffer and the extract must be clarified by filtration and/or

centrifugation to remove debris, fibers, and other particulates. Aqueous two-phase partition is a useful initial purification step to remove polyphenols and cell debris from crude plant extracts [75, 76]. The removal of polyphenols is critical to prevent fouling of the chromatography media used in subsequent purification steps [74, 77]. After clarification, the product may be captured from the feed if a suitable affinity chromatography resin is available, allowing a high level of purification in a single step. A wide range of natural affinity ligands and an increasing number of synthetic ligands (e.g., mercaptoethylpyridine, MEP HyperCel™) are available, particularly for the capture of antibodies [78]. After capture, polishing is usually achieved by the application of two or more orthogonal separation methods to achieve maximal purity and contaminant removal, for example, ion exchange, hydrophobic interaction, hydroxyapatite, and size exclusion chromatography [79]. If a capture step is not possible, these chromatography methods may be used for intermediate purification prior to polishing. Purification can be facilitated by engineering the physicochemical properties of the target protein through genetic fusions, although the fusion tag must be removed after purification to yield the authentic protein as a final product (a protease cleavage site adjacent to the tag can be used to achieve separation). A fusion with the hydrophobic plant protein oleosin enables enrichment of the target protein by floating centrifugation [80]. Alternatively, fusing the target protein to elastin-like polypeptides (ELPs) allows the target protein to be isolated by thermal phase transition [81].

Potential contaminants include macromolecules such as host cell proteins and nucleic acids, as well as small molecules such as secondary metabolites (e.g., nicotine). The removal of contaminants derived from plant-associated microbes must also be demonstrated, especially endotoxins from *A. tumefaciens* that can cause inflammatory responses in humans. The successful removal of these substances has recently been demonstrated for a monoclonal antibody that has been produced in *N. benthamiana* by magnifection [82].

Biopharmaceuticals produced for human clinical trials must achieve certain quality criteria that are defined by current good manufacturing practice (cGMP). The regulations for biopharmaceutical manufacture are defined by the Food and Drug Administration (FDA) in the USA and by the European Medicines Agency (EMEA) in the European Union. The production of pharmaceuticals using plant suspension cells is very similar in concept to conventional systems based on mammalian cells, but intact plants cultivated in the greenhouse or in the open field are very different in concept and in practice. The FDA and EMEA have published guidance documents covering the production of pharmaceuticals in plants, and these might be refined further in the future [83]. Recently the Fraunhofer Institute for Molecular Biology and Applied Ecology in Aachen, Germany, obtained the first GMP license for the production of a plant-made pharmaceutical protein for clinical phase I trials in Europe. Based on this process the anti-HIV antibody 2G12 was produced in transgenic tobacco plants in the greenhouse.

Plant-Derived Vaccines

Plants have been proposed as an alternative production platform for subunit vaccines, with the added advantage that storage tissues such as cereal grains and potato tubers may be used to keep the vaccine stable without the need for a cold chain and could even be used to administer oral vaccines without processing, thus reducing costs.

Antigens embedded in the plant cell matrix are protected against the acidic conditions in the stomach and are released gradually, allowing the induction of a mucosal immune response. Many antigens that could be used as vaccines in humans or farm animals have been produced in plants including the hepatitis B virus surface antigen [3, 84, 85], the Norwalk virus capsid protein [86–88], the *Escherichia coli* heat labile toxin [89–91], and the rabies glycoprotein [42, 92].

Phase I clinical trials in humans have been conducted for some oral vaccines. The coding sequence for the B-chain of the heat labile toxin from enterotoxigenic *E. coli* (LT-B) has been expressed in transgenic potato and maize. Human volunteers who ingested three 50-g or 100-g doses of peeled raw potato slices containing 0.5–1 mg of LT-B developed anti LT serum IgG antibodies (91%) and half of the vaccinees also produced secretory IgA antibodies in their stools [7]. Similarly human volunteers who ingested 2 g of defatted corn germ meal containing 1 mg of LT-B developed anti LT serum IgG and IgA and sIgA in their stools [93].

Transgenic potato tubers producing the major capsid protein of the Norwalk virus (which causes gastroenteritis) were fed to human volunteers in two or three 150-g doses containing ~500 μg of the protein. Higher levels of IgA antibody-secreting cells were observed in more than 90% of the vaccinees, 20% produced serum IgG or IgM responses, and 30% produced anti-NVCP antibodies in their stools [88].

Two phase I clinical trials with plant-derived hepatitis B surface antigen (HBsAg) have been reported. In the first trial, transgenic lettuce (*Lactuca sativa*) was orally administered to seven seronegative individuals in three 200-g doses containing 0.5–1 μg of HBsAg within 5 weeks. After the third dose, all subjects developed serum anti-HB antibodies of up to 6.3 mIU/ml serum [94]. In the second trial, transgenic potato tubers were fed to individuals who had previously been vaccinated against hepatitis B. The vaccinees received two or three 100-g doses of raw peeled potatoes each containing ~800 μg HBsAg. Higher serum anti-HB titers were observed in 60% of the vaccinee group whereas there was no increase in the control group [95].

Recently, H1N1 and H5N1 influenza virus hemagglutinin (HA) have been transiently expressed in *N. benthamiana* [96]. Both proteins assembled into virus-like particles (VLPs) that budded from the plant plasma membrane, a desirable outcome because VLPs are polyvalent and therefore much more immunogenic than soluble subunit vaccines. Mice parenterally immunized with low doses (0.5 μg) of the VLPs were protected against a lethal challenge with influenza virus [96]. A phase I dose escalation study to assess the safety of a plant-derived H5 VLP in healthy volunteers was initiated in 2010 (www.clinicaltrials.gov; NCT00984945).

Although most vaccines are intended to induce an immune response against pathogens, their use is not limited to the prevention of infectious diseases. More recently, vaccines have been developed for the prevention or the treatment of certain types of cancer. A plant-derived vaccine for the treatment of non-Hodgkin's lymphoma (NHL) based on idiotype antibodies has been evaluated in a phase I trial [97]. NHL is a clonal disease of the B-cell lineage and the malignant cells carry specific immunoglobulins (idiotypes) on their surface. These idiotypes can be used to trigger a specific immune response. Because the idiotypes are different in each patient the vaccine has to be manufactured individually for each treated person. Currently the patient's tumor cells are expanded from a biopsy as human/mouse heteromyelomas. The monoclonal idiotype antibody is purified and coupled to an immunogenic carrier protein like keyhole limpet hemocyanin (KLH) and injected into the patient usually together with granulocyte-macrophage colony stimulating factor (GM-CSF) as an adjuvant [98]. To shorten the time needed to manufacture the patient-specific vaccine a plant-based production system has been developed in which the coding sequences for the variable domains of the idiotype antibody are cloned from the patient's biopsy and inserted into a viral vector for the expression of a single chain antibody (scFv). *N. benthamiana* plants have been infected with such viruses allowing the scFv to be purified from leaves [99, 100]. Sixteen NHL patients who had previously received chemotherapy were treated with two different doses of the tobacco-derived idiotype vaccine either with or without a GM-CSF adjuvant [17]. Most of the treated patients developed a cellular immune response although only three patients developed a humoral immune response. Recently, the Bayer Group announced another phase I clinical trial with idiotype vaccines for NHL using the transient magnifection technology developed by their subsidiary Icon Genetics. The ongoing study will enroll 30 patients with progressive or relapsing NHL. The patients will receive 12 injections over 16 months, each consisting of 1.0 mg of the personalized vaccine. The study is designed as a safety study to evaluate potential toxicity associated with the therapy but will also analyze the relevant immunological parameters of the patients. The final results of the study are expected in 2012 (www.clinicaltrials.gov; NCT01022255).

Plant-Made Pharmaceuticals in Advanced Development

The most advanced plant-derived pharmaceutical in terms of clinical development is glucocerebrosidase manufactured in transgenic carrot suspension cells (prGCD, taliglucerase alpha, Uplyso™). Patients suffering from Gaucher disease, an inherited lysosomal storage disorder, cannot produce active glucocerebrosidase and need enzyme replacement therapy with recombinant glucocerebrosidase, which is currently produced in CHO cells (imiglucerase, Cerezyme™) [101].

This is currently one of the most expensive biopharmaceuticals, with an annual treatment cost of USD 200,000 per patient [102]. The purified recombinant imiglucerase needs to be processed enzymatically to expose terminal mannose residues that are required for the efficient uptake of the enzyme into macrophages. The plant-derived counterpart, taliglucerase alpha, does not require these additional processing steps because it is targeted to the cell vacuole where the complex type N-glycans are trimmed to the paucimannose form exposing terminal mannose residues [16]. A phase III clinical trial with taliglucerase alpha was completed successfully in 2009 (www.clinicaltrials.gov, NCT00376168) and the substance currently awaits market approval from the FDA. Meanwhile patients can get access to taliglucerase alpha under an expanded access protocol [101].

Another plant-derived protein currently in clinical development is alpha-interferon (IFN-α2b) for the treatment of chronic hepatitis C infections. IFN-α2b has a low molecular weight (19 kDa, no glycan chains) and is therefore eliminated rapidly by renal filtration. Special formulations are required to increase its serum half-life, and this is achieved in the current formulation produced in *E. coli* (peginterferone alpha-2b; PEGIntron™), by attachment to polyethylene glycol. The plant-derived protein (Locteron™) is produced in duckweed and formulated in poly(ether-ester) microspheres to achieve controlled release over a defined period [103]. The plant-derived version has been tested successfully in a clinical phase I/II study (www.clinicaltrials.gov, NCT00593151) to establish its safety, tolerability, and efficacy compared to PEGIntron™. Currently two phase IIb clinical trials are underway to determine the optimal dose for the treatment of hepatitis C patients and its efficacy in combination with the antiviral compound ribavirin (www.clinicaltrials.gov, NCT00863239, NCT00953589).

The first recombinant biopharmaceutical on the market was insulin, which received regulatory approval in 1982. A large number of diabetes patients depend on insulin therapy so current demand for the protein exceeds 8 metric tons per year. This demand is currently met by production in *E. coli* and *Saccharomyces cerevisiae* [104, 105]. The successful production of active recombinant human insulin has also been demonstrated in oilseeds, where oleosin fusion can be used to facilitate purification. As stated above, oleosin is a hydrophobic protein component of the seed oilbodies and fusion proteins become concentrated in the oilbodies allowing their purification by floating centrifugation, enzymatic cleavage, and then standard polishing chromatography methods [106]. For large-scale insulin production, the Canadian company SemBioSys Genetics Inc. uses safflower (*Carthamus tinctorius*) plants [107]. The company recently announced the successful completion of a phase I/II clinical trial with healthy volunteers demonstrating that the safflower-derived insulin (SBS-1000) is equivalent to the recombinant insulin currently on the market [108].

Future Directions

Many biopharmaceutical proteins have been produced successfully in plants and plant cell cultures, clearly demonstrating the utility of plant-based production platforms. The demand for biopharmaceuticals is predicted to rise in the future based on the large number of ongoing clinical trials that involve recombinant pharmaceutical proteins, but current fermenter-based production platforms are already struggling to meet the demand. The enormous flexibility offered by different plant production systems and their specific advantages in terms of cost, safety, and scalability, means that plants could provide an alternative source for recombinant biopharmaceuticals when the capacity of current platforms is exhausted.

To become more competitive with the currently established protein production platforms an increase in productivity for the plant cell factories is mandatory. Therefore future research will aim to boost the protein accumulation levels in plant cells. To achieve this goal different strategies are pursued including, among others, gene amplification, high throughput screening for elite events, targeting the protein of interest to suited storage organelles or even to create them artificially, and to shield the target protein against proteolytic degradation. Systems biology approaches will help to identify cellular targets that can be subsequently engineered to further improve the plant cell as a protein production host. The engineering process itself will be more precise in the future by employing the newly developed techniques to facilitate homologous recombination within the nuclear genome.

Beside the quantity also the quality of the final product will be a major focus of future research and development. Especially the engineering of the glycosylation pattern bears a great potential to optimize the stability and efficacy of the biopharmaceutical product. A critical point with respect to the glycosylation pattern will be the detailed understanding of plant-produced glycoproteins with the mammalian immune system. This will be an important prerequisite for tailoring plant-produced subunit vaccines and to avoid unintended side effects. With respect to oral vaccines reliable formulation and administration protocols have to be defined to ensure the anticipated outcome is achieved.

Further optimization of transient plant expression systems will help to address future needs for the delivery of vaccine, personalized medicine, and biopharmaceuticals for the treatment of orphan diseases. The rapid production cycle will also enable a timely reaction to emerging diseases, pandemics, or biohazards. However, unlike stable transgenic plant production systems, there are currently no specific regulatory guidelines for transient technologies, which are becoming a perceived barrier to their widespread use and commercialization. Therefore, the establishment and harmonization of international regulations for transient expression systems are needed to enable the commercial application of this promising technology.

Bibliography

Primary Literature

1. Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76–78
2. Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. Biotechnology 8:217–221
3. Mason HS, Lam DM, Arntzen CJ (1992) Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA 89:11745–11749
4. Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K, Lehner T (1995) Generation and assembly of secretory antibodies in plants. Science 268:716–719
5. van Rooijen GJ, Moloney MM (1995) Plant seed oil-bodies as carriers for foreign proteins. Biotechnology 13:72–77
6. Zhang XR, Urry DW, Daniell H (1996) Expression of an environmentally friendly synthetic protein-based polymer gene in transgenic tobacco plants. Plant Cell Rep 16:174–179
7. Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med 4:607–609
8. Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S, Fischer R (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. Proc Natl Acad Sci USA 96:11128–11133
9. Cabanes-Macheteau M, Fitchette-Laine AC, Loutelier-Bourhis C, Lange C, Vine ND, Ma JK, Lerouge P, Faye L (1999) N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. Glycobiology 9:365–372
10. Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat Biotechnol 18:333–338
11. Bakker H, Bardor M, Molthoff JW, Gomord V, Elbers I, Stevens LH, Jordi W, Lommen A, Faye L, Lerouge P, Bosch D (2001) Galactose-extended glycans of antibodies produced by transgenic plants. Proc Natl Acad Sci USA 98:2899–2904
12. Koprivova A, Stemmer C, Altmann F, Hoffmann A, Kopriva S, Gorr G, Reski R, Decker EL (2004) Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic N-glycans. Plant Biotechnol J 2:517–523
13. Strasser R, Altmann F, Mach L, Glossl J, Steinkellner H (2004) Generation of *Arabidopsis thaliana* plants with complex N-glycans lacking β1, 2-linked xylose and core α1, 3-linked fucose. FEBS Lett 561:132–136
14. Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 23:718–723
15. Walsh G (2006) Biopharmaceutical benchmarks 2006. Nat Biotechnol 24:769–776
16. Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman JL, Futerman AH, Aviezer D (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol J 5:579–590
17. McCormick AA, Reddy S, Reinl SJ, Cameron TI, Czerwinkski DK, Vojdani F, Hanley KM, Garger SJ, White EL, Novak J, Barrett J, Holtz RB, Tuse D, Levy R (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. Proc Natl Acad Sci USA 105:10131–10136
18. Castilho A, Pabst M, Leonard R, Veit C, Altmann F, Mach L, Glossl J, Strasser R, Steinkellner H (2008) Construction of a functional CMP-sialic acid biosynthesis pathway in Arabidopsis. Plant Physiol 147:331–339
19. Aviezer D, Almon-Brill E, Shaaltiel Y, Galili G, Chertkoff R, Hashmueli S, Galun E, Zimran A (2008) Novel enzyme replacement therapy for Gaucher disease: on-going phase III clinical

trial with recombinant human glucocerebrosidase expressed in plant cells. Mol Genet Metab 93:S15–S15
20. Nicholson L, Gonzalez-Melendi P, van Dolleweerd C, Tuck H, Perrin Y, Ma JKC, Fischer R, Christou P, Stoger E (2005) A recombinant multimeric immunoglobulin expressed in rice shows assembly-dependent subcellular localization in endosperm cells. Plant Biotechnol J 3:115–127
21. Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F, Quendler H, Stiegler G, Kunert R, Fischer R, Stoger E (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J 6:189–201
22. Kohli A, Melendi PG, Abranches R, Capell T, Stoger E, Christou P (2006) The quest to understand the basis and mechanisms that control expression of introduced transgenes in crop plants. Plant Signal Behav 1:185–195
23. De Wilde C, Van Houdt H, De Buck S, Angenon G, De Jaeger G, Depicker A (2000) Plants as bioreactors for protein production: avoiding the problem of transgene silencing. Plant Mol Biol 43:347–359
24. Decker EL, Reski R (2004) The moss bioreactor. Curr Opin Plant Biol 7:166–170
25. Cai CQ, Doyon Y, Ainley WM, Miller JC, Dekelver RC, Moehle EA, Rock JM, Lee YL, Garrison R, Schulenberg L, Blue R, Worden A, Baker L, Faraji F, Zhang L, Holmes MC, Rebar EJ, Collingwood TN, Rubin-Wilson B, Gregory PD, Urnov FD, Petolino JF (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol 69:699–709
26. Durai S, Mani M, Kandavelou K, Wu J, Porteus MH, Chandrasegaran S (2005) Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. Nucleic Acids Res 33:5978–5990
27. Porteus MH (2009) Plant biotechnology: zinc fingers on target. Nature 459:337–338
28. Tovkach A, Zeevi V, Tzfira T (2009) A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. Plant J 57:747–757
29. Bendich AJ (1987) Why do chloroplasts and mitochondria contain so many copies of their genome? Bioessays 6:279–282
30. Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc Natl Acad Sci USA 90:913–917
31. De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19:71–74
32. Daniell H, Lee SB, Panchal T, Wiebe PO (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol 311:1001–1009
33. Fernandez-San Millan A, Farran I, Molina A, Mingo-Castel AM, Veramendi J (2007) Expression of recombinant proteins lacking methionine as N-terminal amino acid in plastids: human serum albumin as a case study. J Biotechnol 127:593–604
34. Lelivelt CL, McCabe MS, Newell CA, Desnoo CB, van Dun KM, Birch-Machin I, Gray JC, Mills KH, Nugent JM (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.). Plant Mol Biol 58:763–774
35. Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat Biotechnol 19:870–875
36. Hagemann R (2002) Milestones in plastid genetics of higher plants. In: Esser K, Lüttge U, Beyschlag W, Hellwig F (eds) Progress in botany: genetics, physiology, ecology, vol 63. Springer, New York, pp 5–51
37. McCabe MS, Klaas M, Gonzalez-Rabade N, Poage M, Badillo-Corona JA, Zhou F, Karcher D, Bock R, Gray JC, Dix PJ (2008) Plastid transformation of high-biomass tobacco variety Maryland Mammoth for production of human immunodeficiency virus type 1 (HIV-1) p24 antigen. Plant Biotechnol J 6:914–929
38. Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U (1999) Towards molecular farming in the future: transient protein expression in plants. Biotechnol Appl Biochem 30:113–116
39. Sheludko YV (2008) Agrobacterium-mediated transient expression as an approach to production of recombinant proteins in plants. Recent Pat Biotechnol 2:198–208
40. Lico C, Chen Q, Santi L (2008) Viral vectors for production of recombinant proteins in plants. J Cell Physiol 216:366–377
41. Pogue GP, Lindbo JA, Garger SJ, Fitzmaurice WP (2002) Making an ally from an enemy: plant virology and the new agriculture. Annu Rev Phytopathol 40:45–74
42. Yusibov V, Hooper D, Spitsin S, Fleysh N, Kean R, Mikheeva T, Deka D, Karasev A, Cox S, Randall J, Koprowski H (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 20:3155–3164
43. Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. Proc Natl Acad Sci USA 101:6852–6857
44. Musiychuk K, Stephenson N, Bi H, Farrance CE, Orozovic G, Brodelius M, Brodelius P, Horsey A, Ugulava N, Shamloul AM, Mett V, Rabindran S, Streatfield SJ, Yusibov V (2007) A launch vector for the production of vaccine antigens in plants. Influenza Other Respi Viruses 1:19–25
45. Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, Klimyuk V, Gleba Y (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci USA 103:14701–14706
46. Saint-Jore-Dupas C, Faye L, Gomord V (2007) From planta to pharma with glycosylation in the toolbox. Trends Biotechnol 25:317–323
47. Bardor M, Faveeuw C, Fitchette AC, Gilbert D, Galas L, Trottein F, Faye L, Lerouge P (2003) Immunoreactivity in mammals of two typical plant glyco-epitopes, core α(1, 3)-fucose and core xylose. Glycobiology 13:427–434
48. Jin C, Altmann F, Strasser R, Mach L, Schahs M, Kunert R, Rademacher T, Glossl J, Steinkellner H (2008) A plant-derived

human monoclonal antibody induces an anti-carbohydrate immune response in rabbits. Glycobiology 18:235–241
49. Bosch D, Schots A (2010) Plant glycans: friend or foe in vaccine development? Expert Rev Vaccines 9:835–842
50. Tekoah Y, Ko K, Koprowski H, Harvey DJ, Wormald MR, Dwek RA, Rudd PM (2004) Controlled glycosylation of therapeutic antibodies in plants. Arch Biochem Biophys 426:266–278
51. Sriraman R, Bardor M, Sack M, Vaquero C, Faye L, Fischer R, Finnern R, Lerouge P (2004) Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-α(1, 3)-fucose residues. Plant Biotechnol J 2:279–287
52. Weise A, Altmann F, Rodriguez-Franco M, Sjoberg ER, Baumer W, Launhardt H, Kietzmann M, Gorr G (2007) High-level expression of secreted complex glycosylated recombinant human erythropoietin in the Physcomitrella Δ-*fuc-t* Δ-*xyl-t* mutant. Plant Biotechnol J 5:389–401
53. Schähs M, Strasser R, Stadlmann J, Kunert R, Rademacher T, Steinkellner H (2007) Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. Plant Biotechnol J 5:657–663
54. Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, Peele CG, Black A, Passmore D, Moldovan-Loomis C, Srinivasan M, Cuison S, Cardarelli PM, Dickey LF (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nat Biotechnol 24:1591–1597
55. Gasdaska JR, Sterling JD, Regan JT, Cox KM, Sherwood SW, Dickey LF (2007) Expression of a glyco-optimized anti-CD20 antibody in the aquatic plant *Lemna* with enhanced ADCC activity. Blood 110:697a–697a
56. Schuster M, Jost W, Mudde GC, Wiederkum S, Schwager C, Janzek E, Altmann F, Stadlmann J, Stemmer C, Gorr G (2007) In vivo glyco-engineered antibody with improved lytic potential produced by an innovative non-mammalian expression system. Biotechnol J 2:700–708
57. Bakker H, Rouwendal GJ, Karnoup AS, Florack DE, Stoopen GM, Helsper JP, van Ree R, van Die I, Bosch D (2006) An antibody produced in tobacco expressing a hybrid β-1, 4-galactosyltransferase is essentially devoid of plant carbohydrate epitopes. Proc Natl Acad Sci USA 103:7577–7582
58. Fujiyama K, Furukawa A, Katsura A, Misaki R, Omasa T, Seki T (2007) Production of mouse monoclonal antibody with galactose-extended sugar chain by suspension cultured tobacco BY2 cells expressing human β(1, 4)-galactosyltransferase. Biochem Biophys Res Commun 358:85–91
59. Sourrouille C, Marquet-Blouin E, D'Aoust MA, Kiefer-Meyer MC, Seveno M, Pagny-Salehabadi S, Bardor M, Durambur G, Lerouge P, Vezina L, Gomord V (2008) Down-regulated expression of plant-specific glycoepitopes in alfalfa. Plant Biotechnol J 6:702–721
60. Erbayraktar S, Grasso G, Sfacteria A, Xie QW, Coleman T, Kreilgaard M, Torup L, Sager T, Erbayraktar Z, Gokmen N, Yilmaz O, Ghezzi P, Villa P, Fratelli M, Casagrande S, Leist M, Helboe L, Gerwein J, Christensen S, Geist MA, Pedersen LO, Cerami-Hand C, Wuerth JP, Cerami A, Brines M (2003) Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. Proc Natl Acad Sci USA 100:6741–6746
61. Wee EG, Sherrier DJ, Prime TA, Dupree P (1998) Targeting of active sialyltransferase to the plant Golgi apparatus. Plant Cell 10:1759–1768
62. Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P, Kunert R, Quendler H, Pabst M, Leonard R, Altmann F, Steinkellner H (2010) In planta protein sialylation through overexpression of the respective mammalian pathway. J Biol Chem 285:15923–15930
63. Karnoup AS, Turkelson V, Anderson WH (2005) O-linked glycosylation in maize-expressed human IgA1. Glycobiology 15:965–981
64. Xu J, Tan L, Goodrum KJ, Kieliszewski MJ (2007) High-yields and extended serum half-life of human interferon α2b expressed in tobacco cells as arabinogalactan-protein fusions. Biotechnol Bioeng 97:997–1008
65. De Muynck B, Navarre C, Nizet Y, Stadlmann J, Boutry M (2009) Different subcellular localization and glycosylation for a functional antibody expressed in *Nicotiana tabacum* plants and suspension cells. Transgenic Res 18:467–482
66. Delannoy M, Alves G, Vertommen D, Ma J, Boutry M, Navarre C (2008) Identification of peptidases in *Nicotiana tabacum* leaf intercellular fluid. Proteomics 8:2285–2298
67. Doran PM (2006) Foreign protein degradation and instability in plants and plant tissue cultures. Trends Biotechnol 24:426–432
68. Schiermeyer A, Schinkel H, Apel S, Fischer R, Schillberg S (2005) Production of *Desmodus rotundus* salivary plasminogen activator α1 (DSPAα1) in tobacco is hampered by proteolysis. Biotechnol Bioeng 89:848–858
69. Kim TG, Lee HJ, Jang YS, Shin YJ, Kwon TH, Yang MS (2008) Co-expression of proteinase inhibitor enhances recombinant human granulocyte-macrophage colony stimulating factor production in transgenic rice cell suspension culture. Protein Expr Purif 61:117–121
70. Komarnytsky S, Borisjuk N, Yakoby N, Garvey A, Raskin I (2006) Cosecretion of protease inhibitor stabilizes antibodies produced by plant roots. Plant Physiol 141:1185–1193
71. Rivard D, Anguenot R, Brunelle F, Le VQ, Vezina L-P, Trepanier S, Michaud D (2006) An in-built proteinase inhibitor system for the protection of recombinant proteins recovered from transgenic plants. Plant Biotechnol J 4:359–368
72. Baur A, Reski R, Gorr G (2005) Enhanced recovery of a secreted recombinant human growth factor using stabilizing additives and by co-expression of human serum albumin in the moss *Physcomitrella patens*. Plant Biotechnol J 3:331–340
73. Roque AC, Lowe CR, Taipa MA (2004) Antibodies and genetically engineered related molecules: production and purification. Biotechnol Prog 20:639–654
74. Menkhaus TJ, Bai Y, Zhang C, Nikolov ZL, Glatz CE (2004) Considerations for the recovery of recombinant proteins from plants. Biotechnol Prog 20:1001–1014
75. Platis D, Drossard J, Fischer R, Ma JK, Labrou NE (2008) New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants. J Chromatogr A 1211:80–89

76. Platis D, Labrou NE (2009) Application of a PEG/salt aqueous two-phase partition system for the recovery of monoclonal antibodies from unclarified transgenic tobacco extract. Biotechnol J 4:1320–1327
77. Bai Y, Glatz CE (2003) Bioprocess considerations for expanded-bed chromatography of crude canola extract: sample preparation and adsorbent reuse. Biotechnol Bioeng 81:775–782
78. Low D, O'Leary R, Pujar NS (2007) Future of antibody purification. J Chromatogr B 848:48–63
79. Drossard J (2004) Downstream processing of plant-derived recombinant therapeutic proteins. In: Fischer R, Schillberg S (eds) Molecular farming: plant-made pharmaceuticals and technical proteins. Wiley-VCH, Weinheim, Germany, pp 217–231
80. Parmenter DL, Boothe JG, van Rooijen GJH, Yeung EC, Moloney MM (1995) Production of biologically active hirudin in plant seeds using oleosin partitioning. Plant Mol Biol 29:1167–1180
81. Floss DM, Schallau K, Rose-John S, Conrad U, Scheller J (2010) Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. Trends Biotechnol 28:37–45
82. Pogue GP, Vojdani F, Palmer KE, Hiatt E, Hume S, Phelps J, Long L, Bohorova N, Kim D, Pauly M, Velasco J, Whaley K, Zeitlin L, Garger SJ, White E, Bai Y, Haydon H, Bratcher B (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. Plant Biotechnol J 8:638–654
83. Spok A, Twyman RM, Fischer R, Ma JK, Sparrow PA (2008) Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants. Trends Biotechnol 26:506–517
84. Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, Yusibov V, Koprowski H, Plucienniczak A, Legocki AB (1999) A plant-derived edible vaccine against hepatitis B virus. FASEB J 13:1796–1799
85. Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci USA 98:11539–11544
86. Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Natl Acad Sci USA 93:5335–5340
87. Santi L, Batchelor L, Huang Z, Hjelm B, Kilbourne J, Arntzen CJ, Chen Q, Mason HS (2008) An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. Vaccine 26:1846–1854
88. Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ (2000) Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis 182:302–305
89. Chikwamba R, Cunnick J, Hathaway D, McMurray J, Mason H, Wang K (2002) A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT). Transgenic Res 11:479–493
90. Haq TA, Mason HS, Clements JD, Arntzen CJ (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268:714–716
91. Rosales-Mendoza S, Soria-Guerra RE, Lopez-Revilla R, Moreno-Fierros L, Alpuche-Solis AG (2008) Ingestion of transgenic carrots expressing the *Escherichia coli* heat-labile enterotoxin B subunit protects mice against cholera toxin challenge. Plant Cell Rep 27:79–84
92. Ashraf S, Singh PK, Yadav DK, Shahnawaz M, Mishra S, Sawant SV, Tuli R (2005) High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J Biotechnol 119:1–14
93. Tacket CO, Pasetti MF, Edelman R, Howard JA, Streatfield S (2004) Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine 22:4385–4389
94. Kapusta J, Modelska A, Pniewski T, Figlerowicz M, Jankowski K, Lisowa O, Plucienniczak A, Koprowski H, Legocki AB (2001) Oral immunization of human with transgenic lettuce expressing hepatitis B surface antigen. Adv Exp Med Biol 495:299–303
95. Thanavala Y, Mahoney M, Pal S, Scott A, Richter L, Natarajan N, Goodwin P, Arntzen CJ, Mason HS (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci USA 102:3378–3382
96. D'Aoust MA, Lavoie PO, Couture MM, Trepanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vezina LP (2008) Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J 6:930–940
97. Arntzen CJ (2008) Plant science. Using tobacco to treat cancer. Science 321:1052–1053
98. Sinha R, Shenoy PJ, Flowers CR (2008) Idiotype vaccine strategies for improving outcomes in follicular lymphoma. Expert Opin Biol Ther 8:1213–1223
99. McCormick AA, Kumagai MH, Hanley K, Turpen TH, Hakim I, Grill LK, Tuse D, Levy S, Levy R (1999) Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants. Proc Natl Acad Sci USA 96:703–708
100. McCormick AA, Reinl SJ, Cameron TI, Vojdani F, Fronefield M, Levy R, Tuse D (2003) Individualized human scFv vaccines produced in plants: humoral anti-idiotype responses in vaccinated mice confirm relevance to the tumor Ig. J Immunol Meth 278:95–104
101. Hollak CE, vom Dahl S, Aerts JM, Belmatoug N, Bembi B, Cohen Y, Collin-Histed T, Deegan P, van Dussen L, Giraldo P, Mengel E, Michelakakis H, Manuel J, Hrebicek M, Parini R, Reinke J, di Rocco M, Pocovi M, Sa Miranda MC, Tylki-Szymanska A, Zimran A, Cox TM (2010) Force Majeure: therapeutic measures in response to restricted supply of imiglucerase (Cerezyme) for patients with Gaucher disease. Blood Cells Mol Dis 44:41–47
102. Kaiser J (2008) Is the drought over for pharming? Science 320:473–475

103. De Leede LG, Humphries JE, Bechet AC, Van Hoogdalem EJ, Verrijk R, Spencer DG (2008) Novel controlled-release *Lemna*-derived IFN-α2b (Locteron): pharmacokinetics, pharmacodynamics, and tolerability in a phase I clinical trial. J Interferon Cytokine Res 28:113–122
104. Chance RE, Frank BH (1993) Research, development, production, and safety of biosynthetic human insulin. Diab Care 16(Suppl 3):133–142
105. Kjeldsen T (2000) Yeast secretory expression of insulin precursors. Appl Microbiol Biotechnol 54:277–286
106. Nykiforuk CL, Boothe JG, Murray EW, Keon RG, Goren HJ, Markley NA, Moloney MM (2006) Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. Plant Biotechnol J 4:77–85
107. Moloney MM, Boothe J, Nykiforuk C, Kuhlman P, Pollock B (2008) Plant-based production of authentic recombinant human insulin: chemical, biochemical and preclinical evaluation. FEBS J 275:62–62
108. Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D, Moloney MM (2010) Seed-based expression systems for plant molecular farming. Plant Biotechnol J 8:588–606

Books and Reviews

Chen Q (2008) Expression and purification of pharmaceutical proteins in plants. Biol Eng 1:291–321
Daniell H, Singh ND, Mason H, Streatfield SJ (2009) Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 14:669–679
De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. Plant Biotechnol J 8:529–563
Faye L, Gomord V (eds) (2009) Recombinant proteins from plants: methods and protocols. Humana, New York
Fischer R, Schillberg S (eds) (2004) Molecular farming: plant-made pharmaceuticals and technical proteins. Wiley-VCH, Weinheim, Germany
Fischer R, Schillberg S, Twyman RM (2009) Molecular farming of antibodies in plants. In: Kirakosyan A, Kaufman PB (eds) Recent advances in plant biotechnology. Springer, Dordrecht, The Netherlands, pp 35–63
Gomez E, Zoth SC, Berinstein A (2009) Plant-based vaccines for potential human application: a review. Hum Vaccin 5:738–744
Hefferon KL (2009) Biopharmaceuticals in plants: toward the next generation of medicine. CRC, Boca Raton
Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422
Karasev AV (ed) (2009) Plant-produced microbial vaccines. Springer, Berlin
Kempken F, Jung C (eds) (2010) Genetic modification of plants: agriculture, horticulture and forestry. Springer, Berlin
Knäblein J (2004) Biopharmaceuticals expressed in plants. In: Kayser O, Müller RH (eds) Pharmaceutical biotechnology. Wiley-VCH, Weinheim, Germany, pp 35–56
Levine MM (ed) (2009) New generation vaccines, 4th edn. Informa Healthcare, New York
Rader R (2007) Biopharmaceutical products in the US and European markets, 6th edn. Bioplan Associates, Rockville
Rybicki EP (2010) Plant-made vaccines for humans and animals. Plant Biotechnol J 8:620–637
Sharma AK, Sharma MK (2009) Plants as bioreactors: recent developments and emerging opportunities. Biotechnol Adv 27:811–832
Shih SM, Doran PM (2009) Foreign protein production using plant cell and organ cultures: advantages and limitations. Biotechnol Adv 27:1036–1042
Tiwari S, Verma PC, Singh PK, Tuli R (2009) Plants as bioreactors for the production of vaccine antigens. Biotechnol Adv 27:449–467
Twyman RM, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10:185–218
Walsh G (2009) Market development of biopharmaceuticals. In: Engelhard M, Hagen K, Boysen M (eds) Genetic engineering in livestock: new applications and interdisciplinary perspectives. Springer, Berlin, pp 69–89
Walsh G (ed) (2009) Post-translational modification of protein biopharmaceuticals. Wiley-VCH, Weinheim, Germany

Plant Molecular Pharming, Veterinary Applications

Doreen M. Floss[1], Udo Conrad[2]
[1]Institute of Biochemistry and Molecular Biology II, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
[2]Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Phytoantibodies, Gatersleben, Germany

Article Outline

Glossary

Companion animal Animal kept for companionship and enjoyment (household animal).

Edible vaccines Antigenic proteins, which are produced in organs of transgenic plants (e.g., fruits, tubers) and can be directly administered to humans or animals without any purification procedure.

ELP Elastin-like polypeptide containing the hydrophobic amino acids valine, proline, glycine, and guest residues, which shows temperature-dependent, reversible self-aggregation.

ELPylation Genetic C- or N-terminal target protein fusion to elastin-like polypeptides.

Homoplasmy Presence of the transgene in all copies of chloroplast DNA.

Livestock animal Domesticated animal raised in an agricultural setting to produce, e.g., food and fiber.

Molecular pharming The large-scale production and purification of pharmaceutical proteins in plants.

Plant-based expression Process by which information from a transgene is used in the synthesis of a functional protein *in planta*. Different plant-based expression systems are suitable (e.g., transgenic plants, transient expression, and plant cell cultures).

Plantibodies Antibody or antibody derivative produced in genetically engineered plants.

Transgenic plants Genetically engineered plants generated by the biolistic method (particle gun) or by *Agrobacterium tumefaciens* mediated transformation. The introduced transgene, which does not occur naturally in the plant, is transferred to the offspring.

Transient expression Expression of transgenes for a short period of time. In the context of plant-based expression infiltration of recombinant Agrobacteria (Agro-infiltration) or the use of plant viral vectors are the methods of choice to produce a desired protein *in planta*.

Transplastomic plants Introduced transgene is targeted to the chloroplast genome using particle bombardment or other physical DNA delivery techniques.

Zoonotic diseases Infectious disease that can be transmitted from wild and domestic animals to humans.

Definition of the Subject

"Molecular Pharming" refers to the large-scale production and purification of pharmaceutical proteins in plants or plant-based expression systems. Since the successful expression of complete antibodies in transgenic plants in 1989 and the first report of plant-based vaccine production in 1992, a large number of different vaccines, antibodies, as well as antibody fragments have been produced in plants for medical or veterinary purposes. However, only two plant-produced vaccine-related products have gone all the way through the production and regulatory hurdles, and only one, a plant-made single-chain variable fragment (scFv), is used in the production of a recombinant Hepatitis B Virus (HBV) vaccine in Cuba. Edible vaccines and novel methods of downstream processing such as "ELPylation" have been developed over the past years to facilitate the development of recombinant protein-based therapeutics.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Introduction

Plant-based expression systems possess advantages over conventional eukaryotic expression systems (yeast, insect cells, and mammalian cells), e.g., the ability to obtain complex, correctly folded, and posttranslationally modified proteins [50]. They compete favorably with mammalian cells for the production of vaccines and antibodies because of distinct advantages over conventional systems including cost, safety, and scalability [57]. However, the cost of downstream processing (protein extraction, protein recovery, and protein purification) for recombinant expression systems in general are approximately the same and can represent over 80% of the overall processing costs [30] with the majority of such costs attributed to chromatography and associated materials, labor, and capital equipment [57]. Savings in the upstream components (no need for expensive fermenters, special culture media, and skilled workers) are some of the major benefits for the production of pharmaceutical proteins in plants. Costs of goods sold (COGS) from mammalian cell culture are estimated to be $300 per gram therapeutic protein, whereas the raw material costs for 1 g recombinant protein from plants are in the order of $0.10–$1 (depending on the expression level; [33]). The main technical bottleneck limiting the commercial production of pharmaceuticals in plants is the high cost and inefficiency of downstream processing including purification [34].

One-third of the approved biopharmaceuticals are glycoproteins [56] and the activity of antibodies, blood factors, and interferons is dependent on their glycosylation pattern. Accordingly, biopharmaceuticals are often produced in heterologous expression systems with glycosylation capabilities. Plant-specific glycosylation differs from mammalian glycosylation (for review see [26]) and this aspect explains the major limitation for the use of plant-made pharmaceuticals in therapy. Recently, progress toward the humanization of protein N-glycosylation in plant cells has been made, which focused on the targeted expression of therapeutic proteins, the knock-out of plant-specific N-glycan-processing genes, and/or the introduction of the enzymatic machinery catalyzing the synthesis, transport, and addition of mammalian sugar residues (for review see [27]).

With the development of "edible" vaccines, which can be orally administered in the form of a transgenic fruit or vegetable expressing the appropriate antigen without any prior processing, low-cost production systems and effective delivery systems are expected [40]. One of the easiest ways to get vaccinated against a disease might be eating a bite of banana, full of the virus proteins, as it was contemplated by researchers at the Boyce Thompson Institute for Plant Research at Cornell University in 1997 [25]. In reality, this anticipated development did not occur. Major problems of this technology are low yields, weak antigenicity of plant-produced vaccines and the lack of buy-in by governments and pharmaceutical companies [43].

In this chapter, the historical development of plant-produced vaccines and antibodies, so-called plantibodies, and the development of different stable plant production systems including down-stream processing with a specific focus on the progress of animal therapeutics will be discussed.

Plant-Based Expression Systems

Since the first report of plant-based antibody production [32], different formats have been generated ranging from single variable heavy-chain domain (VHH) antibodies [4] and single-chain molecules (scFvs; [3, 23]) to Fab fragments [10], and complete immunoglobulins [35]. Despite substantial progress in the production of antibodies in plants for human health (for review see [9]), their application to the veterinary field is rather limited (for review see [18]). The development of passive immunization commenced in 1890 with the identification of serum therapy by Emil Behring and Shibasaburo Kitasato. They identified substances in the blood they called antibodies which were responsible for the immunity against diphtheria and tetanus toxins. Furthermore, the researchers were able to transfer immunity to immunologically naïve animals by injecting serum of animals treated with nonlethal doses of a crude toxin preparation [2]. At the same time, Paul Ehrlich discovered that antibodies can act as so-called magic bullets for the targeting of cancer cells [12]. A century later, the structures of antibodies and the sequences coding for the immunoglobulin chains were elucidated and mouse hybridomas provided highly specific monoclonal antibodies for therapeutic

applications [36]. The development of innovative recombinant DNA technologies greatly enhanced the clinical efficiency and safety of mouse-derived monoclonal antibodies. The ability to generate large antibody libraries, and the simplified antibody backbone of a single-chain antibody made antibody phage display a powerful tool for the development of new therapeutic agents (for reviews see [3]).

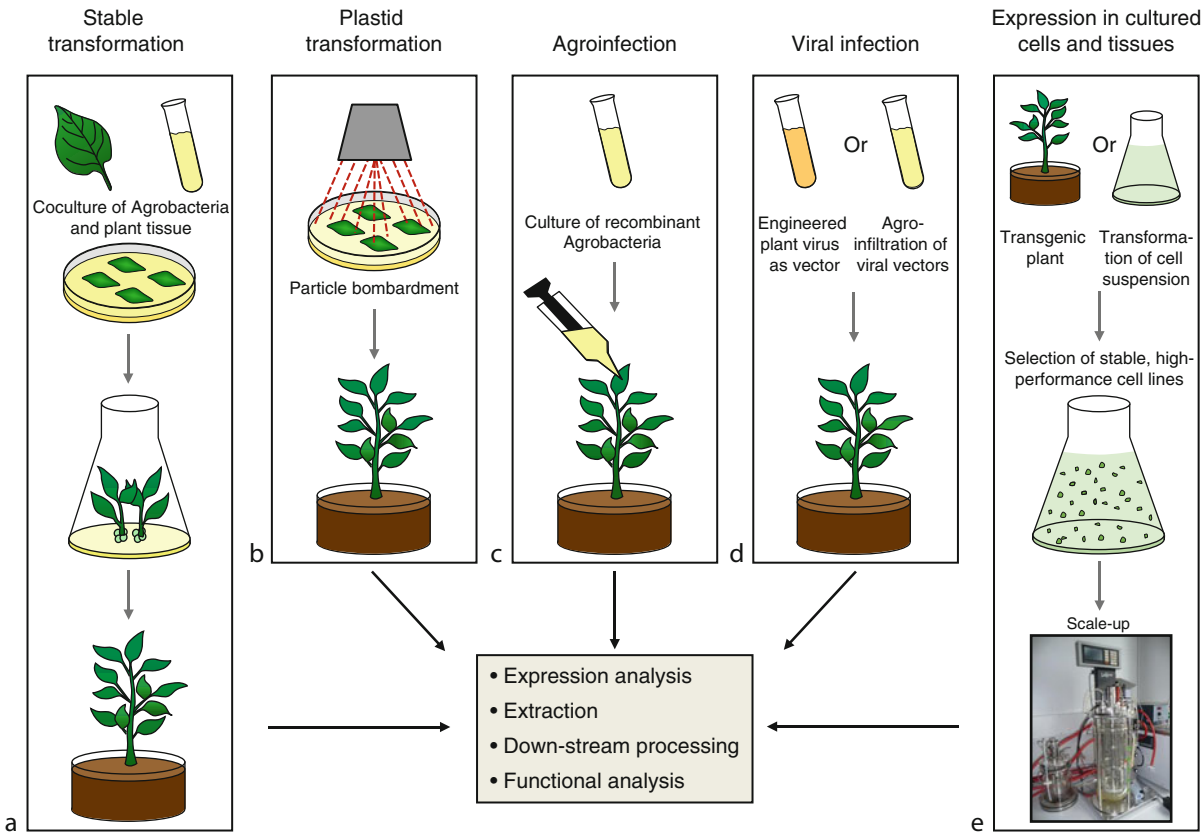
Production shortfalls and high costs are major incentives for further development of alternative antibody production technologies with a focus on active immunization (vaccination). The defining event for the development of "vaccinology" (from the Latin "vacca," meaning cow) dates back more than 200 years. At that time the smallpox vaccine was discovered by Edward Jenner. He inoculated humans with a less virulent, but antigenic related, Cowpox Virus to confer protection against the related human Smallpox Virus. Criteria for the development of veterinary vaccines are different depending on the particular target animals (for review see [38]). Health and welfare of the individual animal are the primary concerns for companion animal vaccines and thus are comparable to those for humans. In contrast, livestock animal vaccines should be inexpensive, prevent, and control infectious diseases of animals used as food to reduce or eliminate health risks to consumers. In some cases, these vaccines are further used to improve the productivity of livestock. To combat zoonotic diseases which are transmittable to humans, e.g., rabies, vaccination of wildlife animals is the method of choice. Furthermore, veterinary vaccines have a significant impact on public health due to the reduction in the administration of veterinary pharmaceuticals such as hormones.

The pioneering work for the expression of vaccines in plants was described in a patent by Curtiss and Cardineau in 1990. They reported the production of the *Streptococcus mutans* surface protein antigen A (SpaA) in transgenic tobacco plants [6]. Subsequently, Mason and co-workers succeeded in expressing the hepatitis B surface antigen in tobacco [37]. In 1993, Usha and co-workers expressed a peptide representing an epitope of the VP1 envelope protein of the Foot-and-Mouth-Disease Virus (FMDV) on the surface of a plant virus particle [55]. This study was the first report of a plant-derived veterinary vaccine. Following this pioneering work, various veterinary candidate vaccines have been produced in a variety of plant species using different expression systems, and they have proven to elicit humoral and mucosal immune responses against toxins, viruses, bacteria, and parasitic pathogens (for reviews see [18, 29, 44, 52, 57]). There are still no plant-derived veterinary vaccines on the market; however, one major step was made at the beginning of 2006 by Dow AgroSciences (DAS, Indianapolis, USA). Their plant cell–expressed vaccine against the Newcastle Disease Virus (NDV), produced in a suspension-cultured tobacco cell line, has gained regulatory approval by the US Department of Agriculture (USDA) Center for Veterinary Biologics – the final authority for veterinary vaccines in the USA [48]. Regrettably, this vaccine has not been introduced to the market. Dow AgroSciences apparently wished to demonstrate that their Concert^{TM} Plant-Cell-Produced system was useful for the production of safe and effective vaccines, fulfilling the approval requirements of the regulatory system [43]. A year prior to the approval of the DAS vaccine, a plant-made scFv, used in the production of a recombinant Hepatitis B Virus (HBV) vaccine in Cuba [41], progressed through the regulatory system and was commercialized. These are the two plant-produced vaccine-related products which have gone through the production and regulatory hurdles, despite nearly 20 years of plant-derived vaccines [43].

Four plant-based expression systems have been developed thus far (Fig. 1):

- Expression in stably transformed transgenic plants including tissue-specific expression (e.g., in seeds or tubers)
- Expression in transplastomic plants
- Transient expression in tobacco leaves (*Nicotiana tabacum, N. benthamiana*) using either plant viruses, *Agrobacterium tumefaciens*, or both to facilitate high accumulation of vaccines and/or antibodies
- Expression in cultured plant cells and tissues, and lower plants including duck weed and mosses

The first plant virus system used was a recombinant Tobacco Mosaic Virus (TMV) where the capsid protein was fused to a malarial epitope [53] followed by others (for review see [43, 57]). "Agro-infection" has been developed as a versatile tool for a rapid production of vaccines and antibodies in transiently



Plant Molecular Pharming, Veterinary Applications. Figure 1

Plant-based expression systems for pharmaceutical proteins. (**a**) Transgenic plants derived by stable transformation, either using *Agrobacterium*-mediated gene transfer [54] or biolistic transformation [1], represent a stable and cheap source for the large-scale production of recombinant proteins. The transgene is genetically fixed and transferred into the next generation. However, the development as well as the selection of a stable transgenic line can take many months. Recombinant proteins may be expressed in the cytoplasm or be localized in other cellular compartments (nucleus, mitochondria, chloroplasts, vacuole, endoplasmic reticulum, or apoplast), or can be produced in different plant tissues (leaves, seeds). (**b**) Transplastomic plants obtained by using particle bombardment often have high yields of the recombinant proteins. However, the system is often not suitable for glycosylated or secreted proteins but this barrier may be overcome soon [27]. (**c**) *Agrobacterium tumefaciens*–mediated transient expression is the standard method for determining if a transgene is expressed *in planta*. Here, a suspension of bacteria is directly injected into the intercellular space of plant leaves either by using a syringe or vacuum. (**d**) Viral vectors can be used for the expression of foreign proteins or of chimeric coat proteins in plants. Two different methods can be used for the delivery of the viral genomes into the plant, either engineered plant viruses (e.g., Tobacco Mosaic Virus) or recombinant Agrobacteria. (**e**) The application of plant cell culture for the production of recombinant proteins is focused on a small number of cell lines, e.g., the tobacco line Bright Yellow-2 (BY-2). Furthermore, transgenic cell lines can be established either from a transgenic plant or by the transformation of cell suspensions either by Agrobacteria or particle bombardment. After selection of stable, high-performance cell lines, the recombinant proteins can be produced in bioreactors under "good manufacturing practice" (GMP)

expressing plant tissues, especially tobacco leaves [15, 16]. Simultaneously, a large number of expression constructs could be tested. This method can easily be scaled up by using vacuum-mediated "Agro-infiltration." Lomonossoff and co-workers positioned a gene of interest (GOI) between the 5' leader sequence and 3' untranslated region (UTR) of RNA-2, thereby emulating a presumably stable mRNA for efficient translation. High-level expression could also be achieved in the absence of RNA-1-derived replication functions using *Agrobacterium*-mediated transient expression. Deletion of an in-frame start codon upstream of the main translation initiation site led to a massive increase in foreign protein accumulation (10–20% of total extractable protein; [47]). The magnICON® system (MagniFection) developed by Icon Genetics (Halle, Germany; now a part of Bayer Innovation GmbH, Düsseldorf, Germany) combined significant mRNA expression enhancement by a TMV-based transient expression vector with systemic delivery based on "Agro-infiltration" [24]. A recent press release announced that Bayer started clinical Phase I study with personalized vaccines from tobacco plants, produced with the magnICON® system, for treatment of non-Hodgkin's lymphoma (http://www.icongenetics.com/html/5954.htm). Stable transformants have been widely used to express antibodies (for review see [9]) and vaccines (for review see [52]) in transgenic plants. The expression of recombinant proteins in storage organs such as seeds [13] resulted in functional and stable products that could be stored at room temperature for extended times without significant loss in amount and activity [51]. Stable expression in dicotyledonous seeds could be significantly boosted by specific regulatory sequences as demonstrated for scFvs [8]. Seed tissues therefore represent a very attractive target for production and extraction of pharmaceutical proteins commercially.

In addition to the expression of recombinant proteins in cultured *Nicotiana* cells (for reviews see [14, 31]), expression in duck weed (*Lemna minor*) and in moss bioreactors are alternative interesting systems providing containment during production. The duck weed system [5] and the moss bioreactors (for review see [11]) provide the possibility of glycan optimization as well. Transgenes could also be targeted to the chloroplast, ensuring that they are embedded in a chloroplast DNA homology region. The number of transgene copies after establishment of homoplasmy was shown to be very high leading to increased expression levels [7]. Epigenetic phenomena (e.g., transgene silencing) are apparently absent in chloroplasts, therefore these plant organelles offer ideal prerequisites for the production of functional vaccine antigens. Moreover, chloroplast DNA is absent in pollen, and thus limits the potential for outcrossing. Unfortunately, chloroplasts lack major post-translational modification machineries such as glycosylation (for review see [57]), and accordingly their utility is limited to molecules which do not require glycosylation.

Edible Vaccines and Purification

The basic idea of edible vaccines was to feed animals with genetically engineered grain directly bypassing purification and complicated and expensive downstream processing.

However, this simple approach has been replaced by plant-derived vaccines because of two main reasons. Firstly, the expression level of the antigen in harvestable parts from the same plant can vary substantially. Secondly, a complete segregation of plants for pharmaceutical or veterinary applications from those meant for human or animal consumption is required [52]. However, another interesting approach making use of high-level expression of recombinant antibodies in legume seeds, e.g., peas [45], was the feeding of neutralizing recombinant antibodies against enterotoxic *Escherichia coli* strains to piglets. These antibodies were sufficiently active in the intestine of the fed animals [28, 46]. A similar approach has been applied for recombinant antibodies against gastrointestinal parasites of chickens, which were expressed in peas [58].

Oral administration is not always the major route for all plant-derived vaccines. In some cases purified antigens are required for injection necessitating the development of specific purification procedures for each product. Two main challenges have to be overcome when purifying proteins from plant material: (1) impurities (proteins, carbohydrates, oils, phenolic compounds, phytic acids, nucleic acids, and other trace products) associated with each plant system must be removed, and (2) low concentrations of the target protein following initial extraction into an aqueous medium have to be avoided. Therefore,

special downstream separation units are required to handle large volumes [39]. Specific methods have to be developed to achieve high amounts of vaccines and/or antibodies in a correctly folded and functional form. The successful development of such methods is also dependent on rather high and stable accumulation of the transgenic proteins *in planta*. Here, fusion to elastin-like polypeptides (ELPs) allows both easy and scalable purification as well as enhancement of the accumulation of the recombinant ELP fusion protein. Elastin-like polypeptides are highly biocompatible proteins. They exhibit the useful property of a thermally responsive reversible phase transition. These characteristics improve the efficiency with which recombinant proteins can be purified. ELP fusion proteins also exhibit reversible phase transition property. This new technology, named "ELPylation," has recently been extended to plant cells and several plant-based expression systems have been evaluated for the production of ELPylated proteins (for review see [22]). The approach has been applied to vaccines [19], complete immunoglobulins [17, 20, 21], and antibody derivatives, scFv [49], as well as VHH [4]. For veterinary purposes, where economical features such as low price and easy-to-handle products are major factors of commercial viability, "ELPylation" is a useful component of enrichment and purification strategies.

Future Directions

Over the past years, plant-based production of recombinant proteins has been developed and 11 plant-derived non-pharmaceutical proteins (avidin, trypsin, β-glucuronidase, aprotinin, lactoferrin, lysozyme, thyroid-stimulating hormone receptor, Hantaan and Puumala viral antigens, peroxidase, laccase, and cellulase) have been brought to the market [52] indicating a huge capability of these expression technologies for the production of diagnostic and therapeutic proteins for both human and veterinary medicine. Six years after the commercialization of the first plant-derived recombinant protein, TrypZean, from corn (ProdiGene, USA), only two plant-derived compounds are in late-stage clinical trials: Interferon α-2b made in aquaculture (*Lemna* expression system, LEX system) for the treatment of hepatitis C infections (Biolex Therapeutics, Pittsboro, USA) and taligurase alfa, a form of the enzyme glucocerebrosidase known as prGCD in development for treatment of Gaucher's disease from Protalix Biotherapeutics (Carmiel, Israel). Recently, Pfizer acquired rights to prGCD produced in carrot cells and became the first big pharma company to commit itself to take to the market a biologic plant-produced drug [42].

In view of the new influenza A H1N1 pandemic, plant-based expression systems represent the fastest production for any influenza vaccine as it was demonstrated by two research groups – at Medicago Inc. (Québec, Canada) and at the Fraunhofer Institute (Plymouth, USA) – via the transient expression of the H1 HA protein in tobacco plants [43]. These results underline the advantages of plant-based expression technologies over traditional expression technologies for the production of antigens. This should be essentially true for veterinary purposes, where costs should be generally lower fitting into economical parameters of animal-based food production. Here, the "old" concept of edible vaccines could be verified much easier, because seeds could be used as a source that is an essential and common component of the feed, which do not need to be treated at harsh conditions as baking or cooking. Farm animals grown in high numbers in confined conditions are a suitable target for future attempts to produce veterinary pharmaceuticals using plant-based expression systems. Nevertheless, further improvement of expression levels and development of easy and cheap downstream processes are still needed before decisions about economic viability of transgenic plant-based pharmaceuticals for animal health could be made.

Acknowledgments

The authors thank Stefan Rose-John and Paul Christou for a critical review of the manuscript.

Bibliography

1. Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15:305–327
2. Behring E, Kitasato S (1890) Ueber das Zustandekommen der Diphtherieimmunitaet und der Tetanusimmunitaet bei Tieren. Dtsch Med Wochenschr 16:1113–1114

3. Conrad U, Floss DM (2010) Expression of antibody fragments in transgenic plants. In: Kontermann R, Dübel S (eds) Antibody engineering, vol 2. Springer, Berlin, pp 377–386
4. Conrad U, Plagmann I, Malchow S, Sack M, Floss DM, Kruglov AA, Nedospasov SA, Rose-John S, Scheller J (2010) ELPylated anti-human TNF therapeutic single-domain antibodies for prevention of lethal septic shock. Plant Biotechnol J. doi:10.1111/j.1467-7652.2010.00523.x
5. Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, Peele CG, Black A, Passmore D, Moldovan-Loomis C, Srinivasan M, Cuison S, Cardarelli PM, Dickey LF (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nat Biotechnol 24:1591–1597
6. Curtiss RI, Cardineau CA (1990) Oral immunization by transgenic plants. World Patent Application, WO 90/02484
7. Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine 23:1779–1783
8. De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, Depicker A, Angenon G (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. Nat Biotechnol 20:1265–1268
9. De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. Plant Biotechnol J 8:529–563
10. De Wilde C, Peeters K, Jacobs A, Peck I, Depicker A (2002) Expression of antibodies and Fab fragments in transgenic potato plants: a case study for bulk production in crop plants. Mol Breed 9:271–282
11. Decker EL, Reski R (2008) Current achievements in the production of complex biopharmaceuticals with moss bioreactors. Bioprocess Biosyst Eng 31:3–9
12. Ehrlich P (1900) On immunity, with special reference to cell life. The Croonian lecture. Proc Roy Soc Lond 66:424–448
13. Fiedler U, Conrad U (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. Biotechnology 13:1090–1093
14. Fischer R, Emans N, Schuster F, Hellwig S, Drossard J (1999) Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. Biotechnol Appl Biochem 30:109–112
15. Fischer R, Schillberg S (2004) Molecular farming: plant-made pharmaceuticals and technical proteins. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
16. Fischer R, Vaquero C, Sack M, Drossard J, Emans N, Commandeur U (1999) Towards molecular farming in the future: transient protein expression in plants. Biotechnol Appl Biochem 30:113–116
17. Floss DM, Conrad U (2010) Expression of complete antibodies in transgenic plants. In: Kontermann R, Dübel S (eds) Antibody engineering, vol 2. Springer, Berlin, pp 489–502
18. Floss DM, Falkenburg D, Conrad U (2007) Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. Transgenic Res 16:315–332
19. Floss DM, Mockey M, Zanello G, Brosson D, Diogon M, Frutos R, Bruel T, Rodrigues V, Garzon E, Chevaleyre C, Berri M, Salmon H, Conrad U, Dedieu L (2010) Expression and immunogenicity of the mycobacterial Ag85B/ESAT-6 antigens produced in transgenic plants by elastin-like peptide fusion strategy. J Biomed Biotechnol 2010:274346
20. Floss DM, Sack M, Arcalis E, Stadlmann J, Quendler H, Rademacher T, Stoger E, Scheller J, Fischer R, Conrad U (2009) Influence of ELP fusions on the quantity and quality of a tobacco-derived HIV-neutralizing antibody. Plant Biotechnol J 7:899–913
21. Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, Fischer R, Conrad U (2008) Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. Plant Biotechnol J 6:379–391
22. Floss DM, Schallau K, Rose-John S, Conrad U, Scheller J (2010) Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. Trends Biotechnol 28:37–45
23. Gahrtz M, Conrad U (2009) Immunomodulation of plant function by in vitro selected single-chain Fv intrabodies. In: Faye L, Gomord V (eds) Methods in molecular biology: recombinant proteins from plants. Humana Press, Totowa, pp 289–312
24. Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection – a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042–2048
25. Goldstein D (1997) Banana vaccines. Sci World 53:4
26. Gomord V, Faye L (2004) Posttranslational modification of therapeutic proteins in plants. Curr Opin Plant Biol 7: 171–181
27. Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 8:564–587
28. Hagemann M (2006) Untersuchungen zur Wirksamkeit oral applizierter Antikörper (produziert in transgenen Hefen und Erbsen) gegen enterotoxische *Escherichia coli* (ETEC) bei experimentell infizierten Absetzferkeln. Dissertation, Tierärztliche Hochschule Hannover
29. Hammond RW, Nemchinov LG (2009) Plant production of veterinary vaccines and therapeutics. In: Karasev AV (ed) Plant-produced microbial vaccines. Springer, Berlin, pp 79–102
30. Hassan S, van Dolleweerd CJ, Ioakeimidis F, Keshavarz-Moore E, Ma JKC (2008) Considerations for extraction of monoclonal antibodies targeted to different subcellular compartments in transgenic tobacco plants. Plant Biotechnol J 6:733–748
31. Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422
32. Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76–78
33. Hood EE, Woodard SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants – myths and realities. Curr Opin Biotechnol 13:630–635
34. Hussack G, Grohs BM, Almquist KC, McLean MD, Ghosh R, Hall JC (2010) Purification of plant-derived antibodies through

direct immobilization of affinity ligands on cellulose. J Agric Food Chem 58:3451–3459
35. Ko K, Brodzik R, Steplewski Z (2009) Production of antibodies in plants: approaches and perspectives. In: Karasev AV (ed) Plant-produced microbial vaccines. Springer, Berlin, pp 55–78
36. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497
37. Mason HS, Lam DMK, Arntzen CJ (1992) Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA 89:11745–11749
38. Meeusen ENT, Walker J, Peters A, Pastoret PP, Jungersen G (2007) Current status of veterinary vaccines. Clin Microbiol Rev 20:489–510
39. Menkhaus TJ, Roseland J (2008) Recovery of proteins from corn and soybean extracts by membrane adsorption. Biotechnol Prog 24:1075–1084
40. Mor TS, Gomez-Lim MA, Palmer KE (1998) Perspective: edible vaccines – a concept coming of age. Trends Microbiol 6:449–453
41. Pujol M, Ramirez NI, Ayala M, Gavilondo JV, Valdes R, Rodriguez M, Brito J, Padilla S, Gomez L, Reyes B, Peral R, Perez M, Marcelo JL, Mila L, Sanchez RR, Paez R, Cremata JA, Enriquez G, Mendoza O, Ortega M, Borroto C (2005) An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification. Vaccine 23:1833–1837
42. Ratner M (2010) Pfizer stakes a claim in plant cell-made biopharmaceuticals. Nat Biotechnol 28:107–108
43. Rybicki EP (2009) Plant-produced vaccines: promise and reality. Drug Discov Today 14:16–24
44. Rybicki EP (2010) Plant-made vaccines for humans and animals. Plant Biotechnol J 8:620–637
45. Saalbach I, Giersberg M, Conrad U (2001) High-level expression of a single-chain Fv fragment (scFv) antibody in transgenic pea seeds. J Plant Physiol 158:529–533
46. Saalbach I, Riehl M, Giersberg M, Kumlehn J, Falkenburg D (2007) Production of recombinant antibodies in pea seeds and their oral application in piglets. In: Xu Z (ed) Biotechnology and sustainable agriculture 2006 and beyond. Springer, The Netherlands, pp 399–402
47. Sainsbury F, Lomonossoff GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol 148:1212–1218
48. Santi L (2009) Plant derived veterinary vaccines. Vet Res Commun 33:S61–S66
49. Scheller J, Leps M, Conrad U (2006) Forcing single-chain variable fragment production in tobacco seeds by fusion to elastin-like polypeptides. Plant Biotechnol J 4:243–249
50. Sharma AK, Sharma MK (2009) Plants as bioreactors: recent developments and emerging opportunities. Biotechnol Adv 27:811–832
51. Stoger E, Ma JKC, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173
52. Tiwari S, Verma PC, Singh PK, Tuli R (2009) Plants as bioreactors for the production of vaccine antigens. Biotechnol Adv 27:449–467
53. Turpen TH, Reinl SJ, Charoenvit Y, Hoffman SL, Fallarme V, Grill LK (1995) Malarial epitopes expressed on the surface of recombinant Tobacco Mosaic Virus. Biotechnology 13:53–57
54. Tzfira T, Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol 17:147–154
55. Usha R, Rohll JB, Spall VE, Shanks M, Maule AJ, Johnson JE, Lomonossoff GP (1993) Expression of an animal virus antigenic site on the surface of a plant-virus particle. Virology 197:366–374
56. Walsh G, Jefferis R (2006) Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol 24: 1241–1252
57. Yusibov V, Rabindran S (2008) Recent progress in the development of plant-derived vaccines. Expert Rev Vaccin 7:1173–1183
58. Zimmermann J, Saalbach I, Jahn D, Giersberg M, Haehnel S, Wedel J, Macek J, Zoufal K, Glunder G, Falkenburg D, Kipriyanov SM (2009) Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. BMC Biotechnol 9:79

Polyculture in Aquaculture

Robert R. Stickney
Texas Sea Grant College Program, Texas A&M University, College Station, TX, USA

Article Outline

Glossary

Benthos Organisms that live on or in the sediments in aquatic environments.

Niche A habitat that provides for the needs to support the life or an organism.

Plankton The community of plants and animals suspended in the water column that drift with the currents. They have limited or no swimming ability but may be able to move vertically.

Phytoplankton The plant component of the plankton community.

Zooplankton The animal component of the plankton community.

Definition of Polyculture

Polyculture is the production of two or more cultured species in the same physical space at the same time, often with the objective of producing multiple products that have economic value. They may be a combination of animals, plants and animals, aquatic species only, or aquatic and terrestrial species.

Introduction

Aquaculture has its roots in China, perhaps as long as 4,000 years ago [1]. Interestingly, polyculture was a part of that early history. The Chinese often stocked multiple species of carp together in ponds to take advantage of all the types of food available. The pond would be fertilized, often with terrestrial animal manure, to promote the growth of the food organisms. In addition, terrestrial vegetation might be added. Thus, each of the species stocked occupied its own ecological niche.

Another form of polyculture that has a long history is rice–fish culture. By modifying rice ponds to provide a refuge for fish when a rice paddy is dewatered (usually a trench down one side or in the middle of the paddy), the rice farmer can obtain two types of crops. The most common fishes employed in rice–fish farming are common carp (*Cyprinus carpio*) and tilapia (*Oreochromis* sp.).

Today, polyculture employs a much wider variety of species than those that have been used for millennia in China, and there have been nuances developed in the polyculture approach. In most cases, the species used in polyculture systems need to be compatible, that is, they need to grow well without interfering with one another. One example is culturing freshwater shrimp (*Macrobrachium rosenbergii*) with tilapia [2]. Exceptions do occur. For example, a predatory species may be stocked to prey on unwanted offspring of the primary culture species. A good example is tilapia culture, where stocked fingerlings may become reproductively active well in advance of reaching desirable market size. A fish predator, such as the snakehead (*Channa* sp.), can be stocked at a size too small to consume the tilapia that are in the system for growout, but large enough to prey upon unwanted tilapia fry.

In marine cage culture, fouling by such organisms as bryozoans and barnacles is often a serious problem. Netting may become so fouled that circulation through the cage is reduced to the point that dissolved oxygen depletions can occur. Depending upon the location of the cage culture operation, such fouling can occur within several days to a few weeks, so frequent cleaning is required. If an animal that will consume the fouling organisms can be found and stocked in the cages, it may be possible to extend the time between manual cleanings. There have also been instances where fish have been stocked in marine cages to consume parasites. One example is the stocking of wrasse (family Labridae) to control sea lice in Atlantic salmon cages in Norway [3]. Another is stocking sea cucumbers (class Holothuroidea) in salmon net pens to feed on fish feces, fouling organisms, and unconsumed feed [4].

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Polyculture is primarily used to enhance total production within an aquaculture facility while maintaining and, in many cases, enhancing water quality. Mussels grown in the vicinity of salmon net pens, for example, remove phytoplankton that take up nutrients associated with the degradation of fish feces and unconsumed feed. Adding a seaweed, such as kelp (order Laminariales), to the system can further reduce the levels of dissolved nitrogen and phosphorus in the water. Polyculture of fish, shellfish, and algae together is an example of integrated multitrophic aquaculture [5]. While Atlantic salmon (*Salmo salar*) is the most common fish cultured using the technique, it has also been used with rabbitfish (*Siganus* sp.) [6].

Another specialized case of polyculture is hydroponics (sometimes called aquaponics), wherein terrestrial plants are grown in a nutrient solution rather than being planted in soil. Hydroponics is usually conducted in greenhouses. The water for the terrestrial plants, which are often a combination of vegetables and/or herbs, is fertilized to provide all the proper nutrients for rapid growth and, of course, provided with sufficient natural or artificial light for photosynthesis. In many cases, one or more aquatic species (fish or shellfish) are incorporated into the system.

Many nations culture fish in ponds that receive avian, livestock, or even human waste as fertilizer. The practice is particularly common in developing countries where inorganic fertilizer is expensive and often difficult to obtain. Common carp and tilapia are often grown in ponds that receive the waste from the terrestrial animals.

Key Principles

Polyculture is a Technique that Produces Two or More Compatible Species Within the Same Culture System

Not every species in a polyculture system needs to be aquatic in nature. Combinations of terrestrial and aquatic species are common. One or more of the species in a polyculture system may be a terrestrial or an aquatic plant.

Polyculture Systems can Increase the Profitability of a Culture System

By culturing two or more marketable species together in the same culture system or in close proximity to one another, it is often possible to increase the amount of income generated by a producer. While it may not be economically possible to rear one of the species profitably alone, by polyculturing it with a high-value species, total revenue can be increased. An example is culturing seaweed that is a good source of agar or carrageenan and that could add value to a culture facility if grown in the vicinity of a higher value crop like salmon [7].

Polyculture Systems can Reduce the Environmental Impacts from Aquaculture Systems

Nutrients released from cultured fish and shellfish-culture facilities can be effectively removed from the water by culturing plants such as seaweeds in close proximity to the animals [7]. An alternative approach would involve the nutrients from such species as fish or shrimp to support phytoplankton that could, in turn, provide a food source for filter feeding Mollusca, such as oysters, scallops, and mussels [5].

Polyculture Successes

As mentioned, polyculture was developed 1000s of years ago [1] and must be considered successful without question since it is still being employed, not only in China, but also in many other countries. The objective of the Chinese system is to stock species that take advantage of the various available food sources in culture ponds. Silver carp (*Hypophthalmichthys molitrix*) consume phytoplankton, bighead carp (*Hypophthalmichthys nobilis*) graze on zooplankton, mud carp (*Cirrhinus molitorella*) feed primarily on detritus, black carp (*Mylopharyngodon piceus*) are mollusc eaters, while grass carp (*Ctenopharyngodon idella*) consume higher vegetation. Grass carp will eat some species of aquatic macrophytes and will also consume various types of terrestrial plants that may be added to the culture pond. Other species that are used in carp polyculture systems are common carp (*Cyprinus carpio*) and tilapia (*Oreochromis* spp.). There are a wide variety of fish, shellfish, and plants that are used in polyculture around the world. In many cases, they are local species, though introduced species are also commonly found – in many cases those were introduced decades ago, such as is the case with tilapia in Asia and the Americas.

Integrated multi-trophic aquaculture systems have a relatively short history, but appear to be highly effective and are a modern approach to polyculture that is being increasingly adopted. Thus, that approach can also be considered a success. The approach typically involves the co-culture of carnivorous species, such as finfish or shrimp, with a filter feeder and a primary producer, typically some kind of algae [8, 9].

Hydroponics can be considered a success, in that the approach can be used to produce a variety of vegetables and other types of plants. While capital intensive, hydroponic systems can be profitable. Various species of aquatic animals have been grown in such systems to provide nutrients that the plants can use and also to produce an additional marketable product. The aquatic animals cannot be counted upon as the only or even the primary nutrient source for the plants, however. The nutrient levels in the aquatic-animal waste products are insufficient in volume and composition, so a nutrient broth needs to be provided.

Future Directions

Biofloc aquaculture is an approach that has been around since the 1970s, but has as yet to be widely adopted. In biofloc systems, the development of high levels of nonpathogenic bacteria in the water is encouraged. Heavy aeration is required to maintain the dissolved oxygen level for the culture species, which is most commonly shrimp, though such systems have also been used in conjunction with tilapia and other fishes. As more experience and research has occurred over the years, the methods for developing and controlling water quality in such systems have advanced. In 2009, one research group was able to produce shrimp at a final density of 9.75 kg/m^3, which is as much as ten times higher than what is typical [10]. It is likely that the approach will be more widely adopted in the future.

Open-ocean aquaculture is expanding rapidly around much of the globe. In the USA, marine aquaculture is largely confined to production of molluscs in coastal waters and salmon cage and net pen culture in protected waters. Once a regulatory framework for the United States Exclusive Economic Zone has been promulgated, there is a likelihood that offshore aquaculture facilities will be developed. It seems reasonable to assume that a polyculture approach similar to the one that is commonly seen with respect to salmon culture, particularly in Chile, will be employed in conjunction with offshore aquaculture as a means of helping prevent water-quality degradation and increasing profitability. Offshore polyculture may involve rearing molluscs suspended from platforms used as support facilities for sea-cage culture operations. Such support structures may provide housing for the culturists, feed, and equipment storage, and may also be used as hatcheries (oil and gas platforms being appropriate once they are out of production and which have been of interest to some prospective aquaculturists). Seaweeds could also be produced, so an offshore operation might employ the multitrophic approach to culture.

Bibliography

1. Stickney RR (2009) Aquaculture: an introductory text. CABI, Wallingford, United Kingdom p 304
2. Brick RW, Stickney RR (1979) Polyculture of Tilapia aurea and Macrobrachium rosenbergii in Texas. Proc World Maricult Soc 10:222–228.
3. Sayer MDJ, Treasurer JW, Costello MJ (1996) Wrasse biology and use in aquaculture. Wiley, New York p 296.
4. Ahlgren MO (1998) Consumption and assimilation of salmon net pen fouling debris by the red sea cucumber *Parastichopus californicus*: implications for polyculture. J World Aquacult Soc 29:133–139
5. Chopin T, Bastarache S (2004) Mariculture in Canada: finfish, shellfish and seaweed. World Aquacult 34(3):37–41
6. Mmochi AJ, Mozes N, Kissil G, Dubi AM, Jiddawi NS, Mwangamilo J (2001) Design and preliminary results of an integrated mariculture pond system (IMPS) at Makoba, Zanzibar. Tanzan Mar Sci Devel In Tanzan East Afr 1:431–450
7. Troell M, Nilsson C, Buschmann A, Kautsky AH, Kautsky N (1997) Integrated marine cultivation of *Gracilaria chilensis* (Gracilariales, Bangiophyceae) and salmon cages for reduced environmental impact and increased economic output. Aquacult 156:45–61
8. Neori A, Troell M, Chopin T, Yarish C (2007) The need for a balanced approach to blue revolution aquaculture. Environ 49:37–43
9. Ridler N, Wowchuk M, Robinson B, Barreington K, Chopin T, Robinson S, Page F, Reid G, Szemerda M, Sewuster J, Boyne-Travis S (2007) Integrated multi-trophic aquaculture (IMTA): a potential strategic choice for farmers. Aquacult Econ Manage 11:99–110
10. Samocha T, Correia E, Hanson T, Wilkenfeld J, Morris T (2010) Opeation and economics of a biofloc-dominated zero exchange system for the production of Pacific white shrimp, *Litopenaeus vannamei,* in greenhouse-enclosed raceways. In: Proceedings of the AES issues forum, Roanoke, 18–19 Aug 2010

Poultry Breeding

Yoav Eitan, Morris Soller
Department of Genetics, The Silberman Life Sciences Institute, The Hebrew University of Jerusalem, Jerusalem, Israel

Article Outline

Glossary

Breeding value The deviation of trait value of an individual from the overall population mean that is due to genetic factors and hence can be transmitted to the progeny.

Breeding nuclei The closed and pedigreed flocks at the top of the breeding pyramid within which selection is practiced and from which genetic gains are disseminated to the commercial populations.

Feed conversion ratio The weight of food in kg required to produce 1 kg of body weight to market.

Fitness The ability of the animal to reproduce and survive.

Functional traits Traits that contribute to fitness, e.g., reproductive performance, resistance to pathogens and toxins.

Genetic variation Variation in trait expression that is due to variation in structure of the genetic material.

Heritability The proportion of total variation in trait expression that is due to genetic variation.

Index selection Selection based on combination of estimated breeding value of an individual for a number of production and functional traits weighted according to economic importance and genetic correlations to other traits.

Mass selection Selection based on the performance of the individual itself, without consideration of performance of close relatives.

Metabolic heat and water Heat and water produced as a by-product of body metabolism.

Pleiotropic effects Effects of an individual genetic locus on multiple traits.

Primary breeders The commercial organizations that maintain and improve breeding nuclei.

Production traits Traits that contribute directly to production of the animal product that reaches the consumer, e.g., JWfA, FCR, and PBM.

Secondary effect An unintended effect of selection for one trait on some other trait.

Definition of the Subject

Animal breeding today stands on the verge of a methodological revolution that may greatly increase the rate of genetic improvement in production traits. This will modify the physiology of the animals and the genetic architecture of the population at an unprecedented rate. What will be the broader consequences of such extreme modification? What pitfalls and dangers may be encountered as this process unfolds? Beginning about 60 years ago, the broiler chicken has been subject to intense and effective selection for juvenile growth rate (60 generations), feed conversion ratio (40 generations), and body composition (20 generations). This entry describes the ways in which the production and functional traits of the individual bird and

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

accompanying management practices have responded to this unremitting selection, with the objective of suggesting what the future might hold for other agricultural animal species as they enter this new stage of intense and highly effective selection for production traits.

Introduction: Animal Breeding in Transition

Animal breeding today stands in the midst of a methodological revolution based on the availability of microarrays that are capable of genotyping tens and hundreds of thousands of polymorphic markers in a single run, and of low-cost whole genome sequencing that will enable the leading animals in the population to be fully sequenced [1–3]. This has enabled development of deoxyribonucleic acid (DNA)-level diagnostic procedures for highly accurate evaluation of breeding values for production and functional traits. Since DNA can be obtained readily from all animals from birth (and even before) these procedures allow highly effective and intense selection to be implemented at an early age with very high accuracy, and with equal efficiency in males and females; encompassing sex, age, or phenotype-limited characters such as milk and egg production, longevity, and disease resistance. The result will be a quantum leap in the rate of genetic improvement in production traits with consequent far-reaching modification of body development and physiology.

What will be the effects of such intense and effective selection applied to production traits on overall animal performance and capacities? How will management practices and requirements adapt to these changes? The modern broiler chicken, under selection for production traits for over 60 chicken-generations [4] provides an intimation of what to expect [5–9]. The main broiler production traits such as juvenile growth rate, feed conversion ratio, and body composition come to expression in the juvenile bird, are expressed equally in males and females, and have moderate heritabilities, thus enabling highly effective mass selection based on individual phenotype. Introduction of advanced biometrical methods of selection such as Best Linear Unbiased Predictor (BLUP) and Individual Animal Model has made this phenotype-based selection even more effective. Because of the high fecundity of the female chicken, selection for broiler production traits has been intense, and the response to selection has been enormous, converting the broiler chicken from an expensive luxury to a low-cost major meat supplier for the growing human population worldwide [10]. The historical response of the broiler chicken and its management to this selection, models what can be expected from the application of intense and highly effective selection for production traits to other populations of agricultural animals.

Brief History of Broiler Breeding and Broiler Production

The First Commercial Incubator

Keeping a small number of chickens was always a part of the family farm and rural urban household. These were raised primarily for home egg consumption and minor income from sale of surplus eggs and meat. Until the invention of the first commercial incubator in 1875, replacement chicks had to be hatched by the hens, so that each farm reared its own replacements. With the availability of commercial incubators, specialized breeding farms and hatcheries came into being to supply high-quality chicks to the family farms. Prior to the development of vent sexing by the Japanese in the 1930s, and of sex-linked feather sexing in the 1960s, it was not possible to distinguish male and female chicks by gender until they were a few weeks of age. Consequently, when rearing chicks for egg production, all chicks had to be reared to the age of a month or more when cockerels could be recognized. Having invested this much in the cockerels, it was economically rewarding to rear them to market weight. Thus, each year with the renewal of layer flocks by chicks hatched in the early spring, cockerels for sale provided an important secondary income stream. At this time, therefore, chicken meat was a rather rare and costly commodity generally available only in the spring coincident with layer flock renewal. However, recognizing the potential for additional economic return, some farms began raising an extra hatch of chicks in the summer months for sale as meat birds.

Salmonella pullorum Free Flocks, Vitamin D

Large flocks dedicated to poultry meat production as a main source of farm income do not seem to have become widespread until the 1920s, primarily because of high chick mortality due to *Salmonella pullorum*

when large numbers of chicks were reared together. In the early 1920s, a test was developed to identify birds carrying the pathogen, enabling breeders to develop *S. pullorum* free flocks that supplied *S. pullorum*-free chicks. Also about this time (1926), it was found that adding vitamin D to the chick diet in the form of cod liver oil could prevent the development of Rickets in winter broilers. Until then, lack of sunshine had prevented rearing of winter broilers in the Northern states. These two developments made it possible to establish a year round broiler industry [11].

Cornish Breed Males, the "Chicken of Tomorrow Contests," Chick "Sexing"

Growth of the broiler industry was rather slow, and chicken meat remained an expensive specialty item because of the necessity for hand plucking of the feathers. In 1940 the first commercial feather plucker was invented, enabling plucking, evisceration, and packing of broiler carcasses on the same production line. This opened the way to development of a dedicated broiler industry. The use of Cornish breed males to cross with dual-purpose females to produce broiler chicks with higher proportion of breast meat (PBM) was introduced in the 1930s, and by 1942 almost all commercial broiler chicks were crossbreds of "Cornish" males to females of one of the dual-purpose breeds (White Rock, New Hampshire or Barred Rock). In the 1940s, some of the leading White Rock breeders began to develop a bird with high juvenile growth rate specifically for broiler production, while the colored stocks were still bred for the dual-purpose market. From 1946 to 1951, the A&P supermarket chain sponsored a series of "Chicken of Tomorrow" contests [12] in which chicks of leading breeders were reared to 12 weeks of age and compared. By the third of these contests it was clear that the White Rock female, specifically bred for broiler production crossed to Cornish type male was the leading broiler chicken, widely preferred to crosses to dual-purpose colored females. In addition to superior growth rate, the White Rock crosses also gave a clean attractive plucked carcass, in contrast to the unsightly dark-colored pinfeathers remaining on the plucked carcass of the dark-feathered birds.

Thus, by 1952 all the components of the modern broiler chicken were in place. In this year for the first time, specially bred broilers surpassed surplus farm cockerels of the layer flocks as the main source of chicken meat in the USA. With the advent of specialized broiler lines, it was no longer economical to rear layer strain cockerels for meat, as the cockerel of the layer lines, even of dual-purpose breeds, could not achieve the economic returns of the specialized broiler lines. Vent and later feather sexing enabled the male chicks of the layer flocks to be identified at hatch, so that there was no economic incentive to rear these chicks. As a direct consequence, the poultry breeding industry was able to separate into the distinct and completely independent layer and broiler components found today, and intensive broiler breeding focused on the traits of importance for low-cost broiler production commenced in earnest.

Roadmap of the Entry

Broiler breeding experienced three stages with respect to the production traits that were the primary selection objectives. In the first stage, the primary production objective was rapid juvenile growth rate, with some attention to breast conformation. In the second stage, increasing attention was paid to feed conversion ratio in addition to juvenile growth rate [13]. In the third stage, continuing to the present time, increasing the proportion of breast meat in the carcass was added as a third major production goal. At all stages except the very first, good reproductive performance of the female line and fertility of the male line were strong secondary selection objectives. The need to maintain female reproductive performance led in the 1970s to the introduction of crosses between independent White Rock female lines to provide a heterotic lift to performance. Thus, the typical commercial broiler chick today is the product of a three-way cross: A pair of purebred "female lines, of dual-purpose White Rock origin," is crossed to produce the female parent, and this in turn is crossed to males of a purebred "male line of Cornish breed origin" to produce the commercial chick. With the passage of time, selection for health and welfare traits that were negatively affected by the intense selection for production traits has become an increasingly important component of the broiler selection programs.

Each of these three major stages in genetic development of the modern broiler will be considered in turn, describing first the achievements in terms of the

primary production goals, followed by a description of the associated secondary genetic effects on reproductive performance (hatchability and egg numbers) and health (with emphasis on leg and metabolic disorders), and of the management modifications needed to accommodate both the primary genetic effects and their secondary consequences. A common feature of the secondary consequences was inability to predict them in prospect, but ability to explain their appearance in retrospect as a plausible consequence of the changes in primary traits. This will be followed by an overall evaluation of the relative contributions of the breeder and of management to performance of the modern broiler, and a review of possible sources of the genetic variation that has enabled the continuous long-term response to selection for the three major production traits. Welfare issues raised by the genetic and management changes will be addressed, and a framework presented for interpreting the accompanying genetic and management changes in terms of a "resource allocation" model.

Stage 1: Selection for Juvenile Weight for Age (JWfA)

Importance of Juvenile Weight for Age

A day-old chick of one of the dual-purpose breeds that served as the founders for broiler development, typically required up to 90–120 days to reach market weight [4]. This long grow-out period resulted in high feed, labor, veterinary, and capital costs per chick and increased losses by mishap or disease. The number of days to reach market weight is primarily determined by juvenile growth rate – birds growing more rapidly reach market weight earlier. Consequently, increasing juvenile growth rate was the first objective in the development of the modern broiler. The heaviest birds at any given age are obviously the birds that would have reached target weight earliest, so that selection for "age to reach a given target weight" (age for weight) could be achieved by selection for the much more conveniently measured "weight at a given age" (weight for age).

Rapid and Powerful Response to Selection for JWfA

Juvenile weight for age (JWfA) turned out to be an ideal target for selection. The trait had high heritability, meaning that much of the variation in the trait was due to genetic factors. It was easily measured by simply weighing the birds at a given age. It came to expression at an early age equally in males and females. Due to the very high fecundity of hens, only a small proportion of the female chicks and an even smaller proportion of the male chicks produced by a hen had to be retained to renew the breeding population. Consequently, selection was very intense. The response to selection for JWfA was magnificent and continues to the present time yielding a decrease of 1 or 2 days in age for market weight per generation of selection [14] so that the modern broiler reaches market weight at about 35–40 days. This tremendous reduction in market age has reduced broiler-rearing costs in a corresponding manner, bringing chicken from a high-cost luxury food to the lowest-cost meat producer on the market [10]. It should be noted that even during this early period, attention was also paid to breast conformation of the broiler chick [6]. This was needed in order to produce a more attractive whole carcass with a less prominent keel bone on the supermarket shelf. Usually this was done by "touch," i.e., simply handling the birds already selected on JWfA and rejecting those with the sharpest keel bone.

Secondary Effects of Selection for JWfA (I): Excess Adiposity

The first secondary effect of the selection for JWfA, a marked increase in the adiposity of the commercial broiler at slaughter, was noted by the mid-1960s [6, 15]. This was unexpected, since deposition of a gram of fat is more than twice as costly in caloric terms as deposition of a gram of muscle. Thus, the heaviest birds would be expected to be those that had deposited the least fat. Consequently, selection for JWfA should have reduced the proportion of food intake devoted to fat stores, and in this way reduced the proportion of fat content of the carcass. The situation turned out to be more complex. Depositing body mass in a growing bird requires an excess of food intake over body maintenance requirements. Given unlimited access to feed, the magnitude of this excess is a function of appetite. Thus, JWfA is primarily a function of appetite and gross food intake. The birds with the highest intake put on the most weight. However, this high intake introduces a second

factor into the picture. Any excess of intake over maintenance in the juvenile is partitioned between lean body growth (muscle and internal organs) and fat stores. It turns out that the coefficient of partition is itself a function of the excess. When excess is small, almost all goes to build lean body mass (low fat:lean coefficient) but as the excess increases, a greater proportion goes to fat stores (higher fat:lean coefficient). Thus, the animals presenting highest JWfA are primarily those with the highest food intake but these also have the highest fat:lean coefficient, and hence show increased adiposity. The increase in carcass fat content made the carcass unsightly and unattractive to consumers. In addition, the abdominal fat-pad that was discarded in the slaughterhouse represented a waste of feed and was clearly reducing feed conversion ratio on a salable meat basis. This led in the early 1970s to various attempts to select directly against excess fat, e.g., by measuring skin thickness. These approaches were not successful, but control of fat content eventually came from an unexpected direction, as will be seen later.

Secondary Effect of Selection for JWfA (II): EODES in Broiler Breeder Females

A second secondary effect of the intense selection for JWfA was not long in coming and presented as the first of a series of metabolic disorders (see Box 1 for definition and explication) that have afflicted the broiler industry. The disorder manifested as a marked reduction in reproductive performance of the female broiler parent. Egg production dropped precipitously, from over 200 hatching eggs per hen to 120 and below, accompanied by a reduction in fertility and in the hatchability of fertilized eggs. Egg-laying pattern was erratic with many eggs laid outside of the usual time window, and there was a marked increased in double-yolked or otherwise defective eggs. The condition as a whole was termed "Erratic Oviposition Defective Egg Syndrome, EODES" [17]. An outstanding feature was the presence of multiple hierarchies of large yellow follicles in the ovary. The broiler industry was on the verge of collapse, when it was found that restricting (i.e., limiting) feed intake of the broiler pullet during her growth period could completely prevent the problem [18–20]. With this discovery, feed restriction became an essential part of broiler breeder

Box 1 Metabolic Disorders

Metabolism refers to all of the myriad chemical reactions taking place in the cells of a living organism that utilize the chemical and energetic content of ingested food to build and maintain normal body structure and function. These cellular chemical reactions are actively coordinated with one another across the body of the organism and also with the external environment through an intricate network of sensors and regulatory feedback loops so as to maintain life (i.e., stable body function). An outstanding feature of the metabolic system is the ability to actively adapt and modify body structure and function so as to maintain life even under challenging environments.

The metabolic system consists of many thousands of structural, functional, and regulatory components composed of protein or ribonucleic acid (RNA) molecules. All of these are constructed according to instructions coded in the hereditary material of the cell, i.e., its DNA. Mutations in DNA can change the structure or function of the components of the metabolic system. When this happens, the metabolic system may not be able to preserve normal function, and development terminates in the death of the embryo, or in birth of an individual defective in one or more aspects of normal structure and function. Metabolic derangements of this sort are termed "Metabolic Diseases," and there are many hundreds of different hereditary diseases that fall into this category.

Normal metabolism may also be disrupted when the diet does not include essential components that cannot be synthesized by the body but are needed to build the elements of the metabolic system, or conversely, when the diet contains various elements or compounds in toxic excess. Metabolic derangements of this sort are termed "Metabolic deficiency or toxicity disorders". Examples of broiler "Deficiency Disorders" are Rickets, resulting from Vitamin D or mineral deficiencies, and Crazy Chick Disease, resulting from Vitamin E deficiency. Examples of broiler "Toxicity Disorders" are Fluoride and Vanadium toxicity caused by phosphate contamination of feeds, or Selenium toxicity caused by feeding cereals grown on Selenium-rich soil.

The Metabolic Syndrome is a novel form of Metabolic Disorder, which has recently become very

prominent in human populations [16]. This is a constellation of pathologies that increase the risk of artherosclerotic vascular disease and Type II (late onset) diabetes. Risk factors for development of Metabolic Syndrome include obesity, psychosocial stress, sedentary lifestyle, and age. There are no identified genetic lesions, dietary deficiencies, or toxins. Thus, all elements of the metabolic system are present and at an individual level appear to be functioning normally. The central role of obesity in the etiology of the syndrome is of special interest. A moderate degree of adiposity is a normal part of body structure. The very high adiposity defining obesity is thus a normal body structure taken to extreme. Apparently, this excess alone unbalances the metabolic system and imposes a regulatory burden to maintain stable function. When the adiposity load is further exacerbated by the regulatory load imposed by psychosocial stress, sedentary life style, and age, the metabolic regulatory system is unable to cope and the pathologies characteristic of the Metabolic Syndrome emerge. This example indicates that whenever some body structure or function is brought to extreme development, this may impose an overload on the metabolic regulatory system resulting in manifestation of Metabolic Regulatory Disorders, with specific pathology depending on the type of overload.

management, both for males and females. With the passage of generations, it has been necessary to increase the degree of feed restriction from 70% of *ad libitum* initially to about 35% of *ad libitum* today. Since EODES is elicited by *ad libitum* feeding and prevented by restricting feed intake, it is plausible that the proximal cause of this disorder was the increased adiposity of the *ad libitum* fed broiler pullet at entry into lay. Excess adiposity is known to interfere with proper reproductive function in many species. The excess adiposity in turn is due at least in part to disruption of the normal homeostatic control over appetite as a consequence of which *ad libitum* fed female-line broiler pullets continue to eat to excess during the entire pullet rearing stage. The hypothalamic appetite control center may not be fully functional in broilers, since lesions of the hypothalamic appetite control center that lead to hyperphagia in layers, do not increase feed consumption in broilers [21].

The reduced fertility that accompanied the reduction in egg number apparently resulted in part at least, from impairment of male mating performance, perhaps due to sheer physical difficulty of the obese males and females to handle the mechanics of courting and mating or possibly due to hormonal effects on semen quality of the excess adipose tissue in the male. Feed restriction corrected the reduced fertility as well, so that under pullet and male feed restriction, egg production and fertility returned to high levels.

Secondary Effect of Selection for JWfA (III): Increase in Egg Weight and Hatchability Problems

A third secondary consequence of selection for JWfA was an overall increase in egg weight [14] particularly in older hens. The primary reason for this was the increase in mature size of the broiler female, even under feed restriction, as a direct consequence of selection for JWfA. In addition, there may have been indirect selection for large egg size, as a larger egg delivers a larger chick at hatch and higher final weight at market age. The increase in egg size had important effects on incubator management, deriving from the dual function of the incubator in embryo development. The small early stage embryo generates very little internal metabolic heat and must be warmed by the incubator, while the larger and rapidly growing later-stage embryo generates large amounts of metabolic heat and water, both of which must be removed by the incubator (see Box 2). Achieving these multiple goals requires careful control of incubator temperature, humidity, and ventilation (THV). Matters are complicated by the fact that even under the best conditions there is variation in the specific THV conditions at different points within the incubator due to variation in airflow over the eggs, and location of the egg with respect to incubator inlets and outlets. There is also variation in egg size and in shell conductance for water and heat. All of these contribute to variation in internal embryo temperature and water content among the population of eggs within the incubator at any given time. As a result, while most eggs will be comfortably within their optimum temperature and water content, others may lie close to outside limits for one or both of these factors.

Larger egg size impacts negatively on all of the multiple incubator functions. At the early embryo

Box 2 Incubator Management

During the early part of incubation, the embryo generates very little metabolic heat, and must be warmed by the incubator. But during the final week of incubation, the large and rapidly growing embryo generates large amounts of metabolic heat, and must be cooled by the incubator. Both objectives are achieved by setting the incubator air temperature at about 37.5°C. This is sufficient to warm the early embryos, and at the same time, with adequate air movement, will cool the later embryos. Thus, the same incubator (called a "multistage" incubator) was able to accommodate eggs in different stages of development, warming the early embryo eggs and simultaneously cooling the late-embryo eggs. In fact with the multistage incubator, the excess heat produced by the late embryos was used to warm the early embryos.

In addition to producing metabolic heat, the developing embryo also produces water and carbon dioxide. Both carbon dioxide and water escape through the eggshell. However, loss of water must be adjusted carefully. Loss of too much water will dehydrate the chorioallantoic membrane, interfering with importation of oxygen and exportation of metabolic waste, resulting in death of the embryo. It is equally important that the embryo lose more water than it generates (net loss of about 12% of egg weight from beginning to end of incubation). Otherwise, there will be insufficient air space formed for the chick to take its first breath and the chick itself will remain too large to move around inside the shell when trying to pip. In this case, the chick may be unable to break the membrane between itself and the shell, suffocating or drowning to death. Water loss by the embryo is adjusted primarily by adjusting the humidity of the incubation chamber; 50% humidity is close to optimal.

stages, the larger egg makes it more difficult to achieve optimal temperature throughout the egg mass. At the later embryo stages, the larger egg accommodates a larger embryo that generates more metabolic heat and water toward the end of embryonic development. At the same time, the surface/volume ratio of the larger egg is reduced making it more difficult for the embryo to dissipate excess metabolic heat and water [22]. Taken together with variability in TMV conditions within the incubator, and in size and shell conductance of the eggs, an increasing proportion of eggs find themselves outside of the optimal parameter range for development and hatching, yielding grade B chicks, and lower overall hatching proportions. This is exacerbated by the increased difference in egg size between young and old breeder hens that increases variability in egg size and shell conductance within the incubator when eggs of different-aged flocks are incubated together. Thus, as egg weight increased, incubators had to be equipped with more effective ventilation systems, and conditions had to be monitored more closely than in the past to ensure minimum variation and close adherence to optimal temperature and humidity throughout the incubator interior.

Secondary Effect of Selection for JWfA (IV): Skeletal Problems in Commercial Broiler

Initially, broiler chicks were allowed to free access to feed during daylight hours from the first day post-hatch, with the feed provided in the form of coarsely ground grain ("mash" type feed). However, by the 1970s as growth rate increased, the chicks were unable to consume the amounts of feed needed to meet their growth potential. This was solved by compressing and heating the mash to form pellets, enabling the bird to ingest more feed in a shorter time; and by extending hours of light to 23 h per day to provide more time for feeding. It was later found that equivalent results at lower electricity cost could be obtained by alternating 1–2 h of light with 2–4 h of dark ("intermittent light") with the added advantage of reducing energy expenditure for locomotor activity by the growing broiler. Obviously implementing a light-control program of this sort required a shift to light-controlled housing.

However, as chick growth rate increased, the skeleton could not develop rapidly enough to accommodate the large chick body, resulting in many skeletal and leg problems [13, 23, 24]. These include: infected hocks (from staphylococcus, coliform, or viral infections); twisted legs presenting as valgus distortion (knock-kneed: hocks in feet out) or varus distortion (bow-legged: hocks out, feet in); and tibial dyschondroplasia, in which failure of normal chondrolysis and ossification leaves the end-bone epiphyseal plates at the

femoral–tibial junction prone to fracture, infection, and abnormal development resulting in lameness, and swelling of the femoral–tibial joints. These problems did not appear during rearing of broiler parent flocks from day old to maturation with restricted feeding, indicating that the skeletal and leg problems were a result of the very rapid and unbalanced growth rate of the broiler chick, rather than being innate to the animal. This led to more tailored feeding and lighting programs for young broiler chicks, holding growth below the potential in the early part of the growth period, by returning to mash type feed or otherwise decreasing the nutritional content of the diet, and by decreasing hours of light [23, 25]. In the final weeks of the broiler growth period, after the skeleton and body frame were more solidly formed, photoperiod was increased to maximize feed intake, and diet was shifted back to nutritionally dense pelleted form to allow full compensatory growth. Skeletal abnormalities were a leading cause of mortality and carcass condemnations in broiler production in the 1970s. At the present time, however, due to genetic selection to reduce their incidence and formulation of suitable management protocols, skeletal deformities are well controlled.

Stage 2: Combined Selection for JWfA and Feed Conversion Ratio (FCR)

Random sample tests of broiler chickens from different breeding firms were instituted in the 1960s to provide unbiased information as to broiler chick quality. In addition to differences in JWfA, these also showed large differences in feed conversion ratio (FCR) among the various breeders, independent to an appreciable degree of juvenile growth rate. This was surprising, since the metabolic efficiency of feed utilization was thought to be highly correlated with evolutionary fitness and hence it was anticipated that there would be little genetic variation in the trait. Because of the economic importance of FCR, starting in the 1970s breeders included this in their breeding programs as a second major objective along with JWfA, with considerable success.

Further consideration led to the realization that although the metabolic efficiency of catabolism and anabolism may not vary, variation in motor activity, carcass adiposity, and possibly protein turnover rate [14, 26, 27] may all contribute to genetic variation in FCR. Consequently, a positive albeit unexpected secondary effect of selection for reduced FCR was a gradual but steady reduction in carcass adiposity [6, 9], apparently by reducing the fat:lean partition coefficient at high intake. Thus, at the present time, excess broiler fat content of the commercial broiler is no longer considered a problem by the broiler industry.

Secondary Effect of Continued Selection for JWfA and FCR (V): Adverse Effects on Reproductive Performance of Broiler Female and Male Parents

The late 1970s and 1980s, however, brought about a number of additional problems, apparently secondary effects of the continued selection for JWfA and FCR. First of all, female and male reproductive performance again became problematic. As noted, to control EODES, female pullets were reared under feed restriction. Initially, birds of the reproductive flock were shifted to *ad libitum* feeding to induce entry into lay, and remained on *ad libitum* feeding during the entire reproductive period. However, in the 1970s while peak production remained high, post-peak production dropped rapidly. This was apparently due to continued effects of the loss of appetite control that led to overeating and fat accumulation during the laying period, by both females and males. As a consequence, feed restriction was extended into the laying period itself [18, 19]. This continues to the present time and is effective in maintaining egg production.

The extension of feed restriction to the laying period had a further positive effect in that egg weight is closely related to actual body weight and feed consumption, so that egg weight can be closely controlled by monitoring body weight and feed intake during the reproductive period. Control of egg weight in this manner, however, is limited by the ever-increasing optimum mature body weight of the female parent even under feed restriction. This is due in all likelihood to an increase in threshold weight for sexual maturity that has all along accompanied the increase in JWfA.

Secondary Effect of Continued Selection for JWfA and FCR (VI): Increased Water Consumption, Nipple Drinkers

The increased daily feed consumption of the rapidly growing broiler chick required a proportional increase

in water consumption to enable the gizzard and digestive system of the bird to deal effectively with the dry feed. Drinking the additional water from the standard "Bell drinker" resulted in increased water spillage and wet litter causing a host of veterinary and welfare problems, such as foot pad and breast dermatitis, and ammonia production interfering with proper breathing and contributing to pulmonary hypertension syndrome (PHS). This was solved in the early 1980s by the introduction of the "nipple drinker" in which water drops are drawn directly into the chicks' throat in this way greatly reducing spillage and the attendant problems with wet litter.

Secondary Effect of Continued Selection for JWFA and FCR (VII): Delayed Entry into Lay

In the 1980s, a new set of problems arose related to entry of the birds into lay. Under natural conditions, onset of sexual maturity in chickens is controlled by day length (photoperiod). Male and female birds enter sexual maturity in the spring, under the stimulus of gradually increasing photoperiod. Normally, chickens will not enter lay in the fall, due to the negative stimulus of the naturally decreasing photoperiod at this season. In order to have a steady supply of broiler chicks, however, it is necessary to have males and females enter sexual maturity and lay throughout the year. For birds maturing in the fall or winter, this was achieved by extending the photoperiod through the use of supplemental artificial light, added at both ends of the natural day. This produced an artificial spring-type light pattern, and brought the birds into sexual maturity in a reliable manner throughout the year. Beginning in the 1980s, however, onset of sexual maturity was delayed for birds maturing in the fall season of the year, even under the stimulus of supplemental artificial light. Some of the females did not enter lay at all; in others, onset of lay was delayed and peak lay was lower. A management solution was found in the form of "Stimulatory lighting." For this, the chicks are reared in fully enclosed so-called dark-out houses in which photoperiod is under total artificial control, under a regime of 16 h total darkness and 8 h dim light until it is time for them to enter lay. As noted above, during this period they are also under quantitative feed restriction. To bring the birds into lay (generally, at about 6 months of age), they are moved to the laying pens, and exposed to a photoperiod of 14–16 h of daylight, while at the same time feed quantities are increased rapidly (within a few weeks from 100 g/day to over 160 g/day). The combined stimulus of increased photoperiod and feed quantity brings the birds into sexual maturity and lay.

Initially, dark-out rearing and stimulatory lighting were required only for birds entering lay in the fall. However, with the passage of generations, and continued selection for juvenile growth rate and FCR, problems on entering lay appeared even in birds coming to sexual maturity in the spring of the year under optimal natural lighting. At present, therefore, stimulatory lighting to bring the birds into sexual maturity is required at all seasons of the year [28]. Experimental studies showed that the need for stimulatory lighting was due to reduced innate photosensitivity of the broiler chicks [29], as well as to a direct effect of feed restriction per se on photosensitivity. Apparently the control systems of the bird interpret feed restriction as indicating that environmental conditions are not yet suitable for chick rearing, and delay onset of lay accordingly.

Secondary Effect of Continued Selection for JWfA and FCR (VIII): Male Overfeeding

In the mid-1980s, growth and appetite patterns of males and females began to diverge sufficiently, so that even under feed restriction, the males, when fed together with the females, became overweight and lost fertility. This was resolved by the use of special feeders and feed composition for the males, while using barriers preventing the males from having access to the female feeders.

Secondary Effect of Continued Selection for JWfA and FCR (IX): Heat Distress

Heat is produced by all aspects of body metabolism, including digestion and growth. Producing a given total mass of body weight, therefore, generates a more or less fixed total quantity of metabolic heat to digest the food required to grow this body mass and convert the digested food into body mass. This metabolic heat must be dissipated to avoid symptoms of heat distress. When the grow-out period is long, the daily production of metabolic heat for growth and digestion per

unit time is low and readily dissipated by the bird. However, as age to market weight decreases with each generation of selection, this same total metabolic heat is produced in a shorter and shorter grow-out period, so that in each generation the metabolic heat generated per unit time increases [30]. This is particularly true for the final weeks of the grow-out period, when the bulk of body mass is produced.

Generation of metabolic heat interacts strongly with stocking density, a major management determinant of broiler costs through its effect on fixed costs of housing per unit of product. Generally, birds are stocked at a density that gives almost complete ground cover toward the end of the rearing period. Under these conditions, there is maximum radiant heat transmission between birds, and stagnant hot air is trapped between birds and between the birds and the litter. Broilers with slow to moderately rapid growth can be reared in open sheds, with air circulation controlled by opening and closing window flaps or curtains, as the weather dictates. At optimal ambient temperature of 18–22°C, normal air circulation adjusted in this manner is sufficient to dissipate metabolic heat and the birds are comfortable. As ambient temperature increases, the birds cope by generating evaporative heat loss through panting [31]. For broilers with slow to moderately rapid growth, this is ordinarily sufficient to deal with the stress of normal summer peak heat. When peak heat exceeds ability of the bird to cope by panting, metabolic heat can be reduced by reducing food intake during the hottest part of the day, and by artificially "fogging" or "misting" the birds to increase evaporative heat loss when humidity is less than 70%. However, with the increase in growth rate achieved by the early 1980s, metabolic heat production increased to the point where these palliatives were no longer sufficient and the birds exhibited excessive panting and heat distress under conditions that were previously adequate. Heat distress was particularly acute at high stocking densities toward the end of the growing period, when floor cover by the birds was maximal. This led to reduction in growth rate since the birds reduced feed intake during the hottest part of the day, and in some cases to collapse and death of the bird from hyperthermia. To deal with this, fans were introduced into the chicken sheds to create additional circulation of air inside the sheds and to increase exchange of air between the inside and outside of the sheds. This system worked well, and in combination with misting or fogging when outside temperatures were high, enabled rapid growth to continue even under hot summer weather conditions with good FCR and low mortality.

Secondary Effect of Continued Selection for JWfA and FCR (X): Pulmonary Hypertension Syndrome and Sudden Death Syndrome

In 1974, a new pathological condition was reported in broiler birds reared at high altitudes. The condition manifested as sudden death, accompanied by "ascites" (accumulation of protein-rich fluid in the body cavity). Initially, death was attributed to the ascites *per se*, and hence the condition was termed "ascites syndrome." However, further studies showed that the ascites was a secondary consequence of pulmonary hypertension and the disease is now termed "Pulmonary Hypertension Syndrome" (PHS). With time, PHS manifested in broiler flocks growing at lower altitudes causing high mortality particularly in males of rapidly growing broiler lines from 4 weeks of age. The etiology of the disease has been worked out in detail (Box 3), and it appears to be a direct consequence of continued selection for rapid JWfA, exacerbated by selection for reduced FCR.

At about the same time that PHS manifested, a second metabolic disorder, "Sudden Death Syndrome" (SDS), also became prominent [32, 34, 35]. SDS presents as sudden convulsions, with squawking, violent flapping, and loss of balance. Death ensues within less than a minute from the onset of symptoms, with the bird lying on its back with one or both legs extended. Greatest losses are from 3 to 6 weeks of age, primarily in males. The syndrome is associated with the same factors that induce PHS, namely, high carbohydrate intake, dense housing, very rapid growth, and low feed conversion ratios; but most commonly manifests at an earlier age than PHS. Also similar to PHS, under inducing conditions, SDS can be precipitated by any sudden movements or noises that cause a stress response in the birds. It is plausible therefore, that SDS is an early pathological response to the hypoxia that accompanies rapid growth in broilers (see also Box 3).

From the above, it is clear that incidence of mortality due to PHS and SDS will be increased by any

Box 3 Etiology of Pulmonary Hypertension Syndrome and Sudden Death Syndrome

The causal sequence leading from selection for JWfA and FCR to PHS is thought to be as follows: Selection for rapid juvenile growth rate increases metabolic rate for growth resulting in increased need for oxygen in tissues. At the same time, selection for decreased FCR caused the relevant regulatory, circulatory, and hormonal systems to reduce oxygen consumption by the tissues resulting in effective hypothyroidism, and also reduced the proportion of oxygen-supplying organs (lung and heart) relative to oxygen-demanding tissue (muscle). The combination of these two factors results in tissue hypoxia that leads, via the kidney and erythropoietin, to increased production of red blood cells that increases blood viscosity, and also to constriction of pulmonary arterioles to ensure blood flow to all parts of the lungs so as to increase pulmonary oxygen exchange. The result of these two factors was increased lung arteriole pressure leading to increased workload on the heart and hypertrophy of the right (pulmonary) ventricle. This in turn resulted in incomplete closure of the right arterial valve with consequent backup of blood pressure to the hepatic and portal veins generating pulmonary hypertension and impaired uptake of fluid by the lymphatic system. Pulmonary hypertension results in fluid leakage and accumulation of fluid in the lungs (hypertensive lung syndrome) and in the abdominal and pericardial cavities (Ascites), and inability of the heart to supply body oxygen needs through left ventricle heart failure. Any of these final syndromes can result in death of the chick from PHS.

The detailed etiology of SDS differs [34, 35]. Electrocardiograms of broilers in the last stages of SDS show the immediate cause of death in SDS to be acute cardiac arrhythmia terminating in ventricular fibrillation. It is thought that under conditions of hypoxia the myocardium may become hyperirritable, serving as a secondary pacemaker and interfering with normal cardiac rhythms. Indeed, studies of rapidly growing broilers show that they manifest a high rate of cardiac arrhythmia under "normal" high growth-rate conditions, and are highly susceptible to stress-induced cardiac arrhythmia. Thus, under normal high growth-rate conditions, any additional stress may be sufficient to tip the heart from simple arrhythmia to ventricular fibrillation and sudden death [32, 33].

management factor that increases oxygen demands of the animal, such as social stress, low ambient temperature, feeding high-energy food in pellet form that stimulates growth rate; or that limits oxygen supply, such as overcrowding and poor air circulation or avian respiratory disease. Although a major problem in the 1980s, and still a problem today when circumstances combine, by the mid-1990s PHS and SDS were brought under control by a combination of family-based genetic selection against the condition, and careful management minimizing the external factors that contribute to hypoxia.

Stage 3: Combined Selection for JWfA, FCR, and Proportion of Breast Meat (PBM)

From the early 1970s, selection of birds with a less-pronounced keel bone was a part of broiler selection, with primary aim of producing a finished product having a fuller appearance that was more attractive to the consumer. This had a secondary result of selecting birds with more breast meat. With an increasing percentage of birds sold as individual parts, a major price differential between chicken breast "white" meat and chicken leg and thigh "dark" meat became apparent. This meant that the income of the broiler producer was primarily a function of the amount of breast meat produced. In the mid-1980s, realization of this economic fact by the breeders led to the addition of specific selection for a high proportion of breast meat in the broiler carcass as a third major breeding goal in addition to JWfA and FCR. This continues to the present time.

Secondary Effect of Selection for JWfA and PBW (XI): Heat Stress (Again)

The major secondary effect of selection for PBW in the broiler was an additional increase in heat stress effects, due to the increased metabolic load induced by the high metabolic cost of muscle mass synthesis. As a result, the modern broiler could no longer cope with even small deviations from the neutral temperature zone without a significant loss of meat yield. It became clear that the typical broiler-rearing shed of minimal structure with open sides plus fan-ventilation could no longer deal successfully with the enormous metabolic heat produced by the modern broiler flocks.

This led the industry to adopt a closed shed and a shift to "tunnel ventilation" with further option of cooling the air by drip technology. During the 2000s, "tunnel ventilation" with "drip cooling pads" for broilers became an obligatory feature of broiler rearing.

Secondary Effect of Selection for PBM (XII): Quasi-EODES at Entry to Lay

A secondary effect of the selection for PBM was the appearance of a new "quasi-EODES" syndrome at the onset of lay in the broiler breeder female parent, with symptoms very similar to classical EODES but to a less-pronounced degree [36]. This "quasi-EODES" syndrome was characterized by high mortality at entering lay, caused mainly by cloacal prolapse, internal lay, and inflammation of the oviduct. For the surviving birds, the quasi-EODES syndrome has marked negative effects on peak lay and eggshell quality resulting in a lower proportion of hatching eggs out of all eggs and lower hatch of healthy chicks from fertile eggs. The overall result is a marked reduction in chick production. Practical experience of hatchery flock managers, supported by later research revealed that the quasi-EODES syndrome is induced by even minor "overfeeding" during the critical weeks of forced sexual maturation. The modern female breeder became very sensitive to even a slight deviation from optimal feed restriction.

Two factors appear to have combined to generate the quasi-EODES syndrome. The first, is the continued increase in the degree of feed restriction during the pullet growth period, needed for preventing EODES, going, as noted above, from about 70% of *ad libitum* consumption during the 1970s to about 35% of *ad libitum* consumption at present. The second is the continued increase in threshold weight for onset of sexual maturity of the female broiler breeder, as a direct result of the selection for higher JWfA and PBM. For example, recommended 24-week body weight for out-of-season "Cobb 500" female broiler breeders increased from 2700 g in 1987 to 3160 g in 2005 [37] with a higher proportion of body mass as muscle tissue. Thus, the actual gap between the pullet lean mass attained at the point where light and feed stimulation is initiated, and the threshold lean mass required for entering lay has progressively increased, and with it the time required from initiation of light and feed stimulation to actual onset of lay. Attempting to cover this gap more rapidly by increasing feeding levels exceeds the ability of the pullet to rebuild breast mass and results in a surplus of nutrients causing the ovary to behave as if fed *ad libitum* and leading to development of quasi-EODES. Thus, the modern broiler breeder pullet entering lay is delicately balanced at a feeding schedule that will induce lay as rapidly as possible, yet will not induce quasi-EODES. Consequently, the bird is very unforgiving of any deviation from optimum feeding schedule in the direction of overfeeding.

Secondary Effect of Selection for PBW (XIII): Hatchability Problems (Again)

A further secondary effect of the selection for increased proportion of breast meat was a reemergence of hatchability problems. These appeared even though continued selection for rapid JWfA did not result in appreciable further increase in egg weight. Stability of egg weight was achieved due to increasing feed restriction of the broiler mother targeted specifically at controlling egg weight (which responds very rapidly to feed restriction) and to conscious selection against excessive egg size by the breeders. Thus, the situation was unchanged throughout the 1990s. However, the intense selection for increased muscle mass that began in the mid-1980s resulted in an increase in the proportion of muscle mass in the late developing embryo. As noted, the metabolic energy expended in development of muscle is greater than for other body tissues, because of the high metabolic cost of protein synthesis. Hence, this resulted in a further increase in metabolic heat production by the late embryo [38–41]. This additional excess heat was beyond the capacity of multistage incubators, no matter how carefully managed. Consequently, hatchability began to fall again, owing to increased late mortality and decreased yolk uptake leading to an increased proportion of weak chicks, and more grade B chicks [42], and negative effects on broiler bone development and leg health [43, 44].

To meet this challenge, the industry began shifting to "single-stage" incubators [40]. In these incubators, all eggs from flocks of about the same age and same average egg weight are loaded into the incubator on the same day. They are then incubated together until they hatch. Thus, they are all at the same stage of

development at any given time (hence the name "Single-Stage" incubator). This makes it possible to better adjust incubation temperature, according to the stage of the embryos [42] – somewhat warmer at the early embryo stages, when the embryo requires external heat to reach desired internal temperature, and cooler at the later embryo stages, when the embryo needs to rid itself of excess heat. Similarly, humidity can also be adjusted so as to achieve optimal egg water loss across the incubation period [41]. This has proven to be a satisfactory solution, and hatchability and chick quality have improved accordingly.

Secondary Effect of Selection for PBW (XIV): Reduced Locomotor Activity

The very large breast of the modern broiler chick results in an animal that is unbalanced between breast weight and leg and thigh muscles [45]. Consequently, it is difficult for the animal to move, and locomotor activity is much reduced. Toward the end of the growth period, the broiler chick spends over 90% of its time sitting and lying, resting its overdeveloped breast on the litter, and basically moving only to eat and drink. Selection for reduced FCR may also have contributed to the reduction in locomotor activity, since this is one way to reduce metabolic expenditure of the animal. As a result, the animal is very susceptible to contact dermatitis, including footpad lesions and breast blisters. These conditions can be well controlled by meticulous attention to litter quality, specifically litter temperature and humidity, but are exacerbated and can reach high levels when litter quality is poor.

The Role of Breeding and Management in the Improvement in Performance of the Modern Broiler Chicken

Achievements of the Modern Commercial Broiler with Respect to the Three Main Production Traits and the Associated Secondary Traits

The extraordinary improvement in broiler performance for the three main production traits (JWfA, FCR, and PBW) over the past 60 years is best captured by the following table (Table 1), which compares the performance (average of males and females) of representative commercial broiler stocks of 1957, 1991, and 2001.

Poultry Breeding. Table 1 Comparison of performance of broiler stocks of 1957, 1991, and 2001[a]

Trait	1957	1991	2001
Age at sale (d)	84	42	42
Live weight at sale (g)	1646	2132	2672
Feed conversion ratio	3.26	2.04	1.63
Breast meat (%)	12.9	15.0	20.0
Carcass fat (%)	14.7	14.1	13.7
Heart+lungs (%)	1.242	ND	1.023
Mortality to market (%)	2.52	9.70	3.57

[a]Based on [6–9]; data for 1957 average of [6 and 8 and 7 and 9] for birds fed the commercial feed formulations of 1991 [6, 7] and 2001 [8, 9].

It is evident that in the period 1957–2001 enormous improvement has been achieved in the three major production traits. Days to market weight and FCR have been reduced by half, while JWfA has almost doubled, and PBW has increased by almost 30%. Improvement in all traits was continuous, with no indication of reduced rate of gains in the latter decade.

When compared using the feed formulations of 1957, gains in JWfA and FCR were only a bit less. There is no doubt therefore, that genetic selection by the primary breeders was by far the main driving force leading to the gains in these traits. The increase in PBM from 1991 to 2001 is particularly impressive and can be attributed almost completely to the attention devoted to this trait by the primary breeder beginning in late 1980s. However, JWfA and FCR also increased strongly during this period showing the ability of the breeder to deal simultaneously and effectively with the three major production traits.

Along with the phenomenal improvement in production traits, the broiler industry was able to cope successfully with all of the deleterious secondary effects detailed in the preceding sections that accompanied the genetic gains in the three main production traits. Through a combination of genetic selection on the one hand, and continual adjustment and innovation in broiler and breeder flock management and physical facilities on the other, the industry succeeded in maintaining male and female reproductive

performance at very high levels, while keeping losses due to heat sensitivity, skeletal problems, and metabolic disorders at very low levels.

Relative Contribution of Genetics and Management to Modern Broiler Performance

The relative contribution of genetics and management to these achievements depends on the specific trait. In particular, it seems clear that the shift to progressively more active ventilation culminating in the tunnel ventilation with drip cooling in general use at present was the main means for coping with the heat sensitivity of the modern broiler. Similarly, changes in incubator management and the more recent shift from multistage to single-stage incubators together with flock management to reduce egg weight were the main factors maintaining high hatchability rates. However, selection played some role in both cases, since families that display low hatchability and high sensitivity to heat stress are generally culled from the breeding population.

With respect to reproductive performance, feed restriction during pullet growth and the hen laying period, and dark-out housing of the parent stock pullets with stimulatory lighting and feeding at entry into lay, were critical management factors contributing to maintaining reproductive performance in male and female broiler parent stock. However, selection for reproductive performance within this overall management scheme played an important secondary role, as attested by the appreciable differences between genetic stocks in reproductive performance under optimal feeding and lighting schedules. In the same way, while appropriate management is essential to control skeletal problems and metabolic disorders, the effective selection applied to these conditions appears to have been crucial in reducing their incidence in well-managed flocks to present-day low single-digit proportions.

Thus, although the improvement in production traits of the modern broiler compared to its original founder populations can be attributed almost entirely to genetic improvement achieved by selection, the bird that results is able to achieve optimal production and functional performance as broiler and parent stock primarily due to major modifications in broiler housing, and in parent flock and incubator management coupled with a greater or lesser contribution by selection, depending on the trait. Be that as it may, one can only stand in awe at the combined ability and synergistic work of the major players in the industry to overcome the challenges as they arose while maintaining continual progress in overall efficiency of the broiler enterprise.

Breeding Methods for Genetic Improvement

In large part, this is due to the fact that the methods practiced by the primary broiler breeders increased in sophistication in accord with the complexity of the breeding challenges that they faced [46]. In the first stage of broiler genetic improvement, JWfA was the main trait under selection, with a minor goal of improved breast conformation. This was achieved by simple two-step tandem mass selection, the first step being selection for JWfA followed by a second step of selection for breast conformation. Flocks were not pedigreed at this stage. With the addition of reproductive performance, FCR and reduction of adiposity and skeletal problems as breeding objectives in the second stage of broiler genetic improvement, simple mass selection was no longer effective and multiple-stage tandem selection no longer feasible. To meet the new challenges, breeders changed to fully pedigreed flocks to provide information on reproductive performance, FCR, and skeletal problems, and to index selection based on individual and close relatives to combine information on the various traits in an optimal manner. Finally, in the third stage of broiler genetic improvement, with the addition of PBM and reduction of metabolic disorders to the breeding objectives, breeders adopted BLUP and Individual Animal Model statistical methodologies for estimating breeding values for the various traits under selection, based on individual data for the entire current and past population. At the same time, the primary breeders adopted phenotyping procedures that increased the completeness and accuracy with which the traits of importance were evaluated. For example, individual skeletal problems are now assessed by x-ray scanning of bird joints and individual cardiovascular function is assessed by blood oximeter machine. At present, over 50 traits are recorded on the individuals in the breeding nuclei, about half of these relating to various aspects of broiler health.

At all stages, breeding nuclei were kept very large in order to avoid inbreeding and maintain genetic variation. With the advent of genome-wide procedures for estimation of breeding values [1–3], all of the major primary breeders have instituted in-house experimental studies to evaluate the use of these methodologies in their practical breeding programs. Although very promising in initial studies, the results in practice in commercial breeding nuclei remain to be seen.

Accounting for the Long-Term Continuous Response to Selection and for the Punctuated and Coordinated Appearance of Secondary Effects

Commercial breeding nuclei have been under intense and effective directional selection for JWfA for 60 generations, for FCR for 40 generations, and for PBM for 25 generations. Two remarkable features characterize the response to this selection. The first and most striking is that the direct response of the target traits to selection has been positive and continuous for all three traits, with no indications of having reached a selection plateau for any of them. This continued response to selection requires explanation. Since selection acts on all relevant loci at the same time, the intense directional selection for the target traits should have exhausted the existing genetic variation in the original dual-purpose chicken founder populations rather rapidly, leading to selection plateaus for the various traits. The second remarkable feature is the rather sudden manifestation of the secondary effects of the response to selection at about the same time period in the stock of all leading breeders, even though their respective breeding nuclei are effectively isolated from one another. This "punctuated" (i.e., "relatively sudden") and "coordinated" (i.e., relatively universal and synchronous) manifestation of the secondary affects requires explanation. If due to genetic correlations or pleiotropic effects of the initial genetic variation of the founder populations, they would be expected to manifest in a more linear and gradual manner. If due to pleiotropic effects of new mutations, they would be expected to manifest in a more sporadic manner, affecting only stock of one or two of the various breeders.

Sources of Genetic Variation for the Long Continued Response to Selection

Based on classical population genetics, three sources can be envisaged with respect to sources of genetic variation to serve as a substrate for selection. The first is the genetic variation present in the original founder populations of the broiler stocks. Recent research in mapping of the loci responsible for genetic variation in production traits in farm animals (the so-called Quantitative Trait Loci or QTL) indicates that tens of QTL may contribute to genetic variation of such traits. This genetic variation present in the founder populations would have provided the basis for the first decades of response. The second source comprises rare alleles with positive effects on the target traits that were present at low frequency in the founder populations. The primary breeders maintain large breeding nuclei, and these could have included an appreciable number of such loci. In the early generations, because of their low frequency these loci would not have contributed materially to genetic variation in the target traits, or to the response to selection. However, after several decades of intense selection, their frequency would have increased to the point where they could serve as useful targets for the middle stages of selection. Finally, new mutations or rare recombinants would supply a steady stream of new genetic variation that would come into play in the later stages of selection.

Punctuated and Coordinated Appearance of Deleterious Secondary Effects

Similarly, a number of explanations can be offered within classical frameworks, for the punctuated and coordinated appearance of deleterious secondary effects. Some of these effects may be due to non-linear secondary effects of selection interacting with downstream threshold effects. For example, adiposity can be presumed to have increased more or less linearly with selection for JWfA, but the effect on egg production was virtually nil for the first 10–15 generations of selection, and then suddenly became very severe – in the few years just before the introduction of feed restriction in the late 1960s, yearly egg production had fallen by almost 100 eggs per laying hen. Such effects would be expected to manifest in flocks of all

breeders at about the same time, as their breeding populations reached more or less the same degree of expression of the primary traits. Other secondary effects may be due to the introduction of new primary target traits. Thus, an entire group of new secondary effects manifested following the introduction of FCR, and again following the introduction of PBM as targets for selection. Since the primary breeders introduced the same new target traits for selection at more or less the same time, these secondary effects would be expected to manifest in flock of all breeders at about the same time.

Difficulties with the Explanations Within the Classical Framework of Population Genetics

Rare mutations are often those that are held at low frequencies because of deleterious pleiotropic effects on fitness. Similarly, new mutations generally affect numerous traits, with deleterious fitness effects on one or more of them. Intense selection can increase the frequency of genes with strong positive effect on the target trait in spite of negative effects on fitness, but in a long-term selection program this should result in reduced overall functional fitness of the population. Furthermore, because of their low frequency in the founder populations, rare alleles would not distribute uniformly among different breeding populations derived from the same founders. The same would certainly hold for the genes that were affected by mutation. Consequently, fitness traits should be differentially affected in different breeding populations, according to the specific low-frequency genes that were inherited or mutated in each population. This does not appear to have been the case. Similarly, although in almost all cases it was possible to trace in retrospect a causal chain leading from selection for the primary trait to the manifestation of the secondary trait it is somewhat unexpected that the specific form of these manifestations (e.g., PHS, SDS, quasi-EODES, reduced photosensitivity) was the same in stocks of all breeders. If much genetic variation is due to initially rare or mutated loci that are necessarily specific and different in different breeding populations, specific manifestations of secondary traits would be expected to differ as well.

Selection-Induced Genetic Variation (SIGV)

The difficulties with the explanations proposed within the classical framework for long-term response to selection and for punctuated and coordinated appearance of the secondary manifestations have led to a recent proposal for a new previously unrecognized source of genetic variation in ongoing selection programs: selection-induced genetic variation (SIGV) [47]. The SIGV hypothesis is based on the observation that the individual QTL determining genetic variation in production traits often exhibit strong epistatic interactions with one another and with the genetic background. Consequently, a segregating locus with zero effect on the target trait in one genetic background might have a strong effect on the trait in a different genetic background.

The intense continued directional selection, changes the frequency of many alleles in many loci and by means of "hitchhiking effects" of their closely linked neighboring genes as well. This changes the genetic architecture of a population to a marked degree. Thus, some loci that were neutral in their effect on the target trait at the beginning of the selection program, may transform into sources of genetic variation as the selection program progressively modifies the genetic architecture of the population. As the positive alleles at these loci are caught up and brought to high frequency by the ongoing selection, the genetic architecture of the population becomes modified again, and new previously neutral loci come into play as sources of genetic variation. In this way, the population under selection would continually generate new genetic variation to serve as a substrate for the next phase of the directional selection process, with no obvious limit in place.

Since the loci involved in the SIGV process are loci that were present at appreciable frequencies in the original founder populations, they would be present in all breeding stocks, and would be expected to come into play at about the same stage in the selection process in the individual flocks. Thus, genetic progress in the various breeder populations would not depend on rare alleles or new mutations, which would be specific to each population, but on segregating loci already present in the population at useful frequencies, that come into play in a programmed manner, as the genetic architecture of the population changes. On this

hypothesis, the genetic architecture of all breeding populations under the same general selection regime would be similar at all stages of the selection process, and hence the specific form of any secondary effects would also be similar in the different populations. Strong secondary effects on fitness would not be expected, since the loci involved are loci historically present in the populations, and hence have survived the screening effect of natural selection.

Thus, the SIGV hypothesis provides plausible explanations both for the long-term response to selection and for the punctuated and coordinated appearance of secondary effects. Indeed, recent experimental studies analyzing crosses between layers and broilers, and between two-way single-trait selection lines for JWfA, provide significant experimental support for this hypothesis [48, 49]. The SIGV hypothesis emphasizes the importance of epistatic interactions in genetic variation and encourages the development of statistical and genomic methodologies that can exploit these interactions.

Genetic Improvement of Broiler Production Traits and Broiler Welfare

In the previous sections, it was seen that the modern broiler is greatly modified developmentally and physiologically from the original dual-purpose breeds from which it was developed. Furthermore, to achieve optimal economic performance the modern broiler and its parent flocks must be reared, managed, and housed under conditions very different from the husbandry conditions of the founder parent lines. Various aspects of these genetic and management changes impinge on animal welfare, in the sense that they can lead to distress or pain of the individual. These can be considered in two main categories. Those where the distressful situation is unavoidable, and those where the distressful situation is avoidable under good management, but manifests under less than optimal management. There is a third category, which considers aspects where the bird is comfortable and not in distress, but where it is thought that its life might be more joyful if living under different conditions, for example, on free range or scrounging for food in the jungle or farmyard, instead of in a closed shed. This latter raises more profound issues of the morality and ethics of animal agriculture in general and industrial animal agriculture in particular, and will not be considered here.

Unavoidable Distress

The negative effect of excess adiposity on reproductive performance and survival of the modern broiler parent female or male is dramatic and extreme. Consequently, stringent feed restriction of the parent flock pullets and males during the rearing and laying period is essential, if the birds are to achieve optimal reproductive performance. This appears to be the only aspect of the broiler enterprise that causes distress, but is unavoidable. An animal that is fed only 35% of its *ad libitum* consumption, and can be seen to eat ravenously when feed is available, must surely be feeling hunger pangs. The period of stringent feed restriction is rather short, however, from about 4–14 weeks of age, and while it can be presumed to be uncomfortable, does not affect the overall health of the individuals in any way. Indeed, allowing the animal to eat *ad libitum* and become grossly obese would probably entail much more distress over the entire life span of the animal including mortality. The much milder feed restriction that is in effect during lay would not seem to pose a welfare problem, as it is ordinarily sufficiently generous to even allow for some increase in body weight over the course of the laying period.

Avoidable Distress

A review of the preceding sections, however, reveals a considerable list of conditions to which the modern broiler is susceptible due to its changed genetic constitution. These include: quasi-EODES at entry into lay that can result in death from cloacal prolapse or from internal lay; heat stress, skeletal abnormalities, PHS which often manifests as ascites, SDS, and various forms of contact dermatitis. SDS, in which death of the animal occurs within less than a minute from the onset of first symptoms of distress, would seem to occur too rapidly to be a source of significant pain or distress. But the other conditions can be presumed to be moderately to severely painful, according to their respective natures and degree of severity. As emphasized in the previous sections, thanks to the selective work of the breeder on the one hand, and innovative management of the industry on the other, when broiler

commercial or parent flocks are managed with care and attention to detail, all of the above can be kept at very low single-digit levels [46, 50].

Importance of Stocking Density

In this context, stocking density, an aspect of management that was not previously emphasized becomes important [45]. Stocking density interacts with the susceptibilities of the modern broiler in two ways: increasing heat stress and decreasing litter quality with consequent increase in the incidence of contact dermatitis. When stocking density is high, the bodies of the chicks cover the entire floor area, forming a barrier between the litter surface and the ventilated area of the shed. In addition, higher stocking density provides additional nitrogen and moisture to the litter increasing heat production due to microbial growth and metabolism. Taken together, these two factors can generate an appreciable differential between temperature at broiler level and temperature a meter above the floor. High stocking density also limits air movement and increases radiant heat transfer between birds, further increasing heat stress. The increase in litter temperature, humidity, and ammonia concentration resulting from high stocking density also interacts with the limited locomotor activity of the broilers to increase the incidence of contact dermatitis. Accordingly, proper control of stocking density is essential for optimal broiler welfare, and may be less than the economic optimum.

From Farm to Market

The handling of the bird on its final path from shed to slaughterhouse is another case in point. Because of the young age of the broiler at market weight, and its minimal locomotor activity, the long bones of the legs and wings are fragile relative to body weight. Moving the birds from the grow-out shed to the slaughter line is low-wage work with high personnel turnover and great pressure to get the work done with minimal labor costs. Consequently, the birds may be roughly handled at this time resulting in leg and wing bone breakage when they are captured and moved from the pen to the transport crates, and again from the transport crates to the slaughter line [51, 52]. Such breakage results in direct losses to the farmer and hence normally efforts are expended to reduce this to a minimum through mechanization, proper design of facilities, and training of personnel [53].

Welfare and Society

Apparent from the above paragraphs is the critical dependence of broiler welfare on meticulous flock and personnel management. Due to the work of the breeder, with proper management the susceptibilities of the bird remain latent. But there is little room for error. The bird is unforgiving of even slight deviations from optimal management, and will react to such deviations by manifesting one or other of its innate susceptibilities. The modern chicks that manifest any of the conditions above are a net loss to the farmer, and management conditions conducive to such manifestation are often inimical to optimal growth and reproduction. Consequently, for the most part, the welfare of the chick and the economic interests of the farmer correspond. However, achieving optimal management has its costs as well, and economic maximization may result in a management program that is somewhat less than optimal for the bird.

Society as a whole has benefited greatly from the improved production characteristics of the modern broiler, which has added a low-cost highly nutritious staple to the menu at minimal environmental footprint. Along with these benefits comes responsibility for the welfare of the bird in those instances where economic interests of the farmer and the quality of life of the broiler are not perfectly aligned. This is true throughout the broiler enterprise; nowhere more than in the final hours of the chick's life. Happily, society is accepting these responsibilities by designing facilities based on understanding of livestock behavior [53], and by defining acceptable production standards, and embodying these in appropriate regulatory legislation [54, 55].

Future Directions

One stands in awe of the magnificent achievements of the primary broiler breeders who have succeeded in improving the production characteristics of the modern broiler manifold relative to the initial dual-purpose founder populations, while at the same time dealing successfully with the many secondary functional problems that arose along the way, maintaining high reproductive performance together with very low incidence

of health problems. This was not the achievement of the breeder working alone, of course, and at all stages involved combined contributions by all components of the industry in devising novel management programs and physical facilities such as feed restriction and stimulatory photoperiods, dark-out housing, and tunnel ventilation that enabled optimal functional performance of the birds.

The Resource Allocation Model

These results are all the more remarkable, because they differ from what would be expected on the widely accepted resource allocation model for interpreting the effects of selection for production traits on functional traits [56, 57]. The basic assumption of this model is that under the conditions of intensive agriculture, the energy resources available to an animal are fixed. The animal must then allocate these resources to maintenance, to production traits including growth, and to functional traits including reproduction, regulation of physiology and metabolism, and defense against pathogens and toxins. Selection for production traits diverts resources preferentially to these traits, leaving less for the functional traits. Consequently, functional traits are expected to suffer. Indeed, many experimental studies show that production traits suffer when resources are preferentially allocated genetically or experimentally to functional traits, and functional traits suffer when resources are preferentially allocated to production traits. Amazingly, the broiler industry appears to have been able to circumvent this negative correlation, achieving high levels in both production and functional traits.

Management and Biological Costs of the Performance Achievements of the Broiler Industry

More detailed consideration, however, shows these achievements have incurred two costs. The first is the cost of more sophisticated physical facilities and complexity of management. These include sheds with tunnel ventilation and drip cooling for broiler rearing; dark-out houses for pullet rearing; feed restriction, single-stage incubators; and all of the many management and nutritional modifications required for optimum performance. The second cost is the apparent loss of buffering capacity of the animal regulatory systems, resulting in ever-increasing fragility or sensitivity of the animal to environmental challenges. This sensitivity expresses itself as reduced production and functional performance, and increased incidence of health problems as a consequence of even minor deviations from optimum management and environment. The result is not only loss of economic value, but also harmful impact on animal welfare. Because of the high metabolic cost of protein synthesis, a provocative hypothesis suggests that selection for productivity in general, and FCR in particular may reduce protein turnover rate. Since protein synthesis is essential to meet new conditions, this may be the underlying cause for the general loss in buffering capacity [58]. The primary broiler breeders today are attempting to decrease the sensitivity of their populations to environmental insults by testing under multiple environments and selecting for "robustness", as they would select for any other positive health trait. Will they succeed? Or will the future broiler require an increasingly narrow and complex multidimensional management path for optimal performance, with steep losses outside of this path? The coming years will tell, with strong implications for the broiler industry, and for animal breeding in general.

Conclusions and Interpretation

Be that as it may, the broiler experience teaches that deleterious effects on functional traits are almost certain to arise in the course of selection, and can take unexpected forms. The industry, whatever the species, must be alert to this possibility, so as to seek solutions as early as possible. The broiler experience also teaches that the breeder and the industry acting in concert can indeed find these solutions to these problems, enabling a joint genetic-management program that provides very high productive performance together with high functional performance within a framework of innovative physical facilities and increased management complexity. Based on past experience, therefore, as animal breeding enters a new genomic phase with greatly accelerated improvement in production traits, the future can be looked at with appropriate confidence in the ability of the breeder and industry working together to achieve sustained genetic improvement in production traits while maintaining high levels of functional and health performance.

Abbreviations

BLUP	Best linear unbiased predictor
DNA	Deoxyribonucleic acid
EODES	Erratic oviposition defective egg syndrome
FCR	Feed conversion ratio
JWfA	Juvenile weight for age
PBM	Proportion of breast meat
PHS	Pulmonary hypertension syndrome
QTL	Quantitative trait locus
RNA	Ribonucleic acid
SDS	Sudden death syndrome
SIGV	Selection-induced genetic variation

Bibliography

Primary Literature

1. Hayes BJ, Bowman PJ, Chamberlain J, Goddard ME (2009) Invited review: genomic selection in dairy cattle: progress and challenges. J Dairy Sci 92:433–443
2. Tier B (2010) Editorial: genomic selection: promise and propriety. J Anim Breed Genet 127:169–170
3. Meuwissen T, Goddard M (2010) Accurate prediction of genetic values for complex traits by whole-genome resequencing. Genetics 185:623–631
4. Hartmann W (1989) From Mendel to multi-national in poultry breeding. Worlds Poult Sci J 45:5–26
5. Schmidt CJ, Persia ME, Feierstein E, Kingham B, Saylor WW (2009) Comparison of a modern broiler line and heritage line unselected since the 1950's. Poult Sci 88:2610–2619
6. Havenstein GB, Ferket PR, Scheideler SE, Rives DV (1994) Carcass composition and yield of 1957 vs 1991 broiler when fed "typical" 1957 and 1991 broiler diets. Poult Sci 73: 1795–1804
7. Havenstein GB, Ferket PR, Scheideler SE, Larson BT (1994) Growth, livability and feed conversion of 1957 vs 1991 broiler when fed "typical" 1957 and 1991 broiler diets. Poult Sci 73:1785–1794
8. Havenstein GB, Ferket PR, Qureshi MA (2003) Growth, liveability and feed conversion of 1957 vs 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1500–1508
9. Havenstein GB, Ferket PR, Qureshi MA (2003) Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1509–1518
10. F.A.O. Food outlook global market analysis (2010). http://www.fao.org/docrep/012/ak349e/ak349e00.pdf. Accessed April 2010
11. Anonymous (1996) The history of Hubbard Farms. Hubbard Farms, Walpole
12. National Chicken Council. US chicken industry history. http://www.nationalchickencouncil.com/. Accessed April 2010
13. Emmerson DA (1997) Commercial approaches to genetic selection for growth and feed conversion in domestic poultry. Poult Sci 76:1121–1125
14. Siegel PB, Wolford JH (2003) A review of some results of selection for juvenile body weight in chickens. Poult Sci J 40:81–91
15. Soller M, Eitan Y (1984) Why does selection for live weight gain increase fat deposition? A model. Worlds Poult Sci J 40:5–9
16. Wikipedia The metabolic syndrome. http://en.wikipedia.org/wiki/Metabolic_syndrome. Accessed April 2010
17. Jaap RG, Muir FV (1968) Erratic oviposition and egg defects in broiler type pullets. Poult Sci 47:417–423
18. Katanbaf MN, Dunnington EA, Siegel PB (1989) Restricted feeding in early and late feathering chickens 2. Reproductive response. Poult Sci 68:352–358
19. Robinson FE, Robinson NA, Scott TA (1991) Reproductive performance, growth rate and body composition of full fed versus feed restricted broiler breeder hens. Can J Anim Sci 71:549–556
20. Hocking PM, Waddington D, Walker MA, Gilbert AB (1989) Control of the development of the ovarian follicle hierarchy in broiler breeder pullets by food restriction during rearing. Br Poult Sci 30:161–174
21. Nir I, Nitsan Z, Dror Y, Shapira N (1978) Influence of over feeding on growth, obesity and intestinal tract in young chicken of light and heavy breeds. Br J Nutr 39:27–35
22. Boerjan M (2004) Genetic progress inspires changes in incubator technology. World Poult 5:16–17
23. Leach RM (1992) Leg weakness in broilers – complex situation involving many factors. Poult Dig 3:24–27
24. Oviedo-Rondon EO, Ferket PR, Havenstein GB (2006) Understanding long bone development in broilers and turkeys. Avian Poult Biol Rev 17(3):77–88
25. Nicholson D (1998) Research: is it the broiler industry's partner into the new millennium. World's Poult Sci J 54:271–278
26. Tomas FM, Pym RA, Johnson RJ (1991) Muscle protein turnover in chickens selected for increased growth rate, food consumption or efficiency of food utilization: effects of genotype and relationship to plasma IGF-I and growth hormone. Br Poult Sci 32:363–376
27. Skinner-Noble DO, Teeter RG (2003) Components of feed efficiency in broiler breeding stocks: energetics, performance, carcass composition, metabolism and body temperature. Poult Sci 82:1080–1090
28. Lewis P (2009) Proper lighting for broiler and turkey breeders. World Poult 25(12):18–21
29. Eitan Y, Soller M (1994) Selection for high and low threshold body weight at first egg in broiler strain females. 4. Photoperiodic drive in the selection lines and in commercial layers and broiler breeders. Poult Sci 73:769–780
30. Teeter RG (1994) Optimizing production of heat stressed broilers. Poult Dig 53:10–27
31. Yahav S (2010) Alleviating heat stress in domestic fowl: different strategies. Worlds Poult Sci J 65:719–732
32. Leeson S (2007) Metabolic challenges: past, present and future. J Appl Poult Res 16:121–125

33. Decuypere E, Buyse J, Buys N (2000) Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. Worlds Poult Sci J 56:367–377
34. Moghadam HK, NcMillan I, Chambers JR, Julian RJ, Tranchant CC (2005) Heritability of sudden death syndrome and its associated correlations to ascites and body weight in broilers. Br Poult Sci 46:54–57
35. Olkowski AA, Classen HL (1997) Malignant ventricular dysrhythmia in broiler chickens dying of sudden death syndrome. Vet Rec 140:177–179
36. Eitan Y, Soller M (2009) Problems associated with broiler breeder entry into lay: a review and hypothesis. Worlds Poult Sci J 65:641–648
37. Cobb-Vantress (2005, 2008) Cobb 500 breeder management guide. Cobb-Vantress, Arkansas
38. Taylor G (1999) High yield breeds require special incubation. World Poul 15(3):27–29
39. Hill D (2000) Multistage incubation and yield genetics. Poult Dig 10–11:14–20
40. Boerjan M (2004) Single stage incubation is the most natural choice. World Poult 20(7):18–20
41. Tona K, Bamelis F, Coucke W, Bruggeman V, Decuypere E (2001) Relation between Broiler Breeder's age and egg weight loss and embryonic mortality during incubation in large-scale conditions. J App Poult Res 10:221–227
42. Meijerhof R (2002) Incubation by embryo temperature. World Poult 18(6):36–37
43. Oviedo-Rondon EO, Wineland MJ, Funderburk S, Small J, Cutchin H, Mann M (2009) Incubation conditions affect leg health in large high yield broilers. J Appl Poult Res 18:640–646
44. Oviedo-Rondon EO, Wineland MJ, Small J, Cutchin H, McElroy A, Barry A, Martin S (2009) Effect of incubation temperatures and chick transportation conditions on bone development and leg health. J Appl Poult Res 18: 671–678
45. Bessei W (2006) Welfare of broilers: a review. Worlds Poult Sci J 62:455–466
46. Katanbaf MN, Hardiman JW (2010) Primary broiler breeding – striking a balance between economic and well-being traits. Poult Sci 89:822–824
47. Eitan Y, Soller M (2004) Selection induced genetic variation: a new model to explain direct and indirect effects of sixty years of commercial selection for juvenile growth rate in broiler chickens. In: Wasser SP (ed) Evolutionary theory and processes: modern horizons. Papers in honor of Eviatar Nevo. Kluwer, Dordrecht, pp 153–176
48. Deeb N, Lamont SJ (2002) Genetic architecture of growth and body composition in unique chicken populations. J Hered 93:107–118
49. Carlborg O, Jacobsson L, Ahgren P, Siegel P, Andersson L (2006) Epistasis and the release of genetic variation during long-term selection. Nat Genet 38:418–420
50. Flock DK, Laughlin KE, Bentley J (2005) Minimizing losses in poultry breeding and production: how breeding companies contribute to poultry welfare. Worlds Poult Sci J 61:227–237
51. Nicol CJ, Scott GB (1990) Pre-slaughter handling and transport of broiler chickens. Appl Anim Behav Sci 28:57–73
52. Grandin T, Johnson C (2009) Animals make us human - Creating the best life for animals. Houghton Mifflin Harcourt, Boston/New York
53. Grandin T, Deesing M (2008) Humane livestock handling: understanding livestock behavior and building facilities for healthier animals. Storey Publ., North Adams
54. Shane S (2009) Broiler welfare symposium – The balance between producers' and consumers' standards. World Poult 25(12):32–34
55. Blokhuis HJ (2004) Recent developments in European and international welfare regulations. Worlds Poult Sci J 60:469–477
56. Gross WB, Siegel PB (1997) Why some get sick. J Appl Poult Res 6:453–460
57. Siegel PB, Honaker CF (2009) Impact of genetic selection for growth and immunity on resource allocations. J Appl Poult Res 18:125–130
58. Rauw WM, Knap PW, Varona L, Gomez-Raya L, Noguera J (2002) Does selection for high production affect protein turnover rate? Communication No. 10-03, 7th world congress on genetics applications to livestock production, Montpellier, France

Books and Reviews

Falconer DS, Mackay TFC (1996) Introduction to Quantitative Genetics, 4th edn. Longman Group Ltd., Harlow
Weller JI (2009) Quantitative Trait Loci Analysis in Animals, 2nd edn. CAB International, Wallingford

Roots and Uptake of Water and Nutrients

P. Carvalho, M. J. Foulkes
Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Loughborough, UK

Article Outline

Glossary

Fibrous root system Root system formed by various root axis of similar size, typical of cereals.

Lateral roots Lateral roots are the roots formed from the pericycle cells of other roots. The first-order laterals refer to the roots emerging from the primary and secondary root axes. Second-order laterals emerge from the first-order laterals, and third-order laterals from the second-order lateral, and so on. Usually, lateral branching is limited to the fifth-order laterals.

Primary roots Often called seminal roots, these are the first root axes to develop arising from the coleorhizae of the seed.

Rhizosphere Volume of soil immediately adjacent to plant roots (usually between 10 and 20 mm), which is affected by their growth, secretions, respiration, nutrient and water, and associated soil microorganisms.

Root architecture Describes the spatial configuration of the root system as a whole. Since it describes multiple root axes it subsumes both topology and distribution.

Root cap Root cap is the tissue that covers the apex of the root. It protects the apical meristem, acts as gravisensor tissue, and facilitates the passage of the growing roots by producing root mucilage.

Root distribution Root distribution refers to the distribution of different root traits, often morphologic ones (e.g., weight, length, volume), as a function of several factors, the most common being soil depth.

Root hair Specialized projection formed by a modified epidermal root cell. It augments the total surface area of a root system, dramatically increasing its absorption capacity.

Root morphology Root morphology refers to the surface features of a single root axes as an organ. It includes the characteristics of the epidermis such as root hairs, root cap, pattern of appearance of lateral roots, cortical senescence, and diameter. Weight, volume, and area are also part of the morphology.

Secondary roots Secondary roots are the roots that grow from the hypocotyl, the coleoptile, stem, and tillers; they are also called crown, nodal, or adventitious roots. This term is also used to describe the roots emerging from the primary roots; however, first-order laterals is a better term to describe those roots.

Taproot In many gymnosperms and dicotyledons, the primary root axis to arise from the seed, greatly enlarges to become the most prominent root axis of the plant, and is usually referred to as a taproot.

Taproot system Taproot system refers to the root systems formed from a central and usually relatively large root axis, the taproot.

Topology Describes the branching pattern of the individual root axes.

The main quantitative root traits and resource capture variables discussed in this entry are summarized in Table 1.

Definition of the Subject and Its Importance

The UN forecasts that the world population will reach 9.4 billion by 2050. The world must therefore develop the capacity to feed 10 billion within the next 40–50 years [1, 2]. The increase in production has to come

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Roots and Uptake of Water and Nutrients. Table 1 List of physiological variables, their definitions and units

Physiological variable (symbol)	Definition	Units
Nitrogen-use efficiency (NUE)	Grain DM at harvest (kg) per N available (soil + fertilizer) (kg)	Dimensionless
Nitrogen-uptake efficiency (UPE)	Above-ground N (kg) at harvest per N available (soil + fertilizer) (kg).	Dimensionless
Water-use efficiency (WUE)	Above-ground DM per water used (soil evaporation + crop transpiration).	g l^{-1}
Transpiration efficiency (TE)	Above-ground DM (g) per water used (l) through crop transpiration.	g l^{-1}
Radiation-use efficiency (RUE)	Above-ground dry mass (g) per intercepted global radiation (MJ).	g MJ^{-1}
Root to shoot ratio (R:S)	Ratio between the root and above-ground shoot dry mass.	Dimensionless (g g^{-1})
Root mass ratio (RMR)	Ratio between the root dry mass (g) and total plant dry mass (g).	Dimensionless (g g^{-1})
Root mass (RM)	Dry mass (g) of the total root system.	g
Root length (RL)	Total length (cm) of all roots present.	cm
Root length density (RLD)	Root length (cm) per unit of soil volume (cm^3).	cm cm^{-3}
Root volume (RV)	Total volume of the root system (cm^3).	cm^3
Root diameter	Average diameter (mm) of an individual root; commonly assumed to be a cylinder.	mm
Root fineness (RL:RV)	Ratio of the total root length (mm) to total root volume (mm^3).	mm mm^{-3}
Root depth	Maximum depth reached by a plant root system (m)	m
β Parameter	Describes the cumulative root distribution with depth. Estimated from: $p = 1 - \beta^d$, where p is the proportion of roots accumulated from the surface to a depth, d.	Dimensionless
k Parameter	Resource capture coefficient. Estimated from $\Phi = 1 - e^{-k.RLD}$, where Φ is the proportional resource captured (resource captured/ available resource).	m^2
Specific root length (SRL)	Ratio between root length (m) and root weight (g).	m g^{-1}
Root tissue density (RM:RV)	Ratio between root dry mass (mg) and root volume (mm^3).	mg mm^{-3}
Root front velocity(RFV)	Downward extension rate of the root front (mm) per day.	mm day^{-1}
Root longevity	Length of time of which an individual root is present in the soil.	days

from greater yields on existing cropland; but also without proportionate increases in the use of water or fertilizer, and within the context of climate change [3, 4]. A substantial increase in the effectiveness with which available water and nutrients are used is therefore required to ensure food security and environmental protection in future decades. Water is recognized as the most limiting factor in crop production worldwide, though nutrient shortages may often be as important as water scarcity and worldwide recovery of nitrogen (N) fertilizer in cereal systems worldwide is on average low at ca. 30–50% [5], with implications for potential

environmental impacts. Crop improvement under these conditions seems likely to be increasingly dependent on breeding for deeper or denser root systems, which promote soil moisture and nutrient capture and high dry matter production in cultivars subjected to water and/or nutrient stresses. In this entry, information is set out on the current understanding of the structure and functions of crop root systems. The avenues for the optimization of root anatomy and morphology traits that could be applied to the genetic and agronomic improvement of crop root systems for more effective below-ground resource capture are considered. Specifically, the determinants of effectiveness of capture by root systems of two key resources, water and nitrogen, according to their structure and function are considered.

Introduction

All higher plants have roots and the root fraction of the plant's total dry weight varies widely, both between and within species [6]. Although roots encounter many environmental fluctuations that affect their growth, they have a capacity to adjust to these as a whole system that makes them strongly dynamic in their spatial and temporal expansion. Understanding of these modifications in the form and function of the root system and their relationships with resource capture, whether due to environmental response or genetic control, is of importance for sustainable crop production.

Although the influence of canopy characteristics on above-ground productivity of crops is now relatively well understood, due to the difficulty of access and complexity of environment interactions, understanding the role of the root system is less complete. There is no doubt of the importance of the form and function of root systems to water and nutrient capture, and information has increased in the last decades on the role of crop root systems in maintaining yields under abiotic stress [7–9]. Root traits are a relatively new target in crop improvement programs aimed at improving tolerance of abiotic stress (water and nutrients). There are reports that particular root morphology and/or anatomical traits help plants maintain higher grain yields under low resource availability, for example, relatively deeper distribution of roots increasing water uptake under drought in wheat [10] and rice [11], longer root hairs increasing P acquisition under low P availability in barley [12], and narrower root xylem vessels in wheat were associated with increased water uptake during grain filling [13]. Nevertheless, the genetic control of root characteristics is poorly understood in most major staple crops, especially in bread wheat. Future improvement will depend on a better understanding of the morphological and anatomical traits determining below-ground resource capture, as well as the development and application of phenotypic screens to characterize genetic variation in the key traits. In this entry, the prospects for manipulating roots systems for improved resource capture and yield (drought and low nutrient availability) are considered, with a particular emphasis on cereal crops. The root traits that are focused on are principally morphological relating to root proliferation, root biomass and root length density, and their distribution with depth; although some consideration is given to anatomical features, for example, xylem root frequency and diameter. Roots and the uptake of water and nutrients are considered with regard to: (1) root morphology, (2) responses of root systems to water and nutrient stress, (3) the capacity of root systems for resource capture, and (4) prospects for breeding crops with optimized root systems for resilience to abiotic stresses.

Root System Morphology and Anatomy

Due to the difficulty of access and complexity of environmental interactions, roots are still one of the most challenging subjects in plant investigations, but their importance is unquestionable. Anchorage, support, and water and nutrient uptake are the main functions of the plant root system. With regard to crop root systems, the terms morphology and architecture are frequently used. The root system may be characterized according to four main categories of morphology/architecture [14, 15]. "Root morphology" refers to the surface features of a single root axes as an organ. It includes the characteristics of the epidermis such as root hairs, root cap, pattern of appearance of lateral roots, cortical senescence, and diameter. Root weight, volume, and area are also part of the morphology. "Root topology" describes the branching pattern of the individual root axes. "Root distribution" refers to the distribution of root traits, often morphologic ones (e.g., biomass length, biomass, etc.) as a function of several factors, the most common

being soil depth. Finally, "root architecture" relates to the spatial configuration of the root system as a whole.

The root morphology of monocotyledons differs from that of dicotyledons in several important respects. In the monocotyledons, for example, small grain cereals, two types of roots constitute the root system: the primary and the secondary roots [7, 16]. The primary roots (often called seminal roots; usually between three and eight axes) develop first arising from the coleorhizae of the seed [17, 18] and are active throughout all the crop life cycle [18]. Their extension is mainly downward allowing them to occupy the deeper layers of the soil profile [17]. The secondary (often called crown, nodal, or adventitious roots) are the roots that grow from the nodes of the coleoptile, main shoot, and tillers. The onset of tillering is the starting point of the growth of the secondary roots, and their formation is intimately related to tiller formation [19], so that factors favoring tillering will increase secondary root production. In dicotyledons, for example, oilseed rape, for most species the primary root consists of a taproot from which lateral roots and their branches arise. In both monocotyledons and dicotyledons, lateral roots are initiated in the pericycle and grow through the cortex to emerge at the surface of the parent root.

Rooting depth is affected by root-penetration rate and phenology. Generally, the longer a crop is growing, the deeper it roots [20]. Rooting depth (maximum depth reached by the roots) determines the amount of the soil that a plant can explore. Maximum rooting depth is typically 140–200 cm in winter cereals [18, 21] and 80–120 cm in spring cereals [22]. Rooting depth also strongly depends on the soil type and depth as well as below-ground resource availability, but generally the longer a crop grows the deeper the root system. Root growth and rooting depth of crop plants can be restricted because of physical and chemical impediments. Where physical and chemical soil constraints are absent, the maximum depth of rooting on deep soils is genetically determined and differs not only between vegetation types but also between crop species grown under identical conditions [8]. Much of within season variation in maximum rooting depth can be explained by temperature [23].

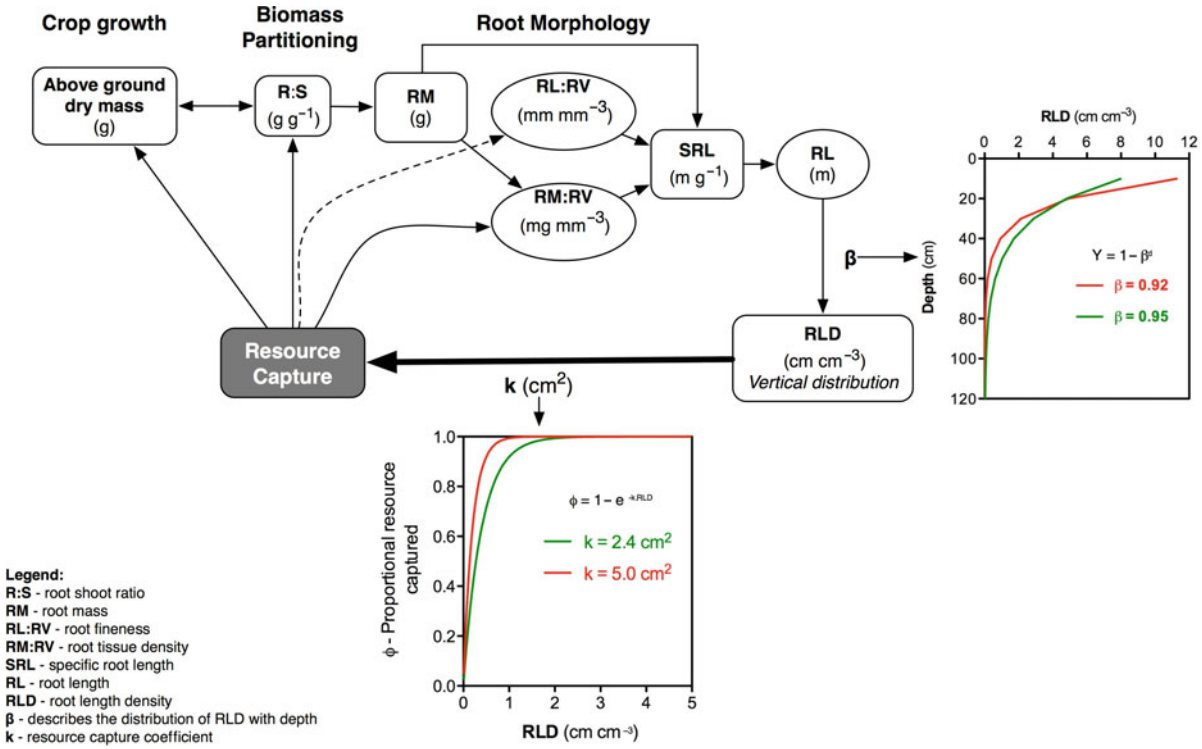
In monocotyledons, immediately after sowing root growth is favored, followed by a gradual decrease in assimilate partitioning to the root in favor of shoot growth after emergence. After flowering, the aboveground growth (fruit and grain formation) is favored, whereas root weight usually remains constant or decreases [24, 25]. Thus, root biomass and total length production generally follow a sigmoidal pattern from sowing to flowering in cereals, at which point further increases are not usually observed [18, 21]. The root dry mass ratio (root DM/total DM; RMR) is ca. 0.3 in wheat and barley during early growth, decreasing to ca. 0.1 at harvest [18, 22]. Another important trait influencing the crop's capacity to capture resources per unit soil volume is the root length density (RLD), which describes the total root length per unit of soil volume. Typical values of RLD in the upper 0.1 m of soil are about 20 cm cm^{-3} in grasses, 5–10 cm cm^{-3} in temperate cereal crops and 1–2 cm cm^{-3} in other crops [8]. The distribution of the RLD through the soil profile typically follows an exponential decrease with depth [26]. The cumulative distribution of RLD with depth (β) can be approximated by the equation described by Gale and Grigal [27] as:

$$Y = 1 - \beta^d \quad (1)$$

where Y is the fraction of the root system accumulated from the soil surface to depth, d, and β is a parameter that describes the shape of the cumulative distribution with depth.

This equation has been widely adopted since (e.g., [28–30]). The distribution of roots of many crops (e.g., cauliflower and winter wheat) is well described by the relationship, but in others (e.g., oilseed rape and sugar beet), this relation is found in the surface layers, but there is a tendency for values of RLD in deeper soil layers to be almost constant [8]. Differences in the distribution of RLD with depth may be associated with the velocity at which roots elongate to depth (root front velocity [RFV]) and the proliferation rate at each soil layer [31]. Root front velocity is closely related with the water and N extracted by the crop [31–33]. Another important trait influencing the potential for water and nutrient acquisition by roots is the mean root diameter (see Root traits and resource capture below).

Specific root length (root length per unit root DM - SRL; km g^{-1}) strongly influences the RLD. Theoretically, a high SRL (thinner roots) would be beneficial especially in resource-deficit situations. Specific root length is also positively correlated with root extension rate [34]. Specific root length varies considerably in the



Roots and Uptake of Water and Nutrients. Figure 1
Interrelationships between these root traits and their relations with resource capture

field and is strongly affected by environment; typical values are 130–250 m g^{-1} for cereals [35–37]. Root tissue density (root weight (RW): root volume (RV)) is highly correlated with root life span but inversely correlated with root expansion [38, 39]. So low RW: RV will may be one strategy to increase SRL of crop root systems [40] and potentially resource acquisition. Specific root length is a complex parameter that is determined by root length, tissue density, and diameter. It influences plant investment in potential resource acquisition (RLD) but also reflects root longevity and root growth rate, and therefore it is of potential interest as a selection criteria in breeding programs for optimized root systems. Maximizing SRL seems to be an advantage particularly in water- and nutrient-limited conditions [39, 41] and is associated with higher RLD. Intuitively, thinner roots would be advantageous for acquiring soil resources, though there may be trade-offs with other root functions such as anchorage, support, and transport [42]. The interrelationships between these root traits and their relations with resource capture are summarized in Fig. 1. Additionally, there are reported effects of root diameter on resource capture, which has been shown to be highly correlated with plant dry mass [43, 44] and the diameter of conducting vessels. The principal root traits described above may be influenced by abiotic stresses during the rapid phase of root growth and expansion in the pre-flowering period with implications for resource capture during later seed filling, and these effects are now considered in the following section of this entry.

Effects of Abiotic Stress and Root Development

Drought and Root Development

Drought overall usually reduces the size of root systems. Water deficits decrease carbon assimilation by the plant due to a reduction of green leaf area, but also due to a decline in net photosynthetic rate. Nevertheless, under drought plants tend to increase the proportion of total carbon allocated to the roots [25, 45]. For example, plants responded to water deficits by

increasing the proportion of assimilate allocated to roots in wheat [46, 47] and barley [48]. Experiments using pulse-labeled 13C in wheat have shown that water deficits increase the allocation of assimilated C to the roots due to a greater reduction of growth in the above-ground than below-ground plant components [49]. Although the relative root dry mass tends to increase with water deficits the absolute weight of both roots and shoots tends to decrease. As the soil dries, there are changes in its physical condition such as increases in soil strength [50]. Shoots are generally more affected by drought than roots, associated with more severe water deficits developing and persisting longer in the transpiring shoots [49, 51, 52]. Thus, roots are typically prioritized during drought to facilitate access to water while decreasing transpiration. The relationship between these two systems is often described as a competition where both roots and shoots compete for carbohydrates, minerals, and water, the most successful being the one nearer the source [24]. Therefore, the growth of the root and shoot systems is an integrative process working in a functional equilibrium [53, 54]. So when light is limited, root growth will be more restricted than shoots and the opposite happens when soil resources are in deficit; this functional balance hypothesis is elegantly explained by Brouwer [24]. In addition, under water-limiting conditions, solutes may accumulate in the root tip attracting the movement of water by diffusion, allowing the cells in the root tip to maintain their turgor and growth [55].

Although water deficits typically increase the percentage of carbon allocated to the roots, there are some reports of contrasting responses of root partitioning to drought amongst cultivars, for example, in glasshouse- [52] and field-grown wheat [56]. Therefore, the root growth response under drought is not simple, since drought not only affects plant and root growth but also the soil structure and N availability [57]. For example, water deficits may have a neutral effect on root weight, but still influence root length and its distribution with depth in wheat [47]; or increase SRL with drought in bread wheat [58] and durum wheat and barley [59]. There is some evidence that thinner roots may themselves be more vulnerable to drought [44, 60]. Therefore, the relatively high diameters reported for irrigated compared to droughted plants [58, 61] might relate to the necessity of the root system to support a larger plant and facilitate faster and greater water uptake and transport in well-watered conditions.

Leaf expansion and senescence are particularly susceptible to water deficiency [62]. The causes for restricted leaf expansion with drought have been discussed extensively, and there are mainly two views on the underlying mechanisms involved. Some authors attribute the cause to water relations (water potential and cell turgor) in the leaf [63, 64], while others attribute it to root chemical signals, such as abscisic acid (ABA), transmitted to the leaves, in response to water depletion in the soil [65–67]. ABA concentration increases in shoots, leaves, and roots in plants grown under water deficits and its exogenous application on well-watered plants mimics many of the drought effects on the plant [68, 69]. The chemical mechanism involves the synthesis of the plant hormone ABA by the roots when sensing the drying of the soil, and the transfer of ABA in the xylem to shoots and leaves inducing stomatal closure hence reducing water uptake and shoot and leaf growth [68, 70–72].

Evidence for water relations as the main cause of the decrease in leaf expansion was described by Boedt & Hensley (1987 in [63]) where leaves of field-grown maize showed visual symptoms of water stress in soil near field capacity. Tazaki et al. (1980 in [63]) in Japan reported similar effects for rice leaves, even though plants were rooted in wet soil. Furthermore, seedling experiments in maize plants using the pressure-pump technique [64] showed that an increase in the water pressure in the roots was quickly and fully transmitted to the base of the leaf increasing the leaf elongation. In contrast with these findings, Passioura [66] growing wheat seedlings in drying soil but maintaining leaf turgidity using the pressure-chamber method, showed a decrease in the relative expansion rate of leaves. Additional evidence for the root chemical signal was given by Gollan et al. [73] where wheat and sunflower plants showed a decrease in stomatal conductance with an increase of water deficits while the pressure in the plant was maintained. Using partial root-zone drying (PRD) techniques where half of the root system is droughted while the other half is irrigated, to maintain the same leaf water status as control plants (full irrigation), results showed a decrease of 65% of leaf area and 70% of water loss in apple plant seedlings subjected to PRD [67].

A more recent hypothesis is that both hydraulic and chemical signals interact and that the importance of one or the other will depend on the timescale considered [74]. Experiments in maize and barley showed that sudden changes in leaf water status by light, humidity, or salinity greatly affect leaf-elongation rate, and that those effects vanished when their roots were placed in a pressure chamber to maintain the xylem and air pressures in equilibrium, showing that hydraulic relations dominated in this response [75]. If the saline or water stress was prolonged, water relations were overridden by chemical signals and pressurization failed to maintain leaf elongation rates [75]. The combination of hydraulic and chemical factors was also demonstrated by differences in the sensitivity of different maize lines under drought to xylem ABA [76].

Nutrients and Root Development

It is well established that plants respond to N and P deficiencies by increasing RMR due to the functional equilibrium between the growth of the root and shoot [24, 77–81]. Crop root systems are plastic and respond by proliferating roots to exploit patches of nutrients where the distribution within the soil is uneven [82]. For example, responses to aqueous fertilizer in wheat have been observed within 24 h of application [83]. Frequently there is a strong association between root length and P uptake. Root proliferation in P-rich patches is, therefore, relatively straightforward to interpret in terms of a "foraging" response. The responses of roots to N- and P-rich patches of soil include proliferation of laterals and stimulation of nutrient inflow (uptake rate per unit root length) within the patch [81]. Nitrate uptake from a N-rich patch may compensate for an uneven supply of nitrate to the whole root system. Localized N application on barley seminal root systems promoted the number and extension rate of both first- and second-order lateral roots [84]. The potential magnitude of the responses to N and P has been demonstrated in barley by Drew and Saker [85, 86]. With regard to genetic effects, Zhang and Forde [87] demonstrated that, in Arabidopsis, the extension of lateral roots in nitrate-rich patches is partly under genetic control. Since irrigation and N fertilizer can cause root proliferation in the surface soil [47, 88], the distribution of the availability of these resources earlier in the crop's growth may alter the relative distribution of roots with depth (β) at anthesis.

For two barley varieties grown in Mediterranean field conditions, RMR increased under low N and P fertilizer supply compared to a control treatment with ample N and P supply [89]. Herrera et al. [90] in wheat showed that high N supply increased the number of roots, and when N was limited root formation ceased earlier. Barraclough et al. [47] observed an increase in RMR with low N supply in N x drought field experiments in winter wheat in the UK. N application effects on SRL are inconsistent, and increases, decreases, or neutral effects are reported for different species [44, 91]. Field experiments on spring barley and durum wheat in Jordan showed no consistent response for SRL for three different levels of N fertilizer [59]. SRL increased with N application under rain-fed conditions for durum wheat, but the opposite was found for spring barley. There appear to be few previous investigations regarding the effects of N fertilization on SRL and its components in cereals in field conditions. N application has been observed to increase mean root diameter in cereals but to decrease RW:RV [44, 91].

Root Traits and Resource Capture

The primary root traits for improved below-ground resource capture would appear to be root morphology (root axis number, rooting depth, rooting length density), root extension rates, root longevity, and root function along the length of the root system [9, 92, 93].

Water Capture

The importance of water for plants is unquestionable; it performs a varied number of physiological and structural functions. Water constitutes on average 80–90% of the fresh weight of herbaceous plants providing a continuous liquid phase in which gases, minerals, and other solutes enter the cells and move from one cell to another and within the different plant organs [94]. Water is a reactant or substrate in most of the plant's biochemical reactions (e.g., photosynthesis) and it maintains the plant turgor essential for cell growth, enlargement, form and movement of various plant structures, like the stomata opening [8, 94].

Crop production is closely related to water transpired; therefore, maintaining an uninterrupted supply

of water to leaves is essential to maximize yields. Water capture is intimately related with root size, usually measured, as surface area, volume, or length. According to the theoretical model of van Noordwijk [95], the rate of water uptake by the plant is mainly limited by the transport in the soil toward the root (soil–root interface). Therefore, the density of roots, measured as length per unit soil volume (root length density – RLD, cm cm^{-3}), is the most suitable parameter to describe water uptake by plant roots. Prolific root systems are more effective at capturing water than sparse systems, but inter-root competition sets a natural ceiling on optimum RLD in cereals, above which further increases require excessive roots which do not have measurable effects on water uptake [95]. Theoretical calculations predict a critical root length density (C_{RLD}) of about 1 cm cm^{-3} for water uptake. This figure broadly concurs with the values reported for water uptake of Gregory and Brown [96] and Barraclough et al. [47] who showed that a RLD of 1 cm cm^{-3} was associated with the abstraction of all of the available water by both spring barley and winter wheat, respectively. However, for upland rice, values of C_{RLD} between 1.5 and 1.6 cm cm^{-3} have been reported [97, 98] and in controlled environment conditions values as low as 0.30 cm cm^{-3} [99].

RLD distribution with depth is principally determined by time for growth (residence times are greater in the topsoil than the subsoil), soil porosity and strength, and water availability [20]. Root length density in wheat is typically below the C_{RLD} of ca. 1 cm cm^{-3} at soil depths below ca. 80 cm [18, 21, 36, 100]. A modeling study concluded that distributing roots relatively deeper in the soil profile and decreasing SRL would confer greater water capture and yield under low water availability in wheat [30]. Experimental evidence also supports the strategy of distributing roots relatively deeper to improve water capture under drought. Synthetic derivative wheat lines showed increased water uptake associated with a root system that was distributed relatively deeper in the soil compared with recurrent parents [10], and the drought tolerance of spring wheat SeriM82 was related to its relatively deep root system compared to the check cultivar Hartog [101]. Further root traits which could be beneficial in boosting water capture include enhanced post-anthesis root longevity and root penetration ability [102], although there is relatively little information on genetic variation in these traits in cereals.

In rice under flooded conditions, attempts to optimize the root system through plant breeding methods must additionally allow for the complicated interplay between adaptations for internal aeration and those for efficient nutrient acquisition. A recent model developed by Kirk [103] provides a coherent representation of the rice root system in submerged soil and predicted that a system of coarse, aerenchymatous primary roots with gas-impermeable walls conducting O_2 down to short, fine, gas-permeable laterals provided the best compromise between the need for internal aeration and the need for the largest possible absorbing surface per unit root mass.

Nutrient Capture

Water and nutrient uptake should really be considered together since nutrients become less available as the soil dries. Nitrate is readily leached down the soil profile and consequently rooting depth is an important attribute for soil N acquisition. For a long time, due to its high mobility in soil, N supply was considered independent of the root system characteristics, assuming that only mass flow and diffusion were the relevant mechanisms for the uptake of N by the plant [90]. The role of crop root systems in capturing N is still a topic of debate. Findings of Robinson et al. [104] indicated only 4–11% of the total root length is involved in N uptake. On the other hand, Palta and Watt [9] demonstrated through 15N labeling experiments on wheat that vigorous root systems captured ca. 60% more N in the top 0.2 m of the soil profile than non-vigorous root systems. Furthermore, a positive correlation was found between nitrate and water uptake and root length density in maize [105–107] and in several catch crop species [108]. These studies indicated that greater root length densities are generally more effective in N acquisition. However, root length is probably more important for the uptake of relatively immobile ions such as phosphates [109, 110]. However, some investigations have found uptake rates of phosphate, calcium, and potassium from solution are poorly related to root length [111, 112], possibly because root length is only significant if the uptake of these nutrients is limiting [7]. In addition, several

investigations have shown that nitrate capture depends on the ability of the root system to respond to spatial and temporal nitrogen supply [81, 113].

The ability to capture N depends mainly on the amount of nitrate present in the soil relative to the morphology of the root system. Nitrate is supplied to the root system by mass flow (ions carried along in the transpiration stream) and diffusion (ions moving down a concentration gradient, either through bulk soil water or along water films surrounding particles). About 50% of the N taken up by wheat crops may be transported by mass flow [16]. As for water uptake, inter-root competition sets a natural ceiling on optimum RLD in cereals of about 1 cm cm^{-3} [95]. RLD distribution with depth is principally determined by time for growth (residence times are greater in the topsoil than the subsoil), soil porosity and strength, and nutrient and water availability [20]. The modeling study of King et al. [30] concluded that distributing roots relatively deeper in the soil profile and increasing SRL would confer greater N capture and yield under low N availability. The particular properties of each nutrient in the soil impose different RLD requirements for effective uptake. For example, due the low mobility of phosphorous (P) in the soil, a higher RLD of ca. 10 cm cm^{-3} is required for effective P uptake compared to water and/or nitrogen [114, 115]. Similar to water uptake, root traits that could be beneficial in boosting N capture include enhanced root longevity post-anthesis and root penetration ability [102], although there is relatively little information on genetic variation in these traits in wheat. Barraclough et al. [47] in N × drought field experiments in winter wheat in the UK found that water uptake increased with N due to a higher RLD and higher ground cover reducing soil evaporation. Positive correlations between nitrogen capture and RLD have also been found in maize [106, 107] and durum wheat and barley [59]. Although higher N supply tends to increases total RLD and N uptake by the crop, this generally results in a decrease in N uptake efficiency (crop N uptake/N available) [116, 117] leading to potentially greater losses of nitrate to the environment. A recent modeling exercise suggested that higher RLD and deeper rooting depths would reduce residual nitrate in high leaching soils [118]. Forde & Clarkson [119] concluded that there was no strong evidence for significant age-dependent changes in capacity of roots to absorb nitrate or ammonium ions.

Nutrient uptake may also be influenced by root membrane transporter systems. With regard to N uptake, recent work in *Arabidopsis* indicates nitrate is actively transported across the plasma membranes of plant cells, but net uptake is a balance between active influx and passive efflux. Two distinct gene families of nitrate transporters, *NRT1* and *NRT2*, have been identified [119–122] in the *Arabidopsis* genome. Some members of both *NRT1* and *NRT2* gene families are nitrate inducible, and are expressed in the root epidermis and in root hairs, and are likely to be responsible for the uptake of nitrate from the soil (e.g., [123–126]). There are prospects for transferring this information to wheat for improving efficiency of N uptake in the long term if the root screens used for *Arabidopsis* could be adapted to the larger and structurally different root system of wheat. An extensive review of this area is beyond the scope of this entry. Fortunately, excellent reviews are recently available in this topic area [127, 128].

With regard to the carbon costs of roots, it seems there is likely only a limited capacity to reduce root partitioning below current values of ca. 10% at anthesis in cereals in high yield potential ideotypes, due to the trade-off with water and N capture required for future biomass gains. However, a deeper relative distribution of roots while maintaining RMR could comprise part of an ideotype to maximize yield in future breeding programs.

Application of Rooting Traits in Breeding for Tolerance of Abiotic Stresses

Genetic variation in root system size has been widely reported in grain crops (e.g., [6, 45, 129]), but root distribution varies strongly with soil characteristics such as water and nutrient availability and mechanical impedance [54]. The RMR of wheat or barley is typically ca. 30% during early vegetative growth decreasing to ca. 10% by anthesis [18, 96, 130]. Effects of increasing plant height on root partitioning have been studied using isolines and are generally either neutral or negative in wheat [22, 130–132].

Breeding cultivars better adapted to particular conditions of drought and/or low nutrient availability will play a major role for the future of crop production for

adaptation to climate change. Breeding for more effective use of water or nutrients while maintaining, or ideally, increasing yield potential is a difficult task. It will be important in the most efficient crop systems to combine optimized agronomy and new cultivars efficient at acquiring below-ground resources. To date there are relatively few examples of root morphological or anatomical traits that have been successfully selected for crop breeding programs to result in improved performance. In a recent review on this topic, Palta and Watt [9] cited five examples of rooting traits directly related to below-ground resource capture in breeding, including long root hairs for increased P uptake in barley [12], reduced xylem vessel diameter in seminal roots of wheat [13], and increased root density at depth through faster root extension rate in wheat [133]. In addition, improved resource capture has been achieved by alleviating other stresses on roots, for example, resistance to cereal cyst nematode in wheat [134]. Another encouraging example of introgression of rooting traits in crop breeding can be found in rice. When near isogenic lines (NILs) obtained through marker-assisted backcrossing for four QTLs for root length were field-tested, they outperformed the recurrent parent for yield and biomass [135]. In order to introgress rooting traits into elite genotypes, it will be necessary to identify genetic diversity for the key traits as well as developing methods for rapid high-throughput screening of lines in breeders trials.

Field phenotyping methods for roots in cereals were reviewed by Manske et al. [136] and Polomski and Kuhn [137], including the use of rhizotrons and assessments of root parameters from soil cores (root washing and root counts/image analysis). Although several screening tests have been designed to generate accurate and robust data from seedling plants grown under artificial conditions, these phenotypes can rarely be extrapolated to field conditions because of the pronounced plasticity of root growth and development processes [138]. Field phenotyping for root traits in breeding programs is currently infeasible, so genetic progress will depend on the development of high-throughput controlled-environment screens or molecular markers for root traits for marker-assisted selection (MAS). The use of root-observation chambers and a nondestructive digital imaging technique offers some promise [139], but may be less suitable for screening of root traits that are expressed at later stages of crop development.

Future Directions

The existence of significant genetic variation for rooting traits has not resulted to date, except for a few exceptions as mentioned above, in the incorporation of rooting traits in conventional breeding. Nevertheless, future genetic progress for resistance to abiotic stresses should be accelerated by the fact that the genetic control of rooting traits can now be revealed through the application of rapidly emerging genetic resources facilitating the fine mapping of root QTL. Indeed, the cloning of the first root QTL is ongoing. However, successful exploitation of genomics tools and strategies in plant breeding requires extensive and precise phenotyping of agronomic traits for breeding materials and mapping populations. The capacity for precise phenotyping under reliable conditions probably represents the most limiting factor for the progress of genomic studies on root traits underlying resilience to abiotic stresses. There is a need for a high precision because the differences may be small, and detailed physiological measurements (e.g., of growth rate) are difficult when a large numbers of genotypes are involved.

Physiological perspectives that require more attention are analyses to measure the full carbon costs of the turnover of root material (and therefore of the root system), which are presently poorly quantified especially in environments where soil stresses are common. The role of organic substances in the rhizosphere secreted by the root that are able to modify the environment to secure improved water uptake requires more attention. Future research should focus on the importance of root plasticity for nutrient capture rather than simply measuring the size of the response. More studies at the plant community level rather than on single plants are required to translate fundamental studies on root growth and function to the improved water and nutrient capture at the crop scale.

Additionally, future work should aim to address the potential use of marker-assisted backcrossing for root QTLs and to exploit findings in *Arabidopsis* where root screens for mutants have identified genes such as AUX1 and LAX3 that regulate important root architectural

traits such as lateral root development [140]. There is a continuing need to integrate "omics" technologies with plant physiology, agronomy, breeding, and disciplines related to the rhizosphere. In the future to meet the challenge of raising biomass and yield potential as well as improving resilience to abiotic stresses it will be crucial that new root research fosters collaborations between breeders, geneticists, physiologists, crop physiologists, and soil scientists (among others) to translate the genetic data generated from the new genomics resources into improved crop performance.

Bibliography

Primary Literature

1. Lutz W, Sanderson W, Scherbov S (2001) The end of world population growth. Nature 412(6846):543–545
2. Hamilton R (2009) Agriculture's sustainable future: breeding better Crops. In: Scientific American: http://www.scientificamerican.com/article.cfm?id=agricultures-sustainable-future
3. Hirel B, Le Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot 58(9):2369–2387
4. Cattivelli L, Rizza F, Badeck FW, Mazzucotelli E, Mastrangelo AM, Francia E, Marë C, Tondelli A, Stanca AM (2008) Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. Field Crops Res 105(1–2):1–14
5. Raun WR, Solie JB, Johnson GV, Stone ML, Mullen RW, Freeman KW, Thomason WE, Lukina EV (2002) Improving nitrogen use efficiency in cereal grain production with optical sensing and variable rate application. Agron J 94:815–820
6. O'toole JC, Bland WL (1987) Genotypic variation in crop plant-root systems. Adv Agron 41:91–145
7. Hoad SP, Russell G, Lucas ME, Bingham IJ (2001) The management of wheat, barley and oat root systems. Adv Agron 74:193–246
8. Gregory PJ (2006) Plant roots: growth, activity, and interaction with soils. Blackwell, Oxford
9. Palta J, Watt M (2009) Vigorous crop root systems: form and function for improving the capture of water and nutrients. In: Crop Physiology – Applications for genetic improvement and agronomy, Elsevier, San Diego
10. Reynolds M, Dreccer F, Trethowan R (2007) Drought-adaptive traits derived from wheat wild relatives and landraces. J Exp Bot 58(2):177–186
11. Yoshida S, Hasegawa S (1982) The rice root system its development and function. In: O'Toole JC (ed) Drought resistance in crops with emphasis on rice. IRRI, Manila
12. Gahoonia TS, Nielsen NE (2004) Barley genotypes with long root hairs sustain high grain yields in low-P field. Plant Soil 262(1):55–62
13. Richards RA, Passioura JB (1989) A breeding program to reduce the diameter of the major xylem vessel in the seminal roots of wheat and its effect on grain yield in rain-fed environments. Aust J Agric Res 40(5):943–950
14. Fitter AH (1985) Functional significance of root morphology and root system architecture. In: Fitter AH, Atkinson D, Read DJ, Usher MB (eds) Ecological interactions in soil, plants, microbes and animals. Blackwell, Oxford, pp 87–106
15. Lynch J (1995) Root architecture and plant productivity. Plant Physiol 109:7–13
16. Gregory PJ, Crawford DV, Mcgowan M (1979) Nutrient relations of winter wheat. 2. Movement of nutrients to the root and their uptake. J Agri Sci 93:495–504
17. Key JM (1973) The wheat root. In: Sears ER, Sears LMS (eds) 4th International wheat genetics symposium pp 827–842. Agricultural Experiment Station, College of Agriculture, University of Missouri, Columbia, Missouri, Columbia
18. Gregory PJ, Mcgowan M, Biscoe PV, Hunter B (1978) Water relations of winter wheat.1. Growth of root system. J Agric Sci 91:91–102
19. Klepper B, Belford RK, Rickman RW (1984) Root and shoot development in winter wheat. Agron J 76(1):117–122
20. Barraclough PB, Weir AH, Kuhlmann H (1991) Factors affecting the growth and distribution of winter wheat roots under UK field conditions. Dev Agr Manag For Ecol 24:410–441
21. Barraclough PB, Weir AH (1988) Effects of compacted subsoil layer on root and shoot growth, water use and nutrient uptake of winter wheat. J Agric Sci 110:207–216
22. Siddique KHM, Belford RK, Tennant D (1990) Root:shoot ratios of old and modern, tall and semidwarf wheats in a mediterranean environment. Plant Soil 121(1):89–98
23. Barraclough PB (1984) The growth and activity of winter-wheat roots in the field – root-growth of high-yielding crops in relation to shoot growth. J Agri Sci 103:439–442
24. Brouwer R (1983) Functional equilibrium: sense or nonsense? Neth J Agric Sci 31(4):335–348
25. Gregory PJ, Palta JA, Batts GR (1997) Root systems and root:mass ratio – carbon allocation under current and projected atmospheric conditions in arable crops. Plant Soil 187(2):221–228
26. Gerwitz A, Page ER (1974) An empirical mathematical model to describe plant root systems. J Appl Ecol 11(2):773–781
27. Gale MR, Grigal DF (1987) Vertical root distributions of northern tree species in relation to successional status. Can J For Res 17(8):829–834
28. Robertson MJ, Fukai S, Hammer GL, Ludlow MM (1993) Modelling root growth of grain sorghum using the CERES approach. Field Crops Res 33:113–130
29. Zhuang J, Yu GR, Nakayama K (2001) Scaling of root length density of maize in the field profile. Plant Soil 235:135–142
30. King J, Gay A, Sylvester-Bradley R, Bingham I, Foulkes J, Gregory P, Robinson D (2003) Modelling ceral root systems for water and nitrogen capture: towards an economic optimum. Ann Bot 91:383–390
31. Thomas Fukai S, Hammer GL (1995) Growth and yield response of barley and chickpea to water stress under three

environments in Southeast Queensland. II* Root growth and soil wate extraction pattern. Aust J Agric Res 46:35–48
32. Monteith JL (1986) How do crops manipulate water-supply and demand. Philos Trans R Soc Lond Ser Math Phys Eng Sci 316(1537):245–259
33. Robertson MJ, Fukai S, Ludlow MM, Hammer GL (1993) Water extraction by grain sorghum in a sub-humid environment. II. Extraction relation root growth. Field Crops Res 33:99–112
34. Eissenstat DM (1991) On the relationship between specific root length and the rate of root proliferation - a field-study using citrus rootstocks. New Phytol 118(1):63–68
35. Welbank PJ, Gibb MJ, Taylor PJ, Williams ED (1974) Root growth of cereal crops. In: Rothamsted Experimental Station Report, 1973, Part 2 pp 26–66
36. Barraclough PB, Leigh RA (1984) The growth and activity of winter wheat roots in the field: the effect of sowing date and soil type on root growth of high yielding crops. J Agric Sci 103:59–74
37. Van Noordwijk M, Brouwer G (1991) Review of quantitative root length data in agriculture. Elsevier, Amsterdam, Pays-Bas
38. Ryser P (1996) The importance of tissue density for growth and life span of leaves and roots: A comparison of five ecologically contrasting grasses. Funct Ecol 10(6):717–723
39. Wahl S, Ryser P (2000) Root tissue structure is linked to ecological strategies of grasses. New Phytol 148:459–471
40. Valenzuela-Estrada LR, Vera-Caraballo V, Ruth LE, Eissenstat DM (2008) Root anatomy, morphology, and longevity among root orders in *Vaccinium corymbosum* (*Ericaceae*). Am J Bot 95(12):1506–1514
41. Eissenstat DM (1992) Costs and benefits of constructing roots of small diameter. J Plant Nutr 15(6–7):763–782
42. Fitter AH (1996) Characteristics and functions of root systems. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker, New York, pp 1–20
43. Hetrick BAD, Leslie JF, Wilson GT, Kitt DG (1988) Physical and topological assessment of effects of a vesicular-arbuscular mycorrhizal fungus on root architecture of big bluestem. New Phytol 110(1):85–96
44. Ryser P (1998) Intra- and interspecific variation in root length, root turnover and the underlying parameters. In: Lambers H, Poorter H, van Vuuren MMI (eds) Inherent variation in plant growth. Physiological mechanisms and ecological consequences. Backhuys, Leiden
45. Ehdaie B, Waines JG (2003) 1RS translocation increases root biomass in Veery-type wheat isogenic lines and associates with grain yield. In: Pogna NE, Romano M, Pogna EA, Galterio G (eds) Proceedings of the 10th International wheat genetics symposium, ISC Paestum, Italy, Rome, pp 693–695
46. Hamblin A, Tennant D, Perry MW (1990) The cost of stress - dry-matter partitioning changes with seasonal supply of water and nitrogen to dryland wheat. Plant Soil 122(1):47–58
47. Barraclough PB, Kuhlmann H, Weir AH (1989) The effects of prolonged drought and nitrogen-fertilizer on root and shoot growth and water-uptake by winter-wheat. J Agron Crop Sci 163(5):352–360
48. Zenisceva L (1990) The importance of the root-system in adaptation of spring barley genotypes to the conditions of environment. Rost Vyroba 36:937–945
49. Palta JA, Gregory PJ (1997) Drought affects the fluxes of carbon to roots and soil in C-13 pulse-labelled plants of wheat. Soil Biol Biochem 29(9–10):1395–1403
50. Martino DL, Shaykewich CF (1994) Root penetration profiles of wheat and barley as affected by soil penetration resistance in field conditions. Can J Soil Sci 74:193–200
51. Kramer PJ (1983) Water relations of plants. Academic, London
52. Karrou M, Maranville JW (1994) Response of wheat cultivars to different soil nitrogen and moisture regimes: I. Dry matter partitioning root growth. J Plant Nutr 17(5):729–744
53. Davidson RL (1969) Effect of root/leaf temperature differentials on root/shoot ratios in some pasture grasses and clover. Ann Bot 33(3):561–569
54. Gregory PJ (1994) Root growth and activity. In: Boote KJ, Bennett JM, Sinclair TR, Paulsen GM (eds) Physiology and determination of crop yield. Soil Science Soc America, Madison, pp 65–93
55. Sharp RE, Davies WJ (1979) Solute regulation and growth by roots and shoots of water-stressed maize plants. Planta 147(1):43–49
56. Li FM, Yan X, Li FR, Guo AH (2001) Effects of different water supply regimes on water use and yield performance of spring wheat in a simulated semi-arid environment. Agric Water Manage 47(1):25–35
57. Robinson D (1994) Resource capture by single roots. In: Monteith JL, Scott RK, Unsworth MH (eds) Resource capture by crops. Nottingham University Press, Nottingham, pp 53–76
58. Baburai Nagesh AK (2006) The physiological and genetic bases of water-use efficiency in winter wheat. Ph.D. thesis, University of Nottingham
59. Ebrahim NM (2008) Responses of root and shoot growth of durum wheat (Triticum turgidum L. var durum) and barley (Hordeum vulgare L.) plants to different water and nitrogen levels. PhD Thesis University of Jordan, Jordan, 284 pp
60. Fitter AH (1987) An architectural approach to the comparative ecology of plant root systems. New Phytol 106:61–77
61. Li FM, Liu XL, Li SQ (2001) Effects of early soil water distribution on the dry matter partition between roots and shoots of winter wheat. Agric Water Manage 49(3):163–171
62. Turner NC, Begg JE (1981) Plant-water relations and adaptation to stress. Plant Soil 58(1–3):97–131
63. Kramer PJ (1988) Changing concepts regarding plant water relations. Plant Cell Environ 11(7):565–568
64. Hsiao TC, Frensch J, Rojas-Lara BAR (1998) The pressure-jump technique shows maize leaf growth to be enhanced by increases in turgor only when water status is not too high. Plant Cell Environ 21(1):33–42
65. Passioura JB (1988) Changing concepts regarding plant water relations - response. Plant Cell Environ 11(7):569–571
66. Passioura JB (1988) Root signals control leaf expansion in wheat seedlings growing in drying soil. Aust J Plant Physiol 15(5):687–693

67. Gowing DJG, Davies WJ, Jones HG (1990) A positive root-sourced signal as an indicator of soil drying in Apple, *Malus x domestica* Borkh. J Exp Bot 41(233):1535–1540
68. Davies WJ, Tardieu F, Trejo CL (1994) How do chemical signals work in plants that grow in drying soil? Plant Physiol 104(2):309–314
69. Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscisic acid synthesis. J Exp Bot 51(350):1563–1574
70. Zhang J, Davies WJ (1990) Changes in the concentration of aba in xylem sap as a function of changing soil-water status can account for changes in leaf conductance and growth. Plant Cell Environ 13(3):277–285
71. Davies WJ, Zhang J (1991) Root signals and the regulation of growth and development of plants in drying soil. Annu Rev Plant Physiol Plant Mol Biol 42(1):55–76
72. Hartung W, Jeschke WD (1999) Abscisic acid: a long-distance stress signal in salt-stressed plants. In: Lerner HR (ed) Plant responses to environmental stresses. Marcel Dekker, New York, pp 333–348
73. Gollan T, Passioura JB, Munns R (1986) Soil water status affects the stomatal conductance of fully turgid wheat and sunflower leaves. Aust J Plant Physiol 13(4):459–464
74. Aphale SL (2004) Role of root to shoot signalling in coordinating responses to nitrogen deficiency. Ph.D. thesis, University of Nottingham
75. Munns R, Passioura JB, Guo JM, Chazen O, Ramer GR (2000) Water relations and leaf expansion: importance of time scale. J Exp Bot 51(350):1495–1504
76. Stikić R, Davies WJ (2000) Stomatal reactions of two different maize lines to osmotically induced drought stress. Biol Plant 43(3):399–405
77. Novoa R, Loomis RS (1981) Nitrogen and plant production. Plant Soil 58(1–3):177–204
78. Dreccer M, Schapendonk A, Slafer G, Rabbinge R (2000) Comparative response of wheat and oilseed rape to nitrogen supply: absorption and utilisation efficiency of radiation and nitrogen during the reproductive stages determining yield. Plant Soil 220(1):189–205
79. Mengel K, Kirkby EA (2001) Principles of plant nutrition. Kluwer, London
80. Reich PB (2002) Root-shoot relations: optimality in acclimation and adaptation or the: "Emperor's new clothes"? In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker, New York, pp 205–220
81. Robinson D (2001) Root proliferation, nitrate inflow and their carbon costs during nitrogen capture by competing plants in patchy soil. Plant Soil 232(1–2):41–50
82. Robinson D, Hodge A, Griffiths BS, Fitter AH (1999) Plant root proliferation in nitrogen-rich patches confers competitive advantage. Proc R Soc Lond B Biol Sci 266(1418):431–435
83. Jackson RB, Caldwell MM (1989) The timing and degree of root proliferation in fertile-soil microsites for 3 cold-desert perennials. Oecologia 81(2):149–153
84. Drew MC, Saker LR, Ashley TW (1973) Nutrient supply and the growth of the seminal root system in barley: I. Effect nitrate concentration growth axes laterals. J Exp Bot 24(6):1189–1202
85. Drew MC, Saker LR (1975) Nutrient supply and the growth of the seminal root system in barley. II. Localized, compensatory increases in lateral root growth and rates op nitrate uptake when nitrate supply is restricted to only part of the root system. J Exp Bot 26(1):79–90
86. Drew MC, Saker LR (1978) Nutrient supply and the growth of the seminal root system in barley. III. Compensatory increases in growth of lateral roots, and in rates of phosphate uptake, in response to a localized supply of phosphate. J Exp Bot 29(2):435–451
87. Zhang HM, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279(5349):407–409
88. Robinson D (1994) The responses of plants to non-uniform supplies of nutrients. New Phytol 127(4):635–674
89. Brown SC, Keatinge JDH, Gregory PJ, Cooper PJM (1987) Effects of fertilizer, variety and location on barley production under rainfed conditions in northern syria 1root shoot growth. Field Crops Res 16:53–66
90. Herrera JM, Stamp P, Liedgens M (2005) Dynamics of root development of spring wheat genotypes varying in nitrogen use efficiency. In: Foulkes J, Russel G, Hawkesford M, Gooding M, Sparkes D, Stockdale E (eds) Roots and the soil environment II. Association of Applied Biologists, Warwick, pp 197–201
91. Ryser P, Lambers H (1995) Root and leaf attributes accounting for the performance of fast- and slow-growing grasses at different nutrient supply. Plant Soil 170:251–265
92. Foulkes MJ, Hawkesford MJ, Barraclough PB, Holdsworth MJ, Kerr S, Kightley S, Shewry PR (2009) Identifying traits to improve the nitrogen economy of wheat: recent advances and future prospects. Field Crops Res 114:329–342
93. Doussan C, Pages L, Pierret A (2003) Soil exploration and resource acquisition by plant roots: an architectural and modelling point of view. Agronomie 23(5–6):419–431
94. Kramer PJ, Boyer JS (1995) Water relations of plants and soils. Academic, San Diego
95. Van Noordwijk M (1983) Functional interpretation of root densities in the field for nutrient and water uptake. In: Böhm W, Kutschera L, Lichtenegger E (eds) Root ecology and its practical application, Interantinal Symposium. Gumpenstein 1982, Irdning, Bundesanstalt Gumpenstein, pp 207–226
96. Gregory PJ, Brown SC (1989) Root growth, water use and yield of crops in dry environments: what characteristics are desirable? Aspects Appl Biol 22:235–243
97. Pantuwan G, Fukai S, Cooper M, O'toole JC, Sarkarung S (1997) Root traits to increase drought resistance in rainfed lowland rice. In: Fukai S, Cooper M, Salisbury J (eds) Proceedings of Breeding Strategies for Rainfed Lowland Rice in Drought-Prone Environments, vol 77, Canberra, pp 170–179
98. Lilley JM, Fukai S (1994) Effect of timing and severity of water deficit on four diverse rice cultivars I. rooting pattern soil water extraction. Field Crops Res 37(3):205–213

99. Siopongco J, Yamauchi A, Salekdeh H, Bennett J, Wade LJ (2005) Root growth and water extraction response of doubled-haploid rice lines to drought and rewatering during the vegetative stage. Plant Prod Sci 8(5):497–508
100. Ford KE, Gregory PJ, Gooding MJ, Pepler S (2006) Genotype and fungicide effects on late-season root growth of winter wheat. Plant Soil 284(1–2):33–44
101. Christopher JT, Manschadi AM, Hammer GL, Borrell AK (2008) Developmental and physiological traits associated with high yield and stay-green phenotype in wheat. Aust J Agric Res 59(4):354–364
102. Bengough AG, Bransby MF, Hans J, Mckenna SJ, Roberts TJ, Valentine TA (2006) Root responses to soil physical conditions; growth dynamics from field to cell. J Exp Bot 57(2): 437–447
103. Kirk GJD (2003) Rice root properties for internal aeration and efficient nutrient acquisition in submerged soil. New Phytol 159(1):185–194
104. Robinson D, Linehan DJ, Caul S (1991) What limits nitrate uptake from soil. Plant Cell Environ 14(1):77–85
105. Cooper PJM, Gregory PJ, Keatinge JDH, Brown SC (1987) Effects of fertilizer, variety and location on barley production under rainfed conditions in Northern Syria. 2: Soil water dyamics and crop water use. Field Crops Res 16:67–84
106. Wiesler F, Horst WJ (1993) Differences among maize cultivars in the utilization of soil nitrate and the related losses of nitrate through leaching. Plant Soil 151:193–203
107. Wiesler F, Horst WJ (1994) Root growth and nitrate utilization of maize cultivars under field conditions. Plant Soil 163: 267–277
108. Thorup-Kristensen K (1993) Root development of nitrogen catch crops and of a succeeding crop of broccoli. Acta Agriculturae Scaninavica B Soil Plant Sci 43:58–64
109. Gregory PJ (1994) Resource capture by root networks. In: Monteith JL, Scoot RK, Unsworth MH (eds) Resource capture by crops. Nottingham University Press, Nottingham, pp 77–97
110. Nielsen NE, Schjorring JK (1983) Efficiency and kinetics of phosphorus uptake from soil by various barley genotypes. Plant Soil 72(2–3):225–230
111. Gregory PJ (1994) Resource capture by root networks. In: Monteith JL, Scott RK, Unsworth MH (eds) Resource capture by crops. Nottingham University Press, pp 77–97
112. Gao S, Pan WL, Koenig RT (1998) Wheat root growth responses to enhanced ammonium supply. Soil Sci Soc America J 62:1736–1740
113. Fitter AH, Stickland TR (1991) Architectural analysis of plant-root systems. 2. Influence of nutrient supply on architecture in contrasting plant-species. New Phytol 118(3):383–389
114. López-Bucio J, Hernández-Abreu E, Sánchez-Calderón L, Nieto-Jacobo MF, Simpson J, Herrera-Estrella L (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the arabidopsis root system. Plant Physiol 129(1):244–256
115. Poirier Y, Bucher M (2002) Phosphate transport and homeostasis in Arabidopsis. American Society of Plant Biologists, Rockville
116. Cabrera-Bosquet L, Molero G, Bort J, Nogués S, Araus JL (2007) The combined effect of constant water deficit and nitrogen supply on WUE, NUE and D13C in durum wheat potted plants. Ann Appl Biol 151(3):277–289
117. Pask A (2009) Optimising nitrogen storage in wheat canopies for genetic reduction in fertiliser nitrogen inputs. Ph.D. thesis, University of Nottingham
118. Dunbabin V, Diggle A, Rengel Z (2003) Is there an optimal root architecture for nitrate capture in leaching environments? Plant Cell Environ 26:835–844
119. Forde BG, Clarkson DT (1999) Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. In: Ellow JA (ed) Advances in botanical research incorporating advances in plant pathology, vol 30. Academic, San Diego, pp 1–90
120. Crawford NM, Glass ADM (1998) Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci 3(10):389–395
121. Williams LE, Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annu Rev Plant Physiol Plant Mol Biol 52:659–688
122. Hawkesford MJ, Miller AJ (2004) Ion-coupled transport of inorganic solutes. In: Blatt MR (ed) Membrane transport in plants annual reviews 15. Blackwell/CRC Press, Oxford, pp 105–134
123. Lauter FR, Ninnemann O, Bucher M, Riesmeier JW, Frommer WB (1996) Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. Proc Natl Acad Sci USA 93(15):8139–8144
124. Zhuo DG, Okamoto M, Vidmar JJ, Glass ADM (1999) Regulation of a putative high-affinity nitrate transporter (Nrt2;1At) in roots of Arabidopsis thaliana. Plant J 17(5): 563–568
125. Ono F, Frommer WB, Von Wiren N (2000) Coordinated diurnal regulation of low- and high-affinity nitrate transporters in tomato. Plant Biol 2(1):17–23
126. Orsel M, Krapp A, Daniel-Vedele F (2002) Analysis of the NRT2 nitrate transporter family in Arabidopsis. structuer and gene expression. Plant Physiol 129(2):886–896
127. Bucher M (2007) Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytol 173(1):11–26
128. Miller AJ, Shen Q, Xu G (2009) Freeways in the plant: transporters for N, P and S and their regulation. Curr Opin Plant Biol 12(3):284–290
129. Waines JG, Ehdaie B (2007) Domestication and crop physiology: roots of green-revolution wheat. Ann Bot 100(5):991–998
130. Miralles DJ, Slafer GA, Lynch V (1997) Rooting patterns in near-isogenic lines of spring wheat for dwarfism. Plant Soil 197(1):79–86
131. Bush MG, Evans LT (1988) Growth and development in tall and dwarf isogenic lines of spring wheat. Field Crops Res 18(4):243–270

132. Mccaig TN, Morgan JA (1993) Root and shoot dry-matter partitioning in near-isogenic wheat lines differing in height. Can J Plant Sci 73(3):679–689
133. Hurd EA (1974) Phenotype and drought tolerance in wheat. Agric Meteorol 14(1–2):39–55
134. Ogbonnaya FC, Seah S, Delibes A, Jahier J, Lopez-Brana I, Eastwood RF, Lagudah ES (2001) Molecular-genetic characterisation of a new nematode resistance gene in wheat. Theor Appl Genet 102(4):623–629
135. Steele KA, Price AH, Shashidhar HE, Witcombe JR (2006) Marker-assisted selection to introgress rice QTLs controlling root traits into an Indian upland rice variety. Theor Appl Genet 112(2):208–221
136. Manske GGB, Ortiz-Monasterio JI, Vlek PLD (2001) Thecniques for measuring genetic diversity in roots. In: Reynods MP, Ortiz-Monasterio JI, McNab A (eds) Application of physiology in wheat breeding. Mexico, Cimmiyt
137. Polomski J, Kuhn N (2002) Root research methods. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Marcel Dekker, New York, pp 295–321
138. De Dorlodot S, Forster B, Pages L, Price A, Tuberosa R, Draye X (2007) Root system architecture: opportunities and constraints for genetic improvement of crops. Trends Plant Sci 12:474–481
139. Manschadi A, Hammer G, Christopher J, Devoil P (2008) Genotypic variation in seedling root architectural traits and implications for drought adaptation in wheat (Triticum aestivum L). Plant Soil 303(1):115–129
140. Swarup K, Benkova E, Swarup R, Casimiro I, Peret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S, Levesque MP, Carrier D, James N, Calvo V, Ljung K, Kramer E, Roberts R, Graham N, Marillonnet S, Patel K, Jones JDG, Taylor CG, Schachtman DP, May S, Sandberg G, Benfey P, Friml J, Kerr I, Beeckman T, Laplaze L, Bennett MJ (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol 10(8):946–954

Books and Reviews

Bassirirad H (2000) Kinetics of nutrient uptake by roots: responses to global change. New Phytol 147(1):155–169
Beeckman T (ed) (2010) Root development. Blackwell, Chichester
Bingham IJ (2001) Soil-root-canopy interactions. Ann Appl Biol 138(2):243–251
Bingham IJ, Hoad SP (2000) Towards below ground management In: HGCA conference: crop management into the millennium, Homerton College, Conference Centre, HGCA, Cambridge, pp 7.1–7.8
Bingham IJ, Foulkes MJ, Gay AP, Gregory PJ, King JA, Robinson D, Bradley RS (2002) Balancing root and canopy growth. In: HGCA (ed) R & D conference: agronomic intelligence: the basis for profitable production, pp 6.1–6.14
Blum A (ed) (2009) Plant stress http://plantstress.com/
Dakora FD, Phillips DA (2002) Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245(1):35–47
Lynch JP (2007) Roots of the second green revolution. Aust J Bot 55(5):493–512
Lynch J, Brown KM (eds) (2009) Root research methods. http://roots.psu.edu/en/methods
Schachtman DP, Goodger JQD (2008) Chemical root to shoot signaling under drought. Trends Plant Sci 13(6):281–287
Smit AL, Bengough AG, Engels C, Noordwijk MV, Pllerin S, Geijn SCVD (eds) (2000) Root methods. A handbook. Springer, Berlin
Van Noordwijk M, Martikainen P, Bottner P, Cuevas E, Rouland C, Dhillion SS (1998) Global change and root function. Glob change Biol 4:759–772
Waisel Y, Eshel A, Kafkafi U (eds) (2002) Plant roots: the hidden half. Marcel Dekker, New York
Wang E, Smith CJ (2004) Modelling the growth and water uptake function of plant root systems: a review. Aust J Agric Res 55:501–523

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems

Rui Pereira[1], Charles Yarish[2], Alan T. Critchley[3]
[1]CIMAR/CIIMAR – Centre for Marine and Environmental Research, University of Porto, Porto, Portugal
[2]Departments of Ecology & Evolutionary Biology and Marine Sciences, University of Connecticut, Stamford, CT, USA
[3]Acadian Seaplants Limited, Dartmouth, NS, Canada

Article Outline

Glossary

Algae A group of autotrophic organisms, containing chlorophyll a and sometimes other accessory pigments, which are able to convert solar energy into chemical energy via photosynthesis.

Aquaculture The farming of autotrophic and heterotrophic organisms in aquatic systems.

Bioextraction An environmental management strategy by which nutrients are removed from an aquatic ecosystem through the harvest of enhanced biological production, including the aquaculture of suspension-feeding shellfish and/or marine macroalgae.

Ecosystem Is the grouping of all living organisms occupying a particular unit of space and interacting with each other and their environment.

EPA Eicosapentaenoic acid is an omega-3 polyunsaturated fatty acid, sometimes presented with the chemical notation 20:5(n-3).

HDL High-density lipoprotein; composed of a high proportion of protein and relatively little cholesterol; high levels of HDL are thought to be associated with a decreased risk of coronary heart disease and atherosclerosis.

Heteromorphic life histories Life histories in which there are clear morphological differences between the different stages of the life cycle, i.e., individuals of the sporophyte and gametophyte stages are morphologically different and distinguishable. In some cases, such as the genus *Porphyra* and members of the kelps, there are macroscopic and microscopic stages alternating life cycle phases.

IMTA Integrated Multi-Trophic Aquaculture is a form of aquaculture in which organisms from different trophic levels, with complementary resource needs, are produced in the same system. Typically, these aquaculture systems integrate the production of a fed organism, such as fish or shrimp, with that of extractive organic aquaculture such as shellfish and the extractive inorganic aquaculture of seaweed.

Isomorphic life histories Life histories in which there are no distinguishing morphological differences between the different stages of the life cycle, i.e., the individuals of the sporophyte (diploid, 2n) and gametophyte (haploid, n) stages are morphologically identical and can be distinguished only when their respective, characteristic reproductive structures are present, e.g., *Chondrus crispus* and *Palmaria palmata*.

LDL Low-density lipoprotein; a lipoprotein that transports cholesterol in the blood, composed of a moderate amount of protein and a large amount of cholesterol; high levels of LDL are thought to be associated with an increased risk of coronary heart disease and atherosclerosis.

Macroalgae A group of macroscopic algae of which at least one part of their life history is multicellular and visible with unaided eye.

Mariculture Farming of autotrophic and heterotrophic organisms in marine systems, i.e., using seawater.

Polysaccharides Complex structural polymers. They have a structural function in the alga but may be

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

extracted industrially to provide a range of polysaccharides used for their rheological properties, e.g., agar, carrageenan, and alginic acid.

Seaweed A group of macroscopic, marine autotrophic algae.

Sea-vegetables A group of macroscopic, marine autotrophic algae, also called seaweeds, seaplants, or macroalgae; they may be used as vegetables for human consumption or raw materials for a range of industrial, commercially important extracts such as bioactives or polysaccharides.

Definition of the Subject and Its Importance

The production of seaweeds for human foods in land-based aquaculture, is an activity poorly presented by the scientific community. Of the thousands of seaweed species identified, a remarkably small subset is actually farmed in the marine environment (i.e., open water) and even fewer are grown in land-based aquaculture systems. Of those that are used in land-based systems, most are monocultures grown for specific high value uses. For instance, *C. crispus, P. palmata*, and *Saccharina latissima* are grown for human consumption; *Chondrocanthus* and the "*Trailiella*" stage of *Bonnemaisonia/Asparagopsis* for the cosmetic industry; and *Gracilaria* spp., *Palmaria* and *Ulva* spp. as feed for abalone). Given the many centuries history of terrestrial production of land plants for human and animal feed crops and the tremendous efforts given over to the selection and crossbreeding of these plants, by contrast, selection and improvement of seaweed crops is very much in its infancy. Even more so, of all the relatively small number of seaweeds which are domesticated for open water cultivation, even fewer species have actually been tested in land-based culture systems. This is in part due to the lack of reliable, domesticated species and their selected strains suitable for the rigors of on-land cultivation, and in part due to the complexities of life histories and the lack of understanding of the environmental regulation of growth. There is, therefore, a need to test other species since open water systems may not be appropriate for niche cultivation applications. The historical development of open water cultivation and multi-species pond cultivation may have originated in Asia (for discussion and references see later section); however, modern land-based aquaculture of seaweeds began with the work of John Ryther at Woods Hole Oceanographic Institute (WHOI) in the late 1960s through the mid-1970s [1]. Land-based Integrated Multi-Trophic Aquaculture systems (IMTA) may contribute to the development of sustainable fed aquaculture systems by minimizing environmental impacts (i.e., removing excess dissolved inorganic nutrients, *d*issolved *o*rganic *m*atter (DOM), and *p*articulate *o*rganic *m*atter (POM). Furthermore, while the systems may yet have to be optimized geographically and in relation to the species utilized, the controlled production of seaweed biomass in these systems may offer a reliable and safe source of food or ingredients for human consumption, fish feeds, as well as a source of valuable compounds for biotechnological applications. As a special focus, this entry will discuss the importance of land-based seaweed aquaculture systems and their global utilization.

Introduction

Seaweed is a popular term used to collectively describe marine macroalgae. Among this large and diverse assemblage of photosynthetic marine organisms are a number of species with a varied array of uses; when used for human consumption, they are more popularly known as "sea-vegetables." This collective of convenience includes the macroscopic, multicellular, red, green, and brown algae [2]. Seaweeds are often abundant and predominantly found in the near-shore marine ecosystems in all the oceans of the world. As a result of their diverse intercellular compounds including alginic acid, carrageenans, and agar, seaweeds have very important industrial applications [3, 4]. Being important primary producers in marine ecosystems, macroalgae are an integral component of near-shore environment and form a fundamental part of the basis of the photosynthetic food chains, playing a role similar to that of terrestrial plants [5]. In these natural environments, seaweeds often perform a large number of ecosystem services [6] (e.g., nurseries, nutrient cycling, and reduction of coastal erosion among others), which are neither fully costed nor often appreciated by the public or users of the marine environment. Humans have wild harvested (sometimes called "wild crafting") and cultivated seaweeds for several centuries for animal and human consumption

as well as other applications including valuable sources of phycocolloids and most recently, researched as feedstock for biofuels and carbon sequestration [7–11].

Some seaweeds may attain lengths exceeding 90 m or more (e.g., the kelp *Macrocystis pyrifera*), while others may grow only a few centimeters per year. Many seaweeds have isomorphic life cycles (e.g., *C. crispus, P. palmata*, where the gametophyte and sporophyte generations are morphologically similar), while others have heteromorphic life histories (e.g., the genus *Porphyra* and many species of brown algae including the kelps, where the generations are morphologically distinct). The morphology of various seaweeds may include multicellular, highly differentiated kelp with their organs such as blades, complex stipes, and their anchoring structure referred to as haptera. Other multicellular seaweeds may be small and bushy with flat or cylindrical axes (*Gracilaria*), while others may form sheet-like specimens of one or two layers of cells thickness (*Porphyra*, *Ulva*, *Monostroma*). Some macroalgae may be encrusting forms, while yet others may have the ability to precipitate calcium carbonate to varying degrees and be lightly calcified yet remain flexible (e.g., *Padina*) or fully calcareous and occur as prostrate crusts (e.g., *Lithothamnion*, *Phymatolithon*) or fully articulated, e.g., many coralline species such as *Corallina* and *Jania*.

This entry will discuss the advantages for land-based aquaculture of seaweeds for human foods and animal consumption. The first section gives a brief introduction to seaweed uses, both past and present, followed by an introduction as to how seaweed aquaculture has been practiced more recently. Section "Introduction of the IMTA Concept" briefly explains the concept of integrated multi-trophic aquaculture (IMTA), since this can also be an integral component of land-based seaweed aquaculture systems. Section "Physiological Considerations for the Production of Seaweeds in Land-Based Cultivation Systems" presents some of the particularities of intensive, land-based seaweed production, which make it different from the more common extensive, open water seaweed mariculture. Section "Examples of Successful On-Land Cultivation Enterprises" presents success stories of land-based seaweed aquaculture. The entry concludes with a discussion of the potential impacts of the development of land-based seaweed aquaculture as well as future directions and perspectives of research in this area.

Past and Present Uses of Seaweeds

Seaweeds have been used in the human diet since ancient times. Although Asian food culture has seen the most prominent use of seaweed for direct human consumption, there is also recent evidence of the use of seaweeds by prehistoric humans, in other parts of the world. Dillehay et al. [12], in an archeological study conducted in Monte Verde, southern Chile, identified remains of nine species of marine algae, including *Durvillaea antarctica* ("cochayuyo"), *Porphyra* sp. ("luche"), *Gracilaria* sp. ("pelillo"), and *Sargassum* sp. These authors also suggest that some algae may have been burned, suggesting that they had been dried to facilitate transportation and/or storage, or were even cooked and could have been used for their medicinal properties as well. Erlandson et al. [13] discussed the cold water, coastal fringing kelp beds on the Pacific Coast of the Americas as being a route, or a "highway," by which early travelers made their way in northerly and southerly migrations. In Asian and Pacific Island countries, the tradition is to consume seaweeds as raw or cooked sea vegetables [14–16]. In Western countries, the principal use of seaweeds has been as a source of phycocolloids (alginate, carrageenan, and agar) which are structural, thickening, and gelling agents for various industrial applications, including uses in textile, paper, food, toothpastes, shampoos, cosmetics, and pharmaceutical industries [3, 4].

There is presently an increasing interest by the general public regarding the impacts of the human diet in general health and the potential health benefits in the consumption of selective seaweeds in a varied human diet. In fact, the physiological or pharmacological functions of food factors were classified as the third function of food in addition to the nutritional and sensory roles as the primary and secondary function, respectively [17, 18]. Among the bioactive compounds known to have an impact in the human health, there are those described as prebiotic functional ingredients. These are defined as nondigestible, selectively fermented compounds that stimulate the growth

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Table 1 Some examples of seaweeds with their functional ingredients and possible effects on human health (Adapted from Plaza et al. [20]; Kumar et al. [21], Bocanegra et al. [22])

Seaweed	Functional ingredient	Possible health benefits
Sargassum vulgare (B)	Alginic acid, xylofucans	Antiviral activity
Himanthalia elongata (B)	PUFAs	Reduce risk of certain heart diseases
	α-Tocoferol	Antioxidant activity
	Sterols	Reduce total and LDL cholesterol
	Soluble fiber	Reduce total and LDL cholesterol
U. pinnatifida (B)	PUFAs	Reduce risk of certain heart diseases
	Sterols	Reduce total and LDL cholesterol
	Soluble fiber	Reduce total and LDL cholesterol
	Folates	Reduce risk of certain types of cancer
	Sulfated polysaccharides	Antiviral activity
	Fucoxanthin	Preventive effect on cerebrovascular diseases; Increase the metabolism
Porphyra spp. (R)	PUFAs	Reduce risk of certain heart diseases
	Sterols	Reduce total and LDL cholesterol
	Soluble fiber	Reduce total and LDL cholesterol
C. crispus (B)	PUFAs (n-3) fatty acids	Reduce risk of certain heart diseases
	Sterols	Reduce total and LDL cholesterol
	Soluble fiber	Reduce total and LDL cholesterol
Cystoseira spp. (B)	Terpenes	Valuable curative properties
	Sterols	Reduce total and LDL cholesterol
	Sulfated polysaccharides	Regulate the bioactivity of growth factors and cytokines
Ulva spp. (G)	Sterols	Reduce total and LDL cholesterol
Grateloupia filicina (R)	"methanolic extract"	Antioxidant activity
Brown algae (non specified)	Phlorotannins	Detoxification of heavy metals; antibacterial effects
	Fucoidan	Anti-inflammatory, anticoagulant
Colpomenia sinuosa (B)	Fatty acid profile (ω-3)	Increase HDL cholesterol
Hypnea charoides (R)	Fatty acid profile (ω-3)	Decreased LDL cholesterol
A. nodosum (B)	Sodium-binding fiber	Antihypertensive effects

R red, *B* brown, *G* green seaweed

and/or activity of beneficial gut microbiota which, in turn, confer health benefits on the host [19].

Due to their varied nutritional properties, seaweeds are the subject of research seeking new, natural sources of functional ingredients for food. Table 1 presents a summary of that information [20–22]. *Porphyra*, for instance, contains high levels of protein (25–50%), vitamins (higher vitamin C than in oranges), trace minerals, and dietary fibers [23]. This alga contains nearly 17 types of free amino acids, including taurine

which controls blood cholesterol levels and is thought to prevent obesity [24, 25]. Several reviews have been published outlining the nutritional properties of seaweeds (e.g., [21, 22, 26]), the most recent of which include the comprehensive review of Holdt and Kraan [27] and another on antioxidants from macroalgae by Cornish and Garbary [28]. The review of Holdt and Kraan [27] is particularly valuable since it also details the regulatory environment affecting marketing and use of active compounds from seaweeds in human applications. On the other hand, Cornish and Garbary [28] consider the application of seaweed antioxidants in foods, food supplements, nutraceuticals, and medicine from the perspective of benefits to human health. The review provides examples not only from laboratory studies but also from clinical trials where antioxidants derived from seaweeds may provide major health benefits that warrant subsequent investigative studies and possible utilization. Furthermore, those authors advocate that the direct consumption of seaweed products for their antioxidant composition alone provides a useful alternative to nonnatural substances, while simultaneously providing worthwhile nutritional benefits. Finally, the review by Cornish and Garbay [28] includes a comprehensive listing of algal species evaluated for antioxidant activity and potential applications of detected compounds.

Burtin [26] elaborates upon the nutritional value of seaweeds as they are rich in polysaccharides and dietary fibers, minerals, proteins and amino acids, lipids and fatty acids, and micronutrients such as vitamins (vitamin B_{12}, C, and E) and polyphenols (phlorotannins). This author concludes that from a nutritional standpoint, the main beneficial properties of seaweeds are their high mineral (iodine, calcium) and soluble dietary fiber contents, the occurrence of vitamin B_{12} and specific components such as fucoxanthin, fucosterol, and phlorotannins. Burtin also states that seaweeds can be regarded as an underexploited source of health-promoting molecules for food processing and the increasingly important nutraceutical industries [26].

Kumar et al. [21] reviewed the presence and value of various bioactive substances which may be derived from certain seaweeds, namely, polysaccharides and related compounds; proteins and related substances; lipids and related compounds; minerals; vitamins and antioxidant compounds. These authors concluded that seaweeds are a low calorie food source, particularly from the nutritional point of view, since they have high concentrations of certain minerals, vitamins, proteins, and indigestible carbohydrates. Seaweeds also have low lipid content, but the lipids present are of a high quality in terms of their nutritional value. In fact, Blouin et al. [29] suggested that native, Atlantic species of *Porphyra* such as *Porphyra amplissima* and *Porphyra umbilicalis* have potential in foods for North American consumers. They analyzed the fatty acid content of freshly collected *P. umbilicalis* and reported that eicosapentaenoic acid [EPA; 20:5 (n-3)] and palmitic acid were the most common fatty acids. Those authors reported that the concentration of fatty acids found in wild collected *P. umbilicalis* (i.e., 3.2 mg EPA g dry wt^{-1} or 74 mg EPA 100 g fresh wt^{-1}) was not high enough to make this a primary source of daily omega-3 fatty acids, but the favorable n-3/n-6 ratio (2–3:1) in these species constituted an interesting nutritional value. In their review, Kumar et al. [21] concluded that the quality of protein and lipids in seaweeds generally is as acceptable as those present in other dietary vegetables due to high content of essential amino acids and relatively higher levels of unsaturated fatty acids. Furthermore, all of these authors suggested that seaweeds exhibit antioxidant, antimutagenic, anticoagulant, anticancer, and antitumor activity. In many cases, these properties were actually tested and proved in vivo and in vitro, as follows. Zhang et al. [30] showed that a sulfated polysaccharide fraction from *Porphyra haitanensis* could be used to compensate the decline in total antioxidant capacity and activities of antioxidant enzymes. The implications of these findings are that seaweeds and their extracts might play a role in retarding the aging process. In addition, unprocessed powder from the brown seaweed *Fucus vesiculosus* has proven to have strong antioxidant capacities [31]. The authors concluded that the polyphenol (phlorotannin) content of *F. vesiculosus* seemed to provide the main antioxidant properties. Other research using polysaccharides extracted from *Porphyra yezoensis* demonstrated anticoagulant [32] and immune-stimulating activities [33, 34]. Saito et al. [34] showed that *Porphyra* peptides induced a significant reduction in the blood pressure of hypertensive human patients. Various fucoidans, common sulfated polysaccharides of various commercially important brown algae, have been tested

in several studies in rats and in humans, showing beneficial effects as an anticoagulant, antithrombotic, antiviral, and anticancer agent ([35, 36]; see also www.marinova.com). Anticancer properties of seaweeds are also reported in the studies of Teas et al. [37] and Yang et al. [38] among others. In particular, the results of Teas et al. [37] suggested that a diet containing 5% brown seaweed (i.e., *Laminaria*) was effective in delaying the time for chemically induced tumor development in rats. In turn, Yang et al. [38] investigated the association between the intake of *Porphyra* (red seaweed) and *Undaria* (brown seaweed) and the risk of breast cancer, in a case-control study. The authors concluded that the consumption of *Undaria pinnatifida* did not have any significant associations with the disease but the results also suggested that high intake of *Porphyra* may decrease the risk of breast cancer.

Bocanegra et al. [22] reviewed the major physicochemical properties of seaweed fiber, the nutritional properties of the seaweed, and their value as functional foods. In terms of physicochemical properties of the fiber, the authors highlighted the hydration properties and viscosity, the oil retention and fat absorption, the fermentability, and binding capacity (cation-exchange capacity responsible for heavy metal biosorption). As for the nutritional properties of the seaweed, Bocanegra et al. [22] pointed to numerous studies that demonstrated the chemical and nutritional importance of the seaweed, namely in relation to bioavailability, effect on growth and body weight, effects on digestion, excretion and gastrointestinal functions, effects on lowering cholesterol and blood pressure, antioxidant activities and effects on glucose metabolism. Those authors concluded, however, that although some antioxidant compounds are present in algae, other compounds that are also present, such as the arsenic (As), can induce a poor endogenous antioxidant status. Therefore, the use of marine algae in herbal medications or excessive consumption of some of these organisms requires some caution. As with all things, seaweeds should be consumed in moderation as part of a well-balanced diet. Bocanegra et al. [22] also highlighted that, at that point there were no data available on the changes that cooking (e.g., microwave oven, traditional oven, frying, boiling, etc.) might impact on the properties of algal constituents. It is clear that much more work remains to fully realize the full nutritional and health-promoting potential of the consumption of seaweeds. These authors also illustrated that although numerous beneficial health properties can be attributed to seaweed components and extracts, robust studies of potential functional foods containing seaweeds have yet to be carried out, namely, the determination of different matrices affecting their technological and nutritional properties. The points raised by Holdt and Kraan [27], regarding the requirements of various food regulatory agencies, particularly with regard to novel foods and ingredients, should also be carefully considered.

As marine organisms with unique structural and biochemical compositions, seaweeds could be responsibly exploited for their multifunctional properties in the form of food, energy, medicine and cosmetics, and as biotechnological tools. In recent times, the use of seaweeds in a wide variety of biotechnological applications has become more common. Sahoo et al. [39] and Gantt et al. [40] pointed out the advantages of the use of *Porphyra* as a model organism for both applied and basic research. In fact, part of the genome of *P. umbilicalis* and the transcriptome of *Porphyra purpurea* have been recently released by the Joint Genome Institute (U.S. Department of Energy, www.jgi.doe.gov/genome-projects/ Program CSP2008; see also www.porphyra.org) and made available to the public. The aforementioned authors specifically point to the possibility of establishing several pure lines. In particular, the small genome size, which is estimated to be 2.6×10^8 base pairs consisting of three chromosomes and also the short generation time (1–3 months) of the alga are suitable traits for genetic analysis. The "*Porphyra* Genome" project, currently underway, will be one of the first to sequence the full genome of a multicellular red seaweed species and provide valuable information for biotechnological applications [40]. Other macroalgal genome projects include that of *C. crispus*, likely to be published in early 2011 (Jonas Collén, personal communication) and *Ectocarpus* [102]. As has been experienced with microalgal research (see [41, 42] and references therein), such major advances allow for rapid implementation of genetic engineering techniques that may modify seaweeds, thereby increasing their biotechnology applications. Some caution however needs to be applied to

applications of such techniques. Should these "modified" seaweeds be destined for food or food products, then the necessity to label them as GMO (*g*enetically *m*odified *o*rganisms) sources would actually reduce their market acceptance, particularly in Europe and even increasingly in North America.

Seaweed Aquaculture

The largest database reference for seaweed taxonomy (www.algaebase.org) currently has over 10,000 macroalgal species listed, the majority being seaweeds [43]. Despite the variety of life forms and the many thousands of seaweed species, seaweed aquaculture is presently based upon a relatively very small group of less than 100 species worldwide [7]. In fact, only five to seven genera alone (i.e., *Laminaria/Saccharina, Undaria, Porphyra, Eucheuma/Kappaphycus* and *Gracilaria*) account for about 83% of the world seaweed production (Table 2). The basic cultivation techniques of these genera are described in Yarish and Pereira [7] and Pereira and Yarish [44] and the references therein.

The use of seaweeds for food has strong roots in Asian countries such as China, Japan, and the Republic of Korea. For that reason, these are the primary areas where seaweed aquaculture was first developed and, furthermore, the species of seaweed most cultivated are the ones commonly found from those shores. Tseng [46] defined the commercial cultivation of seaweeds as: "the large scale production of macroscopic marine algae for commercial purposes." Doty [47] applied the term "marine agronomy" to define seaweed cultivation as a type of agricultural practice carried out in the sea.

Despite this analogy, marine agronomy is an activity in its infancy, when compared to the traditional terrestrial agronomy, with obvious differences when we compare the developmental status of both activities. While the origin of marine agronomy can be traced back to approximately 200 years ago, the birth of agriculture is still subject of debate among anthropologists but is thought to have happened approximately 10,000–12,000 years ago [48, 49]. In fact, the presently cultivated seaweeds were selected from local flora (i.e., from the wild) and limited "selection and breeding" techniques have been applied to develop domesticated strains, especially when compared to the efforts placed on staple terrestrial crops such as rice, potatoes, wheat, etc. For the latter, agronomic institutions have developed on various continents, which have specialized in their breeding, selection, and improvement, sometimes even using genetic manipulation.

In China, more than 200 years ago, the first methods to manage a seaweed crop, a species of the red marine alga *Porphyra*, consisted of simply cleaning rocky areas in early autumn. This was done just before the mass liberation of algal reproductive spores, so that they had more surface area for attachment and growth [50]. In Japan, a similar approach consisted of inserting bundles of bamboo twigs into sandy/muddy substrata before the spore release season. Net cultivation methods for mass production of "laver" were only introduced in the 1920s, resulting in some increase in productivity but still reliant on the collection of spores released from natural populations [46]. However, the

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Table 2 Main seaweed aquaculture production and value in 2008, according to FAO 2010 [45]

Genera	Production (metric tons)	Value	
		(*1,000 USD)	USD/ton
Laminaria (=*Saccharina*)	4,765,076	2,835,558	595
Eucheuma/Kappaphycus	3,551,273	563,146	159
Undaria	1,755,913	749,213	427
Porphyra	1,389,360	1,345,414	968
Gracilaria	1,418,986	600,223	423
Others	2,661,054	1,262,018	474

substantive development of the aquaculture of this genus came with the description of the life cycle of *P. umbilicalis* by Katherine Drew (Baker), in 1949 [51]. Drew established that the filamentous red alga, "*Conchocelis rosea*," until then considered as a completely separate entity, was in fact the sporophyte phase of the life cycle *Porphyra*. This finding, together with subsequent research [51–54], allowed for the development of methods which could control the life cycle and also the artificial production and collection of spores. These were monumental findings allowing the aquaculture of *Porphyra* to move into a completely different phase of technological developments. Modern commercial cultivation methods were established in the 1960s which led to the very pronounced expansion of cultivation activities and economic development. China is currently the largest global producer of *Porphyra*, with more than 800,000 t, fresh weight, produced in 2008, followed by Japan – 337,000 t and the Republic of Korea – 224,000 t [45].

The brown, kelp species, *Saccharina japonica* (formerly known as *Laminaria japonica*), is presently the largest single species produced in aquaculture. It is grown as a monoculture and the volumes of production exceed any other marine species, including fish, crustacea, and molluscs. More than three million tons fresh weight (FW) are reported to be cultivated on ropes in open coastal waters. The cultivation of *Saccharina* was developed mainly during the second half of the twentieth century, initially using a stone planting technique. Since 1968, a method called "forced cultivation" led to a reduction of the previous 2-year cycle of production to 1 year; the shift to modern methods allowed for a tremendous increase in productivity and commercialization of the kelp and its products [46, 55].

The commercial cultivation of all other seaweed species is even more recent than that of *Porphyra* and *Saccharina*. For instance, the cultivation of the red alga *Gracilaria* probably began as recently as 1967 in Taiwan [46]. Seaweed aquaculture or marine agronomy is, therefore, an activity still in its relative infancy, especially when compared to traditional, terrestrial agriculture.

Despite being a recently developed activity, when compared with the traditional land agriculture sector, seaweed aquaculture has been developing steadily. According to the latest production data from FAO [45], in 2008, total seaweed aquaculture production was more than 15 million tons FW (fresh weight), valued at more than seven billion USD. This corresponds to 23% of the world's aquaculture production and approximately 7% of its global value. Besides the undeniable economic value of the biomass, seaweed aquaculture is nowadays also increasingly recognized for the significant ecosystems services it provides, namely through its extractive process of nutrient removal [56–58]. Chopin et al. [59] argued that evolving aquaculture practices will require a conceptual shift toward understanding the working of food production networks as opposed to simplistic and narrow focus on technological solutions. One of the innovative solutions promoted for environmental sustainability, as well as for economic stability and societal acceptability, is the system coined "IMTA" (or **I**ntegrated **M**ulti-**T**rophic **A**quaculture), which will be discussed in more detail in the next section.

Introduction of the IMTA Concept

In western countries, an interest in integrated aquaculture began toward the end of the twentieth century. After the initial work of Ryther et al. [1], interest in using algae as nutrient scrubbers in an integrated aquaculture system was renewed by a group of like-minded scientists including: Fujita et al. [60], Kautsky and Folke [61], Neori et al. [62], Krom et al. [63], Buschmann [64], Sphigel and Neori [65], Troell et al. [66], Chopin and Yarish [67], Neori and Shpigel [68], Yarish et al. [69], Chopin et al. [70] and Neori et al. [71], among others. In the last decade particularly, numerous papers continued to establish that the concept and implementation of Integrated Multi-Trophic Aquaculture (*sensu* [56, 71–73]) was and will increasingly be of paramount importance for the sustainable development of aquaculture. The advantages are not just important for a sustainable environment, as evidenced by Matos et al. [74], Msuya and Neori [75] and Abreu et al. [76], but also economic, as shown by Troell et al. [77], Whitmarsh et al. [78], Robertson-Andersson [79], Robertson-Andersson et al. [80], and Nobre et al. [58].

The over-riding principle is that in IMTA systems the "wastes" or by-products of animal (fed) aquaculture are used as nutrient source for growth and development of the other trophic component of the system,

such as macroalgae or other extractive, filter or detrital feeding organisms (e.g., bivalves, sea cucumbers, marine worms). The practical result is an added production of biomass which may have a direct economic value in addition to the ecosystem services which are provided by the extractive organisms. At the same time, the concentric alignment of the trophic levels provides substantial reduction of the load of inorganic nutrients in the effluents from intensive aquaculture systems, which in themselves can constitute a potential ecological problem leading to coastal eutrophication and harmful algal blooms (HABs).

Integrated mariculture has been practiced traditionally, although not necessarily intentionally, in China, Japan, and South Korea, where farms of fish net pens, shellfish, and seaweed have been situated in close proximity to one another [71, 81]. The arrangements and ultimate optimal integration of the trophic elements were largely achieved through trial and error and, as a consequence, traditional information regarding quantification and design has seldom been published (e.g., [82–84]). Nevertheless, in Asian countries, macroalgae are naturally considered as nutrient removers. For instance, the production of *S. japonica* (the Japanese kelp) was estimated as 4.765 million tons in 2008 [45]. Considering a very conservative N content of 2.79% DW (dry weight) and a wet to dry ratio of 5:1 [85], it can be estimated that approximately 5.58 kg of N are removed from the water with every ton FW of *Saccharina* produced. Therefore, the annual production of *S. japonica* removed approximately 26,588 metric tons of N from the surrounding seawater in 2008. In contrast, production of *Porphyra* and *Gracilaria*, while lower biomass volumes were produced, their N tissue content can exceed 7% DW for *Porphyra* [86] and 8% DW for *Gracilaria* [76].

On a global scale, the aquaculture of extractive organisms (e.g., seaweeds and shellfish) already removes a significant fraction of nutrients from the oceans [87]. According to Troell et al. [88], the harvests of those organisms already extract roughly 150,000 metric tons of N. However, as those authors also note, extractive and fed aquaculture are very often separated geographically, rarely balancing each other on a regional or local scale. An environmentally sustainable, balanced integrated aquaculture operation creates a mini-ecosystem in which the plant autotrophy balances the animal and microbial heterotrophy, not only in terms of nutrient removal (particularly C, N, and P) but also with respect to oxygen, pH, and carbon dioxide [87, 89]. It is unfortunate that, to date, there are only a few demonstration IMTA systems, in part due to the seasonality of the extractive seaweeds and the lack of "seed-stock." For instance, kelps for extraction of nutrients are only present for part of the cycle. The efficiency of dissolved nutrient removal will improve as alternation of extractive crop species is more clearly understood and refined.

As mentioned in the previous section, seaweed production is presently also recognized for its "ecosystem services" (see http://www.longislandsoundstudy.net/issues-actions/water-quality/nutrient-bioextraction/). Among these ecosystems services, the bioextraction capacity (through which the removal of biomass removes nutrients from the ecosystem) can be key for urban waterways that are not degraded by industrial pollution or don't have restrictions because they are away from sewage treatment facilities. However, as pointed out by Chopin et al. [59], these ecosystems services [90] have an economic benefit that is often ignored both by the industry and the regulators. A recent book sponsored by the World Conservation Monitoring Centre, in its chapter about Marine Systems, says very little about the role and direct economic value of algae in Marine Ecosystems [91]. Chopin et al. [59] argued that to improve the sustainability of anthropogenically derived nutrient-loading practices such as aquaculture, incentives such as nutrient trading credits (NTCs) are required. This would promote nutrient load reduction or nutrient recovery via a "polluter must pay" principle. The question can be posed that if carbon credits are now part of the internalized costs of some industries, why can the same process not be applied to nitrogen (N) and phosphorus (P), released through fed aquaculture, or point source pollution in the coastal marine environment. Neglecting the release of such nutrients in the marine environment can have quite striking consequences such as recently, when N released was associated with eutrophication of coastal waters resulting in massive algal blooms or "green tides" (see [92]). Also P has been discussed as "the next chemical element in global short supply"; therefore, its recovery makes considerable economic and ecological sense [93]. The land-based

cultivation of seaweeds, particularly as part of an IMTA system, may also play an important role on the future recovery of these nutrient wastes.

A particularly interesting initiative, regarding the promotion of IMTA at a national level, was reported by the Australian government under the auspices of the Rural Industries Research and Development Corporation (RIRDC; [94]). The objective of that report was to clearly identify the potential for seaweeds to be cultured in Australia for domestic and export markets. The report identified an enormous potential for growth and development of this activity and defined that targeted markets should include the food and nutritional sectors. The associated health benefits of human consumption of a variety of seaweeds were also indicated in the report.

Following this initiative, the Australian RIRDC moved to support the formation of "Seaweed Australia" – a new organization for the emerging cultivated seaweed industry in that country. Under that context, a new report was prepared [95] to assist all industry and research groups involved in the production, processing, and marketing of seaweed products in a broad range of industries including health and nutrition, aquaculture, animal feeds, nutraceuticals, and pharmaceuticals. The report is the result not just of desktop research but of a series of meetings, workshops, and other forms of extensive consultation of the different stakeholders (for instance, industry and researchers). The conclusions include a clear identification of the priorities for the development of the cultivated seaweed industry in Australia in terms of: market focus; market research; higher value products; regulatory issues, and industry research.

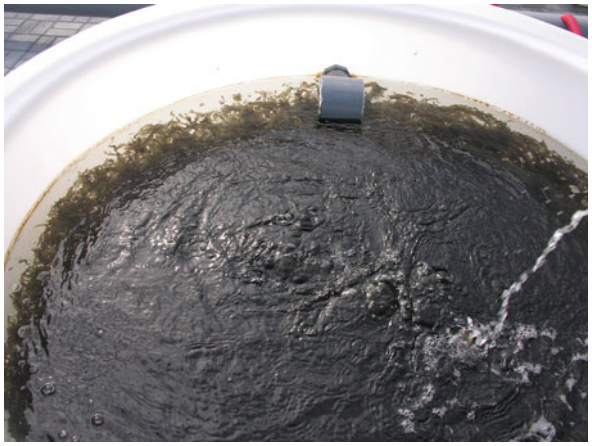
Physiological Considerations for the Production of Seaweeds in Land-Based Cultivation Systems

The physicochemical parameters that affect seaweed physiology in land-based systems are essentially the same as those that affect these organisms in natural populations and in open water aquaculture systems. Factors such as temperature, light and nutrient availability, pH and salinity are always critical for seaweed growth. The work of Craigie and Shacklock in particular was essential for the development of the cultivation of the red seaweed, Irish Moss (*C. crispus*) and that information is a primer for any land-based seaweed aquaculture facility [96]. These authors described the importance of appropriate site selection as a fundamental requirement for the success of any aquaculture undertaking. Craigie and Shacklock [96] confirmed that seawater in the vicinity of the potential site must be of the highest quality, i.e., low sediment and particulate matter, free of agricultural runoff and pollutants from other activities such as industrial, mining, and urban sources. The authors also briefly presented the requirements of the target cultivated species in terms of temperature, pH, salinity, nutrient requirements (i.e., carbon and nitrogen supply), seawater exchange, plant agitation, and interactions with other species. All of this information was the result of integrated basic research conducted for each one of those factors.

The traditional phycological literature, from the past 3–4 decades, contains considerable fundamental research on the physiology of seaweeds in general. However, this research was mainly conducted under laboratory conditions and much less practical work was undertaken in tank systems at scales relevant for economically viable commercial purposes. As with many other organisms, different algal species have different physiological requirements and optima [97]. As pointed out by Troell et al. [88], after reviewing 28 studies on various IMTA systems, where the majority included tank systems, there is a need to:

1. Understand in detail the important biological/biochemical processes in closed recirculating and open seaweed culture systems
2. Conduct research into these advanced aquaculture technologies at scales relevant to commercial implementation or suitable for extrapolation
3. Broaden the focus to include factors affecting seaweed growth and uptake capacity
4. Improve experimental design for statistical calculations
5. Understand the temporal variability in seaweed-filtered mariculture systems
6. Define numerical design parameters critical for engineers in designing commercial recirculation systems with seaweed filters
7. Study the influences of location-specific parameters, such as latitude, climate, and local seaweed strains/species, on seaweed filter performance

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Figure 1
Aspect of the seaweed tank system as part of a pilot scale IMTA in a land-based intensive fish aquaculture. A. Coelho e Castro Lda, Póvoa de Varzim, Portugal



Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Figure 2
Detail of a seaweed production tank (1,200 L) as part of a pilot scale IMTA system in a land-based intensive fish aquaculture. A. Coelho e Castro Lda, Póvoa de Varzim, Portugal

8. Include economic components, considering the added value of seaweeds, their products, and feasibility aspects
9. Analyze the role and function of integrated aquaculture practices for improved environmental, economic, and social acceptability within the broader perspective of integrated coastal management initiatives
10. Develop educational, training, and financial incentive approaches to transfer these novel and somewhat complex technologies of integrated mariculture from the scientists to an industrial scale

Despite the potential benefits for seaweed aquaculture as part of land-based IMTA systems, little progress has been made during the most recent decades, in terms of solving the needs as raised by Troell et al. [88]. The only published and, therefore, known exceptions are the work by Abreu et al. [76] taking into account an appropriate experimental design and scale (Figs. 1 and 2), and the research of Robertson-Anderson [79], Robertson-Anderson et al. [80] and Nobre et al. [58]. The differentiating factor is that Robertsson-Anderson and Nobre et al. performed economic and ecological assessments of a commercial, abalone-seaweed farm in South Africa.

Another less commonly applied method of land-based seaweed production is that of spray cultivation. This method has not been tested extensively on many species, but *Ascophyllum nodosum* [98] and *Gracilaria chilensis* [99] have been grown in such systems with some success. In the spray method, the seaweeds are not fully immersed in seawater but instead are held in adequate containers (often inside a modified greenhouse) and seawater is sprayed continuously or periodically over the target seaweeds. As referred to by Msuya and Neori [75], the documented benefits of seaweed spray culture included: construction and pumping costs, temperature control, gas (CO_2 and O_2) exchange, irradiance, nutrient uptake, and control of pests and epiphytes. Although the reported growth rates for these systems were usually low, Msuya and Neori [75] showed that the performance of *Ulva lactuca* in a spray system was in fact close to that of a standard, air-agitated tank culture system. In that work, *U. lactuca* was spray cultured in a "mattress-like layer," held in air on slanted boards by plastic netting. Fish mariculture effluents were applied by being sprayed onto the algal mattresses. The growth rate, yield, and ammonia-N removal rates were 11.8% day^{-1}, 171 g fresh weight (FW) m^2 day^{-1}, and 5 g N m^2 day^{-1}, respectively, by the spray cultured *U. lactuca*, and 16.9% day^{-1}, 283 g fresh

weight (FW) m^2 day^{-1}, and 7 g N m^2 day^{-1}, respectively, by traditional tank, immersed cultivated materials.

Examples of Successful On-Land Cultivation Enterprises

Acadian Seaplants Limited (www.acadianseaplants.com) is a Canadian company founded in 1981 and is probably the foremost example of an economically successful, land-based seaweed aquaculture enterprise. The company initially began with the collection of wild harvested *C. crispus* (Irish Moss) and progressed to the manual harvesting and processing of Rockweed (*Ascophyllum nodosum*). In particular, harvesting of the rockweed can be regarded as a positive case study for stewardship and successful, sustainable management of a wild resource (see [100, 101]). With respect to *C. crispus*, the company continues to successfully manage wild harvested materials in South West Nova Scotia and Prince Edward Island and also progressed to the land-based production of a specific strain of *Chondrus*.

While the *Chondrus* cultivation enterprise was initially planned to be a source of high-grade carrageenan, the production of *C. crispus* became more sophisticated to produce a value-added, salad product for the Japanese food market. Over the years, this enterprise has grown to become the world's largest, land-based seaweed cultivation system for the production of human food (Fig. 3). The production operation occupies a large site in south-western Nova Scotia, and the seaweeds are grown for the Asian food market and to conduct fundamental and applied research on seaweed extracts.

Spurred on by the needs created by design and construction of the land-based cultivation tanks and the challenges of cultivation of carragenophytes, the company was able to succeed and expand production facilities. The basis for this success was a strong R&D and market development strategies to diversify the company and its products. Presently, the company exports its diversified products to over 70 countries. More than 95% of its products are exported. According to the company managers, the cultivation division of the company has been gaining momentum and future plans include domestication of new species for additional target markets, plus enhancements to the existing edible seaweed product line, new product formats, and additional colors (Fig. 4).

Another example of a land-based seaweed production company is the Sylter Algenfarm GmbH & Co.KG (SAF), founded in 2006 by the marine botanist, Prof. Dr. Klaus Lüning. He spent most of his professional life unraveling the complexities of the environmental and

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Figure 3
Aerial view of Acadian Seaplants Limited seaweed production facilities, south-western Nova Scotia (2010) (Photo courtesy of ASL)

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Figure 4
Hana Tsunomata™ (*C. crispus*) commercial product from Acadian Seaplants Limited. Hana = "flower"; Tsunomata = *Chondrus* (Photo courtesy of ASL)

Seaweed Aquaculture for Human Foods in Land-Based and IMTA Systems. Figure 5
S. latissima seeded by Sylter Algenfarm on 8-mm diameter rope, grown out to harvesting size by Danish cooperator Rasmus Bjerregaard, holding up the harvested kelp (Photo, courtesy of Dr. Klaus Lüning)

internal control of seaweed growth and reproduction [2]. SAF cultivates seaweeds in a land-based seaweed farm at the North Sea island of Sylt, using a seawater source flowing from the oyster tanks of Dittmeyer's Austern-Compagnie. The two main seaweed species cultivated by SAF are young sporophytes of the brown alga *S. latissima* (formerly known as *Laminaria saccharina*) harvested in May, at a blade length of approximately 0.8 m, for the human food sector (Fig. 5) and the red alga *P. palmata* for the cosmetics industry. The niche in the *Laminaria* market occupied by SAF is a result of an opportunity due to the fact that the iodine content of imported *Laminaria* (kombu) from the Far East or from France, with concentrations of 3,000–6,000 mg iodine/kg algal dry weight, is considered too high for safe consumption. In contrast to the imported red alga *Porpyhra* (nori) for European Sushi restaurants, which contains very little iodine, imported kombu from Asia cannot pass the German "veterinary barrier" for human food, while the "young *Laminaria*" produced by SAF contains only 600 mg iodine/kg algal dry weight, probably because of the young age of the thin blades: This has enabled SAF to occupy that niche market and gives *Laminaria* the position as an innovative, marine vegetable in German restaurants.

In order to meet the growing demands for "young *Laminaria*" in Germany, in 2008, SAF began a cooperation with two sea farms in the Northern Baltic Sea (Kattegat Sea area), where *S. latissima* is grown on ropes, in the sea, either together with blue mussels (*Mytilus edulis*), or fish (rainbow trout). Harvesting of the young, thin kelp blades in May secures the low iodine content. Moreover, this co-cultivation of kelp with marine animals provides a further example of integrated multi-trophic aquaculture (IMTA). For the fish cultivators in Denmark, this is important since the Danish state urges them to employ countermeasures against the uncontrolled release of ammonium, nitrate, and phosphate from the fish cages into coastal waters. The total N of 6% kelp dry weight assists the fish cultivators to demonstrate that for each ton of fresh harvested kelp (dry weight is $\approx$ 10% of fresh weight), 6 kg of N are removed from the system. This is yet another advantage of the local production of the kelp. Naturally, this ecosystem service (nutrient removal) would not be performed in the direct vicinity of Danish fish farms if the *Laminaria* or *Saccharina* was imported from Asia. On the other

hand, for SAF, the harvested kelp biomass in the Kattegat provides the sufficient biomass for the German food market.

Another example, although not producing seaweeds directly for human food, is the Big Island Abalone Company, in Hawai. This company cultivates a proprietary strain of *Palmaria mollis* on a significant scale as feed for abalone cultivation. More details are available at their Web site (www.bigislandabalone.com).

Future Directions

Land-based cultivation of seaweeds reduces the pressure on wild harvest of seaplants, particularly those which are difficult to access in time or space or their harvest would be unsustainable and perhaps ecologically damaging. Furthermore, land-based seaweed production allows for the evaluation of numerous species that, due to their size, morphology, and/or particular physiological needs might not necessarily be good candidates for traditional, open water systems, such as those routinely used for *Porphyra*, kelp, and the major phycocolloid-producing seaweeds (i.e., *Kappaphycus* and *Eucheuma*). Another important feature of seaweed production in land-based systems is that they allow for much greater environmental and input controls than would ever be possible in open water seaweed aquaculture. Such high levels of control, or intensive production, is critical to provide the necessary traceability, security of supply, high-quality standards and safety, not just for human consumption as food but especially for nutraceutical and pharmacological applications. Furthermore, the control of some environmental parameters (as well as controlling growth and quality) can also be used to promote the expression of desirable characteristics of the seaweeds, such characteristics may be morphological or biochemical. Further advantages of land-based seaweed production are the possibilities to quarantine foreign species, if grown on land with approved effluent water treatment systems. This feature is definitely not possible in open water cultivation systems, and can allow a land-based facility to work with more species than just those locally available. However, the environmental stewardship responsibilities for the introduction of nonnative species cannot be minimized nor lightly undertaken.

One promising avenue of future research associated with on-land cultivation of seaweeds would be to find the most appropriate culture conditions that maximize the production of particular, valuable biochemical constituents. This would be important for the promotion of a variety of seaweeds as functional foods or food-ingredients. More research is required into the genetics and responses of new target species which, in turn, could provide insight to achieve the high level of control. Unlike terrestrial plants and even microalgae, genetic transformation in seaweeds remains at a very low level. The first fully sequenced genome of a seaweed species are that of *P. umbilicalis*, *P. purpurea*, and *Ectocarpus siliculosus* and were concluded only recently ([40, 102]; the genome of *C. crispus* to follow shortly thereafter). Not forgetting the huge potential that remains in specific, selection of seaweed strains, the genomic and molecular tools now available for seaweeds could provide further insights into algal physiology and metabolism. Even taking into account the caveat of lack of public acceptance of GMO food, just as occurred with their terrestrial counterparts, this knowledge might be used in seaweed research to improve productivity, biofiltration efficiency, disease resistance, and to direct metabolic pathways to produce higher concentrations of desirable metabolites and secondary compounds (these might even play a role in biofuel production from seaweed feed stocks). Overall, these activities and discoveries could contribute to an improved market value of the target seaweeds, including new insights into human nutrition.

In addition, future research should focus on the evaluation and selection of more seaweed species suitable for land-based aquaculture that may be used as the extractive inorganic nutrient component in IMTA systems. In fact, we believe that IMTA systems will play an important role in the overall development of land-based seaweed production in the future. Several factors may account for this:

(a) The availability of water and its quality
(b) The size and morphological characteristics of seaweeds making them suitable candidates for tank cultivation
(c) The diversity of products derived from various target species produced in the same system

The water requirements of intensive animal aquaculture ensure a plentiful supply of water available for seaweed cultivation. In terms of synergies and efficiencies, particularly in engineering, the use of the same water stream allows for sharing of the energetic costs associated with water pumping. On the other hand, since water quality is also important for animal production and health, the seaweed component should have access to high-quality water, free of toxins, heavy metals, and other pollutants. Finally, but equally very important, and the essence of the IMTA concept, the extractive, seaweed production system would have access to nutrient-enriched seawater derived from the animal (fed aquaculture) component of the system. As an example, Burri [103] tested the inclusion of the IMTA-produced red seaweed (*G. vermiculophylla*) in a new form of vegetarian sausages for children. This author verified that the chemical composition of the seaweed chemical was below the permitted values for children consumption in terms of mercury (Hg), lead (Pb), and cadmium (Cd). Even when the initial wild collected stocking biomass had metal values close to or above the limits, these values decreased after 4 weeks in the IMTA conditions. In conclusion, pollutant-free seawater, nutrient-rich and at a free or shared cost, would be available for the seaweed production system when associated with an IMTA system.

As mentioned previously, intensive, land-based seaweed production allows for exploitation of seaweeds which are not necessarily suited to open water techniques such as those applied to various kelps or *Porphyra*. For obvious reasons, open water seaweed production is not the most suited, for instance, for free-floating techniques and vegetative propagation for seaweed cultivation. The free-floating method of tank or pond production can, however, be highly suited to land-based systems, allowing for optimization of water volume in the tanks as well as stocking density, thereby ensuring access to light and nutrient supply. Furthermore, on-land production methods are also highly suited to seaweeds where vegetative, or clonal, propagation is possible, in as much as the seaweed production system guaranties the homogeneity of the biomass (provided that the environmental conditions do not overly influence it). This feature can also decrease the operational costs associated with the production of seaweeds with a complex life cycle.

In terms of the production diversity, land-based systems could be planned to include several independent production units. Even considering the increase in the operation costs, these production units, managed independently from one other but sharing common parts of the infrastructure, could have two main advantages:

1. Produce different species/varieties of seaweed
2. Allow for an easier control of the environmental parameters that can influence the quality of the biomass

Depending on the applications of the biomass, the production units could be scaled to produce either a few kilograms or a few tons per year instead of the thousands of tons required for seaweed such as those used as raw materials for polysaccharide extraction. Such relatively small volumes might be more appropriate for the biomass required for some direct human consumption products or for application as ingredients of functional foods and cosmetic products. In turn, this smaller scale would allow a better control of production parameters and the biomass can be tailored more closely to the needs of the final consumer.

In conclusion, land-based seaweed aquaculture can provide for the production of biomass required for human food and other advanced applications. Rather than focusing on biomass production in the order of thousands of tons, this form of seaweed production can specialize in niche markets, which requires highly specific and tailored biomass. By doing this, land-based seaweed production can contribute to the development of more seaweed or seaweed-based products for human food and health. Finally, when used as part of an IMTA system approach, land-based seaweed production can contribute to the sustainable development of intensive fed aquaculture in an economical and ecological perspective.

Acknowledgments

R. Pereira is sponsored by a fellowship from the Portuguese Foundation for Science and Technology (FCT) through program POCI 2010, with the support of FEDER and FSE(SFRH/BPD/36451/2007). Support to C. Yarish was provided by the Connecticut Sea

Grant (DOC/NOAA/Ocean & Atmospheric Research Contract No. NA10OAR4170095); the Department of Energy's NETL Program, FOA# 0000015 to Gas Technology Institute; NOAA-SBIR Phase I (Ocean Approved, LLC, DOC/National Oceanic and Atmospheric Administration Award/Contract #: AG100206); and National Fish and Wildlife Foundation (contract ##24266).

Bibliography

Primary Literature

1. Ryther JH, Goldman JC, Gifford JE, Huguenin JE, Wing AS, Clarner JP, Williams LD, Lapointe BE (1975) Physical models of integrated waste recycling marine polyculture systems. Aquaculture 5:163–177
2. Lüning K (1990) Seaweeds: their environment, biogeography, and ecophysiology. Wiley, New York, p 527
3. McHugh DJ (2003) A guide to the seaweed industry. FAO Fisheries Technical Paper 441. FAO, Rome, Italy
4. Bixler HJ, Porse H (2010) A decade of change in the seaweed hydrocolloids industry. J Appl Phycol. doi:10.1007/s10811-010-9529-3
5. Graham L, Graham J, Wilcox L (2009) Algae, 2nd edn. Benjamin Cummings (Pearson), San Francisco, p 720
6. Levin SA, Lubchenco J (2008) Resilience, robustness, and marine ecosystem-based management. Biosciences 58(1):27–32
7. Yarish C, Pereira R (2008) Mass production of marine macroalgae. In: Jørgensen SE, Fath BD (eds) Ecological engineering, vol 3 of encyclopedia of ecology, 5 vols. Elsevier, Oxford, pp 2236–2247
8. Goh CS, Lee KT (2009) A visionary and conceptual macroalgae-based third generation bioethanol (TGB) biorefinery in Sabah, Malaysia as an underlay for renewable and sustainable development. Renewable Sustainable Energy Rev 14:842–848
9. Chung IK, Beardall J, Mehta S, Sahoo D, Stojkovic S (2010) Using marine macroalgae for carbon sequestration: a critical appraisal. J Appl Phycol. doi:10.1007/s10811-010-9604-9
10. Kumar P (2010) Biofuels set for a sea-change. Biospectrum November 2010:48–50
11. Notoya M (2010) Production of biofuel by macroalgae with preservation of marine resources and environment. In: Israel A, Einav R, Seckbach J (eds) Seaweeds and their role in globally changing environments, vol 15, Cellular origin, life in extreme habitats and astrobiology. Springer Science+Business Media BV, Dordrecht, pp 217–228
12. Dillehay TD, Ramírez C, Pino M, Collins MB, Rossen J, Pino-Navarro JD (2008) Monte Verde: seaweed, food, medicine, and the peopling of South America. Science 320:784–786
13. Erlandson JM, Graham MH, Bourque BJ, Corbett D, Estes JA, Steneck RS (2007) The kelp highway hypothesis: marine ecology, the coastal migration theory, and the peopling of the Americas. J Isl Coast Archaeol 2:161–174
14. Mumford TF, Miura A (1988) *Porphyra* as food: cultivation and economics. In: Lemby CA, Walland JR (eds) Algae and human affairs. Cambridge University Press, Cambridge, pp 87–117
15. Abbott IA (1996a) Ethnobotany of seaweeds: clues to uses of seaweeds. International Seaweed Symposium XV. Hydrobiologia 326/327:15–20
16. Abbott IA (1996) Limu: an ethnobotanical study of some Hawaiian seaweeds, 4th edn. National Tropical Botanical Garden, Lawai, p 39
17. Konishi T (2008) Integrated functions of diet in anti-aging and cancer prevention. Papers from the 4th international Niigata symposium on diet and health, Niigata, Japan, 20–30 Nov 2008. BioFactors 34(4):261–342
18. Parrot MD, Greenwood CE (2008) Dietary influences on cognitive function with aging: from high-fat diets to healthful eating. Ann NY Acad Sci 1114:389–397
19. O'Sullivan L, Murphy B, McLoughlin P, Duggan P, Lawlor PG, Hughes H, Gardiner GE (2010) Prebiotics from marine macroalgae for human and animal health applications. Mar Drugs 8:2038–2064. doi:10.3390/md8072038
20. Plaza M, Cifuentes A, Ibañez E (2008) In the search of new functional food ingredients from algae. Trends Food Sci Technol 19:31–39
21. Kumar CS, Ganesan P, Suresh SV, Bhaskar N (2008) Seaweeds as a source of nutritionally beneficial compounds. J Food Sci Technol 45(1):1–13
22. Bocanegra A, Bastida S, Benedí J, Ródenas S, Sánchez-Muniz FJ (2009) Characteristics and nutritional and cardiovascular-health properties of seaweeds. J Med Food 12(2):236–258
23. Noda H (1993) Health benefits and nutritional properties of nori. J Appl Phycol 5:255–258
24. Nisizawa K, Noda H, Kikuchi R, Watanabe T (1987) The main seaweed foods in Japan. Hydrobiologia 151/152:5–29
25. Tsuboyama-Kasaoka N, Shozawa C, Sano K, Kamei Y, Kasaoka S, Hosokawa Y, Ezaki O (2006) Taurine (2-aminoethanesulfonic acid) deficiency creates vicious circle promoting obesity. Endocrinology 147(7):3276–3284
26. Burtin P (2003) Nutritional value of seaweeds. Electron J Environ Agric Food Chem 2(4):498–503
27. Holdt S, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol. doi:10.1007/s10811-010-9632-5
28. Cornish ML, Garbary DJ (2010) Antioxidants from macroalgae: potential applications in human health and nutrition. Algae 25(4). doi:10.4490/algae.2010.25.4.000
29. Blouin N, Calder BL, Perkins B, Brawley SH (2006) Sensory and fatty acid analysis of two Atlantic species of *Porphyra*. J Appl Phycol 18:79–85
30. Zhang Q, Li N, Zhou G, Lu X, Xu Z, Li Z (2003) In vivo antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodophyta) in aging mice. Pharmacol Res 48:151–155
31. Díaz-Rubio ME, Pérez-Jiménez J, Saura-Calixto F (2009) Dietary fiber and antioxidant capacity in *Fucus vesiculosus* products. Int J Food Sci Nutr 60(S2):23–34

32. Zhou HP, Chen QH (1990) Anticoagulant and antihyperlipedemic effects of polysaccharide from *Porphyra yezoensis*. J China Pharm Univ 21:358–360
33. Yashizawa Y, Ametani A, Tsunehiro J, Numera K, Itoh M, Fukui F, Kaminogawa S (1995) Macrophage stimulation activity of the polysaccharide fraction from a marine alga (*Porphyra yezoensis*): structure-function relationships and improved solubility. Biosci Biotechnol Biochem 59: 1933–1937
34. Saito M, Kawai M, Hagino H, Okada J, Yamamoto K, Hayashida M, Ikeda T (2002) Antihypertensive effect of nori-peptides derived from red alga *Porphyra yezoensis* in hypertensive patients. Am J Hypertens 5(4, Part 2):210A (Published by Elsevier Science Inc)
35. Béress A, Wassermann O, Bruhn T, Béress L, Kraiselburd EN, González LU, De Motta GE, Chávez PI (1993) A new procedure for the isolation of anti-HIV compounds (polysaccharides and polyphenols) from the marine alga *Fucus vesiculosus*. J Nat Prod 56:478–488
36. Aisa Y, Miyakawa Y, Nakazato T, Shibata H, Saito K, Ikeda Y, Kizami M (2005) Fucoidan induces apoptosis of human HS-sultan cells accompanied by activation of caspase-3 and down-regulation of ERK pathways. Am J Hematol 78:7–14
37. Teas J, Harbison ML, Gelman RS (1984) Dietary seaweed (*Laminaria*) and mammary carcinogenesis in rats. Cancer Res 44:2758–2761
38. Yang YJ, Nam SJ, Kong G, Kim MK (2010) A case–control study on seaweed consumption and the risk of breast cancer. Br J Nutr 103:1345–1353
39. Sahoo D, Tang X, Yarish C (2002) *Porphyra* – the economic seaweed as a new experimental system. Curr Sci 83(11): 1313–1316
40. Gantt E, Berg GM, Bhattacharya D, Blouin NA, Brodie JA, Chan CX, Collén J, Cunninghan FX Jr, Gross J, Grossman AR, Karpowicz S, Kitade Y, Klein AS, Levine IA, Lin S, Lu S, Lynch M, Minocha SC, Müller K, Neefus CD, de Oliveira CM, Rymarquis L, Smith A, Stiller JW, Wu W-K, Yarish C, Zhuang YY, Brawley SH (2010) Porphyra: complex life histories in a harsh environment. P. umbilicalis, an intertidal red alga for genomic analysis. In: Seckbach J, Chapman DJ (eds) Red algae in the genomic age, vol 13, Cellular origin, life in extreme habitats and astrobiology. Springer, Dordrecht, pp 129–148
41. Grossman AR (2007) In the grip of algal genomics. In: León R, Galvin A, Fernandez E (eds) Transgenic microalgae as green cell factories. Landers bioscience Publishers and Springer Science+Business Media, Austin, TX, pp 54–76
42. Grossman AR, Karpowicz SJ, Heinnickel M, Dewez D, Hamel B, Dent R, Niyogi KK, Johnson X, Alric J, Wollman F-A, Li H, Merchant SS (2010) Phylogenomic analysis of the *Chlamydomonas* genome unmasks proteins potentially involved in photosynthetic function and regulation. Photosynth Res 106:3–17
43. Guiry MD, Guiry GM (2010) AlgaeBase. World-wide electronic publication. National University of Ireland, Galway, http://www.algaebase.org; searched on 30 May 2010
44. Pereira R, Yarish C (2010) The role of Porphyra in sustainable culture systems: physiology and applications. In: Israel A, Einav R, Seckbach J (eds) Seaweeds and their role in globally changing environments, vol 15, Cellular Origin, Life in Extreme Habitats and Astrobiology. Springer, Dordrecht, pp 339–353. ISBN 978-90-481-8568-9
45. FAO (2010) FAO fisheries and aquaculture information and statistics service (2010) Aquaculture production 1950–2008, Rome, Italy
46. Tseng CK (1981) Commercial cultivation. In: Lobban CS, Wynne MJ (eds) The biology of seaweeds, vol 17, Botanical Monographs. Blackwell, Oxford, pp 680–725
47. Doty MS (1977) Eucheuma – current marine agronomy. In: Krauss RW (ed) The marine plant biomass of the Pacific Northwest Coast. A potential economic resource. Oregon State University Press, Corvallis, pp 203–214
48. Houyuan L, Zhang J, Liu K, Wu N, Li Y, Zhou K, Ye M, Zhang T, Zhang H, Yang X, Shen L, Xu D, Li Q (2009) Earliest domestication of common millet (Panicum miliaceum) in East Asia extended to 10, 000 years ago. PNAS 106(18):7367–7372
49. Munro ND, Grosman L (2009) Early evidence (ca. 12,000 B.P.) for feasting at a burial cave in Israel. PNAS 107(35): 15362–15366
50. Tseng CK, Chang CF (1956) On China's Porphyra and its cultivation. Shengwuxue Tongbao 3:29–33 Tseng CK (1981) Commercial cultivation. In: Lobban CS, Wynne MJ (eds) The biology of seaweeds. Botanical monographs, Vol 17. Blackwell Scientific Publications, University of California Press, Berkeley and Los Angeles, pp 680–725
51. Drew KM (1949) *Conchocelis*–phase in the life history of *Porphyra umbilicalis* (L.) Kütz. Nature 164:748–749
52. Kurogi M (1953) Study of the life-history of *Porphyra* I. The germination and development of carpospores. Bull Tohoku Reg Fish Lab 2:67–103
53. Kurogi M (1953) On the liberation of monospores from the filamentous thallus (*Conchocelis*-stage) of *Porphyra tenera* Kjellm. Bull Tohoku Reg Fish Lab 2:104–108
54. Kurogi M, Sato S (1962) Influence of light on the growth and maturation of Conchocelis-thallus of Porphyra II. Effect of different photoperiods on the growth and maturation of Conchocelis-thallus of *P. tenera Kjellm*. Bull Tohoku Reg Fish Lab 20:127–137
55. Brinkhuis BH, Levine HG, Schlenk CG, Tobin S (1987) *Laminaria* cultivation in the far east and North America. In: Bird KT, Benson PH (eds) Seaweed cultivation for renewable resources. Elsevier, Amsterdam, pp 107–146
56. Chopin T, Robinson SMC, Troell M, Neori A, Buschmann AH, Fang J (2008) Multitrophic integration for sustainable marine aquaculture. In: Sven EJ, Fath BD (eds) Ecological engineering, vol 3 of encyclopedia of ecology, 5 vols. Elsevier, Oxford, pp 2463–2475
57. Neori A (2008) Essential role of seaweed cultivation in integrated multi-trophic aquaculture farms for global expansion of mariculture: an analysis. J Appl Phycol 20:567–570

58. Nobre AM, Robertson-Andersson D, Neori A, Sankar K (2010) Ecological-economic assessment of aquaculture options: comparison between abalone monoculture and integrated multi-trophic aquaculture of abalone and seaweeds. Aquaculture 306:116–126
59. Chopin T, Troell M, Reid GK, Knowler D, Robinson SMC, Neori A, Buschmann AH, Pang SJ (2010) Integrated multi-trophic aquaculture. Part I. Responsible practice provides diversified products, biomitigation. Glob Aquac Advocate September/October 2010:38–39
60. Fujita RM, Wheeler PA, Edwards RL (1989) Assessment of macroalgal limitation in a seasonal upwelling region. Mar Ecol Prog Ser 53:293–303
61. Kautsky N, Folke C (1991) Integrating open system aquaculture: ecological engineering for increased production and environmental improvement through nutrient recycling. In: Etnier C, Guterstam B (eds) Ecological engineering for wastewater treatment. Bokskogen, Gothenburg, pp 320–334
62. Neori A, Cohen I, Gordin H (1991) *Ulva lactuca* biofilters for marine fish-pond effluents. II. Growth rate, yield and C:N ratio. Bot Mar 34:483–489
63. Krom MD, Ellner S, van Rijn J, Neori A (1995) Nitrogen and phosphorus cycling and transformations in a prototype "non-polluting" integrated mariculture system, Eilat, Israel. Mar Ecol Prog Ser 118:25–36
64. Buschmann AH (1996) An introduction to integrated farming and the use of seaweeds as biofilters. Hydrobiologia 326/327:59–60
65. Shpigel M, Neori A (1996) Abalone and seaweeds intensive cultivation in integrated land-based mariculture system. I. Proposed design and cost analyses. Aquacult Eng 15:313–326
66. Troell M, Halling C, Nilsson A, Buschmann AH, Kautsky N, Kautsky L (1997) Integrated marine cultivation of *Gracilaria chilensis* (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. Aquaculture 156:45–61
67. Chopin T, Yarish C (1998) Nutrients or not nutrients? World Aquac Mag 29:31–33, 60–61
68. Neori A, Shpigel M (1999) Using algae to treat effluents and feed invertebrates in sustainable integrated mariculture. World Aquac 30(2):46–51
69. Yarish C, Chopin T, Wilkes R, Mathieson AC, Fei XG, Lu S (1999) Domestication of nori for Northeast America: the Asian experience. Bull Aquacult Assoc Can 99:11–17
70. Chopin T, Buschmann AH, Halling C, Troell M, Kautsky N, Neori A, Kraemer GP, Zertuche-González JA, Yarish C, Neefus C (2001) Integrating seaweeds into marine aquaculture systems: a key towards sustainability. J Phycol 37:975–986
71. Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Shpigel M, Yarish C (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361–391
72. Kraemer GP, Yarish C (1999) A preliminary comparison of the mariculture potential of *Porphyra purpurea* and *Porphyra umbilicalis*. J Appl Phycol 11:473–477
73. Chopin T (2006) Integrated multi-trophic aquaculture. What it is and why you should care. Northern Aquac July/August 2006:4
74. Matos J, Costa S, Rodrigues A, Pereira R, Sousa Pinto I (2006) Experimental integrated aquaculture of fish and red seaweeds in Northern Portugal. Aquaculture 252:31–42
75. Msuya FE, Neori A (2010) The performance and chemical composition of spray irrigated seaweed *Ulva lactuca* as a crop and as a biofilter of fishpond effluents. J Phycol. doi:10.1111/j.1529-8817.2010.00843.x
76. Abreu MH, Pereira R, Yarish C, Buschmann AH, Sousa-Pinto I. IMTA with *Gracilaria vermiculophylla*: productivity and nutrient removal performance of the seaweed in a land-based pilot-scale system. Aquaculture (in press). doi:10.1016/j.aquaculture.2010.12.036
77. Troell M, Robertson-Andersson D, Anderson RJ, Bolton JJ, Maneveldt G, Halling C, Probyn T (2006) Abalone farming in South Africa: an overview with perspectives on kelp resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. Aquaculture 257:266–281
78. Whitmarsh DJ, Cook EJ, Black KD (2006) Searching for sustainability in aquaculture: an investigation into the economic prospects for an integrated salmon-mussel production system. Mar Policy 30:293–298
79. Robertson-Andersson DV (2007) Biological and economical feasibility studies of using *Ulva lactuca* (Chlorophyta) in recirculating systems in abalone farming. Doctoral dissertation, University of Cape Town, South Africa
80. Robertson-Andersson DV, Potgieter M, Hansen J, Bolton JJ, Troell M, Anderson RJ, Halling C, Probyn T (2008) Integrated seaweed cultivation on an abalone farm in South Africa. J Appl Phycol 20:579–595
81. Rawson MV, Chen C, Ji R, Zhu M, Wang A, Wang L, Yarish C, Sullivan JB, Chopin T, Carmona R (2002) Understanding the interaction of extractive and fed aquaculture using ecosystem modelling. In: Stickney RR, McVey JP (eds) Responsible marine aquaculture. CABI, London, pp 263–296
82. Fang J, Kuang S, Sun H, Li F, Zhang A, Wang X, Tang T (1996) Status and optimising measurements for the culture of scallop *Chlamys farreri* and kelp *Laminaria japonica* in Sanggou Bay. Mar Fish Res (Haiyang Shuichan Yanjiu) 17:95–102 (in Chinese)
83. Sohn CH (1996) Historical review on seaweed cultivation of Korea. Algae 11:357–364
84. Fei XG (2004) Solving the coastal eutrophication problem by large scale seaweed cultivation. Hydrobiologia 512:145–151
85. Egan B, Yarish C (1990) Productivity and life history of *Laminaria ongicruris* at its southern limit in the western Atlantic Ocean. Mar Ecol Prog Ser 67(3):263–273
86. Chopin T, Yarish C, Wilkes R, Belyea E, Lu S, Mathieson A (1999) Developing *Porphyra*/salmon integrated aquaculture for bioremediation and diversification of the aquaculture industry. J Appl Phycol 11:463–472
87. McVey JP, Stickney RR, Yarish C, Chopin T (2002) Aquatic polyculture and balanced ecosystem management: new paradigms for seafood production. In: Stickney RR, McVey JP (eds) Responsible marine aquaculture. CABI, London, pp 91–104

88. Troell M, Halling A, Neori A, Buschmann A, Kautsky N, Yarish C (2003) Integrated mariculture: asking the right questions. Aquaculture 226:69–90
89. Rai LC, Kumar HD, Mohn FH, Soeder CJ (2000) Services of algae to the environmemt. J Microbiol Biotechnol 10:119–136
90. Lubchenco J, Petes LW (2010) The interconnected biosphere: science at the ocean's tipping points. Oceanography 23(2):115–129
91. Ash N, Blanco H, Brown C, Garcia K, Tomich T, Vira B, Zurek M (eds) (2010) Ecosystems and human well-being: a manual for assessment practitioners. Island Press, Washington, DC, p 288
92. Pang SJ, Liu F, Shan TF, Xu N, Zhang ZH, Gao SQ, Chopin T, Sun S (2010) Tracking the algal origin of the *Ulva* bloom in the Yellow Sea by a combination of molecular, morphological and physiological analyses. Mar Environ Res 69: 207–215
93. Vaccari DA (2009) Phosphorus: a looming crisis. Sci Am 300(6):54–59
94. Winberg P, Ghosh D, Tapsell L (2009) Seaweed culture in integrated multi-trophic aquaculture: nutritional benefits and systems for Australia. Rural Industries Research and Development Corporation, RIRDC Publication No 09/005
95. Lee B (2010) Cultivated seaweed and seaweed industry development in Australia. Rural Industries Research and Development Corporation – RIRDC. Publication No. 10/164, Project No. PRJ-004681, p 39. ISBN 978-1-74254-131-0
96. Craigie JS, Shacklock PF (1995) Culture of Irish Moss. In: Boghen AD (ed) Cold water aquaculture in Atlantic Canada. The Canadian Institute for Research on Regional Development, Moncton, pp 365–390
97. Lobban CS, Harrison PJ (1994) Seaweed ecology and physiology. Cambridge University Press, Cambridge, p 366
98. Rheault RB, Ryther JH (1983) Growth, yield and morphology of *Ascophyllum nodosum* (Phaeophyta) under continuous and intermittent seawater spray culture regimes. J Phycol 19:252–254
99. Pickering TD, Gordon ME, Tong LJ (1995) A preliminary trial of a spray culture technique from growing the agarophyte *Gracilaria chilensis* (Gracilariales, Rhodophyta). Aquaculture 130:43–49
100. Ugarte RA, Craigie JS, Critchley AT (2010) Fucoid flora of the rocky intertidal of the Canadian Maritimes: implications for the future with rapid climate change. In: Israel A, Einav R, Seckbach J (eds) Seaweeds and their role in globally changing environments, vol 15, Cellular Origin, Life in Extreme Habitats and Astrobiology. Springer, Dordrecht, pp 73–90. ISBN 978-90-481-8568-9
101. Ugarte R (2010) An evaluation of the mortality of the brown seaweed *Ascophyllum nodosum* (L.) Le Jol. produced by cutter rake harvests in southern New Brunswick, Canada. J Appl Phycol. doi:10.1007/s10811-010-9574-y
102. Cock MJ, Sterck L, Rouzé P, Scornet D, Allen AE, Amoutzias G, Anthouard V, Artiguenave F, Aury J-M, Badger JH, Beszteri B, Billiau K, Bonnet E, Bothwell JH, Bowler C, Boyen C, Brownlee C, Carrano CJ, Charrier B, Cho GY, Coelho SM, Collén J, Corre E, Da Silva C, Delage L, Delaroque N, Dittami SM, Doulbeau S, Elias M, Farnham G, Gachon CMM, Gschloessl B, Heesch S, Jabbari K, Jubin C, Kawai H, Kimura K, Kloareg B, Küpper FC, Lang D, Le Bail A, Leblanc C, Lerouge P, Lohr M, Lopez PJ, Martens C, Maumus F, Michel G, Miranda-Saavedra D, Morales J, Moreau H, Motomura T, Nagasato C, Napoli CA, Nelson DR, Nyvall-Collén P, Peters AF, Pommier C, Potin P, Poulain J, Quesneville H, Read B, Rensing SA, Ritter A, Rousvoal S, Samanta M, Samson S, Schroeder DC, Ségurens B, Strittmatter M, Tonon T, Tregear JW, Valentin K, von Dassow P, Yamagishi T, Van de Peer Y, Wincker P (2010) The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. Nature 465:617–621
103. Burri S (2009) Concepção de um alimento funcional à base de legumes e algas para consumo infantil. Master thesis, Universidade Católica Portuguesa. Escola Superior de Biotecnologia. Porto, Portugal, p 83 (in portuguese)

Books and Reviews

Barrington K, Chopin T, Robinson S (2009) Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. In: Soto D (ed) Integrated aquaculture: a global review. FAO Fisheries and Aquaculture Technical Paper. No. 529. FAO, Rome, pp 7–46

Buschmann AH, Hernández-González MC, Aranda C, Chopin T, Neori A, Halling C, Troell M (2008) Mariculture waste management. In: Jørgensen SE, Fath BD (eds) Ecological engineering, vol 3 of encyclopedia of ecology, 5 vols. Elsevier, Oxford, pp 2211–2217

Critchley AT, Ohno M (eds) (1997/2001). Interactive, computer-based, CD-ROM "Cultivation and farming of marine plants". Expert Centre for Taxonomic Identification (ETI), University of Amsterdam Netherlands. ISBN: 90-75000-31-6, http://www.eti.uva.nl

Critchley AT, Ohno M, Largo D (2006) World seaweed resources: an authoritative reference system, A multimedia, interactive DVD ROM. ETI Bioinformatics Publishers, Univ, Amsterdam. ISBN: 90-75000-80-4

Fei XG, Lu S, Bao Y, Wilkes R, Yarish C (1998) Seaweed cultivation in China. World Aquac 29(4):22–24

Fleurence J (1999) Seaweed proteins: biochemical, nutritional aspects and potential uses. Trends Food Sci Technol 10:25–28

Friedlander M (2008) Israeli R&D activities in seaweed cultivation. Isr J Plant Sci 56:15–28

He P, Xu S, Zhang H, Wen S, Dai Y, Lin S, Yarish C (2008) Bioremediation efficiency in the removal of dissolved inorganic nutrients by the red seaweed, *Porphyra yezoensis*, cultivated in the open sea. Water Res 42:1281–1289

Lembi CA, Waaland R Jr (eds) (1988) Algae and human affairs. Cambridge University Press, London, p 590

Nisizawa L (2002) Seaweeds Kaiso: bountiful harvests from the sea. Japan Seaweed Association, Kochi, p 106

Phang SW, Critchley AT, Ang PO (eds) (2006) Advances in seaweed cultivation and utilisation in Asia. University of Malaya Maritime Research Centre, Kuala Lumpur, p 177

Sahoo D, Yarish C (2005) Mariculture of seaweeds. In: Andersen R (ed) Phycological methods: algal culturing techniques, chapter 15. Academic, New York, pp 219–237

Seckbach J, Chapman DJ (eds) (2010) Red algae in the genomic age, vol 13, Cellular origin, life in extreme habitats and astrobiology. Springer, New York, p 486

Sun S, Wang F, Li CL, Qin S, Zhou MJ, Ding LP, Pang SJ, Duan DL, Wang GC, Yin BS, Yu RC, Jiang P, Liu ZL, Zhang GT, Fei XG, Zhou M (2008) Emerging challenges: massive green algal blooms in the Yellow Sea. Nature Precedings hdl:10101/npre.2008.2266.1

Titlyanov EA, Titlyanova TV (2010) Seaweed cultivation: methods and problems. Russ J Mar Biol 36:227–242

Yuan YV, Bone DE, Carrington MF (2005) Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated *in vitro*. Food Chem 91:485–494

Seed Dormancy and Agriculture, Physiology

Roberto L. Benech-Arnold[1], M. Verónica Rodriguez[2], Diego Batlla[3]
[1]IFEVA – Cátedra de Cultivos Industriales/CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina
[2]IFEVA – Cátedra de Fisiología Vegetal/CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina
[3]IFEVA – Cátedra de Cerealicultura/CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina

Article Outline

Glossary

Dormancy Internal inadequacy of a seed that impedes its germination under, otherwise, favorable thermal, hydric, and gaseous conditions.

Abscisic acid Plant growth regulator that, among other processes, inhibits seed germination and is involved in dormancy imposition.

ROS Reactive oxygen species implicated in tissue aging and, more recently, in seed dormancy relief.

Preharvest sprouting Untimely grain germination in the mother plant due to a combination of low dormancy and damp conditions prior to crop harvest.

Stratification thermal time index An index calculated by summing thermal time below a threshold temperature, which is used to estimate dormancy release as a function of stratification temperature and time.

Definition of the Subject

Dormancy is a common attribute of temperate species. Its adaptive significance is quite evident for species living in the wild: dormancy is a mean for restricting germination to the season when environmental conditions are suitable for plant establishment. From an agricultural perspective, dormancy represents a problem, both when it is long lasting and when it is too short. For that reason, selection against dormancy has been always behind any domestication effort. In some cases, the aim of removing dormancy has not been achieved, and in others, it has gone too far resulting in susceptibility to preharvest sprouting. In addition, the fight against weeds is frequently impaired by the existence of long-lasting seed banks as a result of seed dormancy.

Introduction

A seed can be declared to be dormant when it fails to germinate despite it is given all the "proper" conditions (i.e., enough water and appropriate temperature) for germination to happen. Assuming that there is a living seed (a dead seed would not germinate either), then why does it not germinate? Will it ever germinate? These types of questions were probably first asked in despair by farmers about 10,000 years ago, while staring at their useless recently harvested grains that would not give way to a new crop or allow malting for beer (a process that also relies on germination).

Despite historical human concerns with dormancy, this "internal block to germination" has been positively selected throughout millions of years of evolution in most temperate species, and has obvious ecological benefits in the wild. The main apparent role of seed dormancy is to delay germination until a proper environment is encountered, for example, by keeping track with seasonal changes in temperature to avoid unfavorable conditions for plant establishment [1]. Within the life of most plants, the only chance to travel is as a seed. After separating from the mother plant, the seed is moved by different means (wind, gravity, water, animals) until it is randomly located in a new microenvironment that may prove adequate or not for completing its life cycle. Once germination happens, the small plant will most probably remain attached to that spot until its death. As a consequence of plants being sessile organisms, seeds have developed sophisticated mechanisms to collect information from the

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

environment, and the resulting output is a vital decision: to germinate right away, or not (at least not this time, this place) until new environmental conditions that are good enough are finally met with.

Seed dormancy has been a subject of intensive research during the last decades. Physiological and environmental control of dormancy has been studied deeply and, more recently, its molecular and genetic bases have started to be elucidated. As in other areas of plant science, knowledge on the molecular bases of dormancy has been attained through the use of model organisms. Indeed, work with *Arabidopsis thaliana*, a small winter annual weed preferred by most plant scientists, has revealed the involvement of multiple genes controlling seed dormancy, which is a continuous and complex character. Cultivated species also display different degrees of dormancy, and either the presence or the absence of dormancy can have strong consequences on the utilization of the seeds, and this will be discussed in more detail in this entry. Different strategies exist that are directed to control dormancy through genetic changes (as a result of classical breeding or transgenic events) or to cope with varying degrees of dormancy, such as the development of predictive models. As the level of dormancy a seed will effectively display is the result of its genotype interacting deeply with a changing environment, predictive models have been developed for some species (e.g., malting barley or some weeds). Most of these strategies require a thorough knowledge on the biology of dormancy at different levels.

In this entry, the focus is on what is termed as physiological dormancy, or the lack thereof. The most important challenges facing agriculture, in relation to the presence or the absence of dormancy in both cultivated and weedy species, are also pointed out. Some examples of each type of problem are presented, illustrating on the physiological mechanisms responsible for the expression of the character and commenting how the molecular information coming from studies on *Arabidopsis* and other model species, have started to be used to attain the final aim of solving these dormancy problems.

Dormancy (Classifications and Definitions)

Dormancy is the result of mechanisms operating within the seed that impede germination even when the seed is exposed to an environment known to be friendly for that species (i.e., in which water, oxygen and adequate temperature are available). This impediment or block to seed germination can be determined by both morphological and/or physiological properties of the seed [2]. On the basis of this fact, Baskin and Baskin [3] developed a classification system which includes five classes of seed dormancy: physiological, morphological, morphophysiological, physical, and a combination of physical and physiological. In this entry, mainly physiological dormancy which is the most prevalent dormancy type in temperate seed banks and is also the major form of dormancy in most laboratory model species is referred to [4]. This type of dormancy is caused by a physiological inhibiting mechanism of the embryo that prevents radicle emergence, although other seed structures that cover the embryo can be involved as well [1]. From a physiological point of view, there is enough evidence showing that the mechanism of seed dormancy is mainly regulated by the phytohormones abscisic acid and gibberellins [4, 5].

Dormancy can be also classified in primary and secondary dormancy. Primary dormancy refers to the innate dormancy possessed by seeds upon dispersal from the mother plant. Secondary dormancy refers to a dormant state that is induced in nondormant seeds by unfavorable conditions for germination, or reinduced in once-dormant seeds after a sufficiently low dormancy had been attained.

Primary dormancy is acquired during seed development, and, in most species, is gradually lost after natural seed dispersal (in wild species) or harvest, during post-maturation or after-ripening period. As pointed out before, both the primary dormancy level that seeds present at dispersal or harvest (maturity) and the rate of release from primary dormancy (which determines the time lapse required by a seed population to lose dormancy) are dependent on the genotype, the environment, and the interaction between them. Therefore, although different species or genotypes (i.e., cultivars, inbred lines, etc.) can present more dormant or less dormant seeds at maturity, and faster or slower rates of dormancy release, those traits can be modulated by the environment perceived by the seed during their development-maturation phase (parental environment) and their post-maturation period, respectively. In relation to the parental environment, for example, there is evidence indicating that there are

certain environmental factors tending to have similar effects on different species [6]. For example, low dormancy at seed maturity is generally associated with high temperatures, short days, drought, and nutrient availability during seed development and maturation [7–11]. For the post-maturation period, storage temperature and moisture are the most relevant factors regulating dormancy loss rate in cultivated species, while in natural wild conditions a diverse number of factors can be affecting seed dormancy loss. In wild species (i.e., weeds), the post-maturation process usually takes place in the soil because, after dispersal, seeds usually become part of the soil seed bank [12]. Under these conditions, primary dormancy loss can be affected by many naturally occurring factors, as soil temperature and soil water regime (through changes in seed moisture content), temperature fluctuations, light, etc. [13–15]. Although many factors can be affecting the dormancy level of seeds from cultivated and wild species, there is evidence showing that temperature appears to be primarily responsible for year-to-year variation in dormancy level at maturity [6] and for differences in the dormancy loss rate during post-maturation, though the latter can be modulated by seed moisture content [15, 16].

In many species, nondormant seeds can be reinduced into secondary dormancy after dispersal. There are many factors that can be responsible for induction into secondary dormancy, as specific temperature ranges, changes in light quality and quantity, the gaseous environment, etc. Although induction into secondary dormancy is an uncommon phenomenon in most seeds of cultivated species, it is very common under field conditions for seeds of many wild species, particularly those forming persistence seed banks. Indeed, under natural field conditions, the release from primary dormancy followed by subsequent entrance into secondary dormancy (whenever conditions are given for this entrance) may lead to dormancy cycling. Evidence for dormancy cycling in natural seed soil banks has been obtained for many species.

Dormancy and Its Implications in Agriculture

Seeds in their natural form are still the best way to preserve and propagate most crop species, and their capacity to germinate at the right moment (either in the field or under controlled conditions) is a most important feature. Therefore, any factors (internal or external) affecting the germination capacity of seeds and ultimately their agronomical performance should be understood in order to improve seed quality through breeding and/or crop management strategies. Among the factors that may affect germination, dormancy is probably the most important, as it is a heritable trait that can inhibit germination of a viable seed. Indeed, beyond the fact that dormancy is a highly intriguing biological process and consequently moves the curiosity of scientists, the study of dormancy has strong practical implications. Both farmers and seed companies encounter problems associated either to an anticipated dormancy loss or to a long-lasting dormancy. In the same way, industrial processes like malting, which depends on seed germination, can be largely hampered by the existence of dormancy or, alternatively, face important reductions in the quality of the received lots due to a precocious dormancy termination and pre-germination in the field.

Since dormancy is highly undesirable for agricultural purposes, selection pressure against this trait must have been important throughout the domestication process, particularly in species with temperate origin. In some cases, this pressure must have gone too far, as in the case of cereals, leading to an anticipated dormancy loss which makes some crops susceptible to preharvest sprouting or pre-germination. Nevertheless, intraspecific variability for dormancy exists and "dormancy genes" are still present in some genotypes, although their positive selection through breeding has been difficult due to strong linkage to other undesired traits. In some other cases, selection pressure might have not succeeded in eliminating dormancy, so seeds are not germinable by the time of the next sowing or by the time they need to be "ready" by the malting industry. In any case, dormancy release should take place within a precise "time window." This rarely can be achieved without a solid knowledge of the mechanisms on which dormancy relies in each species.

Dormancy in Cultivated Species

Problems Associated with the *Excess of Dormancy* (Dormancy Persistence)

Sunflower Sunflower is a good example of a crop species with prolonged dormancy. Dormancy

persistence in different sunflower genotypes can vary from several weeks up to almost a year. Also, important variation has been reported on this trait for the same genotype under different environments, indicating a strong interaction with the environment. Sunflower is cultivated in many areas around the globe, and is a summer crop. At harvest time, sunflower seeds are dormant and germinate poorly [17–19]. This dormancy is the result of true embryo dormancy [17, 18] and the inhibitory action of the envelopes [17, 18, 20] including the seed coat and the pericarp, since sunflower "seeds" are achenes. In the case of freshly harvested sunflower seeds, dormancy is expressed at temperatures lower and higher than 25° C [17]. Dormancy expression at low temperatures is attributed to embryo dormancy which is not expressed at high temperatures [17]; conversely, dormancy expressed at high temperatures results from coat-imposed dormancy [17]. The deep dormancy that sunflower grains present at harvest results from the coexistence of coat-imposed dormancy and some remnant embryo dormancy [17]. Embryo dormancy is lost shortly after harvest if the seed is subjected to dry after-ripening, but coat-imposed dormancy persists for longer and may require several weeks of dry after-ripening to be overcome. Consequently, few weeks of dry after-ripening allow seed germination at low temperatures due to termination of embryo dormancy; the acquisition of the capacity to germinate at high temperatures, in contrast, may take several weeks of dry after-ripening [17].

The plant growth regulator ABA appears to be involved in the imposition of embryo dormancy. The inclusion of fluridone (an inhibitor of ABA biosynthesis) in culture media for sunflower embryo development prevents the induction of embryo dormancy [21, 22]. Nevertheless, the pattern of accumulation of ABA in the developing embryo does not coincide with the embryo physiological behavior: during seed development, embryos germinate well at the time when the endogenous ABA level is at its highest (7–12 DAP); thereafter, ABA decreases to a low value when embryo dormancy becomes established [21]. It seems, then, that the ABA peak at early stages is responsible for the imposition of the dormant state that is established immediately after that peak has taken place. Moreover, it appears that ABA needs to be present during a critical time period to induce dormancy. Le Page-Degivry and Garello [22] showed that when young (7 DAP), non-dormant embryos were cultured in the presence of ABA, the hormone produced a temporary inhibition of germination but did not induce dormancy (i.e., embryos were able to germinate when transferred to a basal medium). In contrast, exogenous ABA became effective if applied immediately prior to the natural induction of dormancy. For example, 5 days culture on a medium containing 5×10^{-5} M ABA resulted in partial dormancy in 13 DAP embryos while total induction of dormancy occurred in 17 DAP embryos.

As mentioned before, embryo dormancy can be terminated by dry storage. To identify the process by which dormancy is broken during after-ripening, Oracz et al. [23] focused on the role of reactive oxygen species (ROS) in this phenomenon. After-ripening entailed a progressive accumulation of ROS, namely, superoxide anions and hydrogen peroxide, in cells of embryonic axes. This accumulation, which was investigated at the cellular level by electron microscopy, occurred concomitantly with lipid peroxidation and oxidation (carbonylation) of specific embryo proteins. Incubation of dormant seeds for 3 h in the presence of hydrogen cyanide (a compound that breaks dormancy) or methyl viologen (a ROS-generating compound) also released dormancy and caused the oxidation of a specific set of embryo proteins. In summary, the mechanism proposed by the authors involves ROS production and targeted changes in protein carbonylation patterns [23].

As with other cultivated species such as *Lactuca sativa* [24] and *Arachis hypogea* [25], ethylene (C_2H_4) and etephon strongly stimulate the germination of dormant sunflower seeds [17, 18, 26]. In contrast, gibberellic acid and cold stratification do not overcome dormancy in this species [27] though it was shown that 1 mM GA_3 is effective for overcoming dormancy in some wild sunflowers [28]. Corbineau, Bagniol, and Côme [17] showed that ethylene and its immediate precursor (1-aminocyclopropane-1-carboxylic acid) strongly stimulated germination of primary dormant sunflower seeds; on the contrary, inhibitors of ethylene (i.e., amino-oxyacetic acid and $CoCl_2$) or ethylene action (silver thiosulfate and 2.5 norbomadiene) inhibited germination of nondormant seeds.

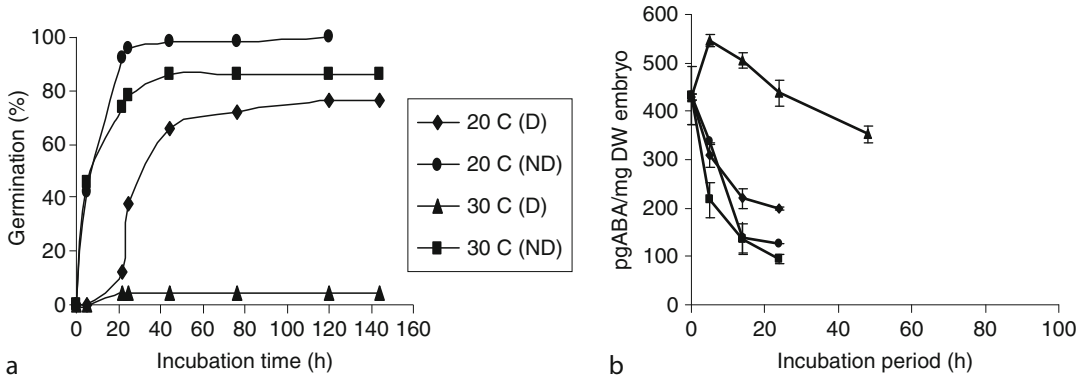
Oracz et al. [29] assessed the possible role of cyanide, which is produced by the conversion of 1-aminocyclopropane 1-carboxylic acid to ethylene, in dormancy release. The beneficial HCN effect on germination of dormant embryos was found to be associated with a marked increase in hydrogen peroxide and superoxide anion generation in the embryonic axes [29].

Malting Barley In addition to impairing a rapid and simultaneous germination after sowing, a persistent dormancy would prevent the utilization of a seed lot for industrial purposes when the process requires germination. This is the case of the malting process which uses barley as the main grain. The malting process itself requires grain germination, so a low dormancy level at harvest is a desirable characteristic because the grain can be malted immediately after crop harvest, thus avoiding costs and deterioration resulting from grain storage until dormancy is terminated. Therefore, breeders have to solve the compromise between obtaining genotypes with low dormancy at harvest, but not with such an anticipated termination of dormancy that leads to sprouting risks (a phenomenon which is referred to in the next section). Dormancy of the barley grain is typically imposed by the seed-covering structures (lemma and palea, pericarp plus seed coat). Indeed, embryos can germinate well from the very early stages of development if they are isolated from the rest of the grain and incubated in water [30]. Limitation of oxygen supply to the embryo by oxygen fixation as a result of oxidation of phenolic compounds in the lemma and palea (hereafter referred to as the glumellae or the hull) has been suggested to be responsible for the dormancy of dressed caryopses of cereals such as barley [31] and oat [32]. In dormant grains of barley, for example, whole intact caryopses germinated with difficulty, even in the air, while de-hulled caryopses were all able to germinate under oxygen tensions of at least 10%, suggesting that oxygen concentration under the covering structures might be less than 10% [31].

Dormancy of the barley grain also appears to be under ABA control: termination of glumellae-imposed dormancy during grain development was shown to be correlated with a sharp decline both in ABA embryonic content and sensitivity [30]. A role for ABA in dormancy maintenance of the barley grain has also been suggested: ABA embryonic content declines during the first hours of incubation of nondormant seeds, whereas it remains at high levels in embryos of dormant grains [33]. In recent years, Benech-Arnold et al. [34] confirmed this role of ABA in dormancy maintenance. The ABA level of the embryo largely decreased during the first hours of incubation and prior to any visible germination in both dormant and nondormant grains at 20° C, the temperature at which dormancy is not expressed, and in nondormant grains incubated at 30° C, suggesting that ABA catabolism and/or conjugation exceeds ABA biosynthesis. By contrast, at 30° C dormancy expression was associated with a maintenance of ABA at high levels: after an initial increase, ABA content decreased very smoothly and was always between two- and fourfold higher than in embryos from grains in which dormancy was not expressed (Fig. 1).

Both the removal of the glumellae and the incubation of de-hulled grains under low oxygen concentrations modified grain germination behavior and ABA content evolution throughout incubation at 30° C [34]. These results indicate that the presence of the glumellae is instrumental for dormancy maintenance because it imposes oxygen deprivation to the embryo which, in turn, promotes ABA synthesis and/or inhibits ABA inactivation. Indeed, removal of the glumellae in dormant grains incubated at 30° C under 21% oxygen suppressed the initial increase in ABA content that was observed in dressed dormant grains incubated at the same temperature. By contrast, incubation under hypoxia (5% oxygen) of de-hulled grains restored it and inhibited germination [34].

Artificially imposed hypoxia also enhanced embryo sensitivity to ABA by several fold [34]. These results suggest that, in addition to interference with ABA metabolism, the presence of the glumellae increases embryo responsiveness to the phytohormone. To explore this possibility, Mendiondo et al. [35] measured the expression of several components of the ABA signaling pathway during incubation of entire and de-hulled grains: the presence of the glumellae enhanced the expression of most of the investigated genes, suggesting that the hull increases the sensitivity to ABA of the enclosed embryos and that this enhancement is effected at the level of gene expression. Again, artificially imposed hypoxia was not able to mimic the effect of the glumellae on the



Seed Dormancy and Agriculture, Physiology. Figure 1
Germination (**a**) and embryo ABA content evolution (**b**) during incubation at 20° C (circles, diamonds) and 30° C (triangles, squares) of dormant (circles, triangles) and nondormant (diamonds, squares) grains. Freshly harvested (dormant, *D*) grains and grains stored dry for at least 3 months at 25° C (nondormant, *ND*). Means of two measurements ± arithmetical spread (germination) and of three measurements ± SD (ABA content) (Redrawn with data originally published in [34])

expression of ABA signaling components, indicating that the apparent enhancement of embryo sensitivity to ABA under low oxygen tensions was in fact due to over-accumulation of ABA as a result of malfunctioning of the enzyme committed to ABA inactivation [34].

Problems Associated with *the Lack of Dormancy* (Preharvest Sprouting in Cereals)

The advantages of having a freshly harvested seed lot with the capacity to germinate rapidly and uniformly under a wide range of environmental conditions are related to the possibility of immediate sowing or malting, thus avoiding financial costs derived from delays and/or storage until germination capacity is good enough. Nevertheless, genotypes that produce nondormant seeds at harvest may already be able to germinate to some degree even before harvest. The main problem related to an early loss of dormancy in crop species is preharvest sprouting (PHS). This phenomenon is characteristic of cereal species like rice, barley, wheat, and sorghum. As these species all exhibit intraspecific variability for the rate of dormancy loss and PHS behavior, genotypes with contrasting sprouting behavior have proved useful for many comparative studies [36–39], in addition to QTL analysis that leads to the identification of several loci related to dormancy [40–43]. When low levels of dormancy during late grain maturation period are combined with rainy or damp conditions in the field, the process of germination is activated while the seeds are still attached to the mother plant, and the resulting emergence of the radicle from the seed coats is called preharvest sprouting (PHS). Depending on the intended purpose for the seeds after harvest, PHS can have serious negative consequences on their quality and this is economically penalized by the industry. Direct economic losses caused by PHS to producers occur in several ways: Desiccation of a sprouted grain leads to its subsequent loss of viability because, together with the activation of metabolism implicated in embryo growth, tolerance to dehydration is lost rendering the sprouted grains useless for sowing or malting. Sprouting also promotes carbohydrate respiration that not only reduces grain yield, but also creates a favorable environment for the attack of saprophytic fungi and bacteria that produce toxins.

Depending on the level of dormancy and environmental cues such as water availability for imbibition and temperature, the germination process can advance to different extents, and not always reaches completion of germination and sprouting (i.e., germination is complete when embryo growth begins, and post-germinative growth leads to visible radicle and/or coleoptile emergence through the seed-covering structures). Even when desiccation occurs before germination is complete, starch degradation may have advanced partially.

This phenomenon is known as pregermination. Pregerminated grains cannot be distinguished visually, but the level of starch degradation is correlated with a decrease in FN values [36] and a reduction in seed lot longevity [44]. Industrial processes based on wheat and barley (flour and malting) are particularly sensitive to sprouting and pregermination. Seed lots are commonly assessed with the falling number (FN) test, with smaller FN values indicating a greater degree of starch degradation. Wheat and barley seed lots can be penalized or even rejected if FN values show moderate incidence of pregermination and sprouting.

The phenomena of PHS and pregermination are closely related with the process of domestication. Cereal crops have a brief period of dormancy as compared to their wild ancestors. Throughout many years, farmers have pressed toward selection of low levels of dormancy along with other traits such as non-shattering, increased grain size, and less pigmentation. The occurrence of PHS depends not only on morphological and physiological traits genetically controlled (such as infructescence structure and permeability of structures surrounding the seeds, and seed dormancy) but also on environmental factors (water availability and temperature). A single genotype may express PHS when grown in some areas but not in others. Seed dormancy is the main heritable factor that contributes to PHS resistance, but the many attempts to control it through breeding programs have shown that dormancy is tightly linked to other interesting traits. Breeding programs that attempted to separate characters associated with seed color, dormancy, and longevity suggest that these characteristics may not always be separable and are referred to as domestication block [45]. For example, in rice, both loci sh-h (for shattering) and Rc (conferring red pericarp) are tightly linked together with a QTL qSDs-7-1 for seed dormancy, implying that this region might represent a domestication block in the evolutionary pathway of rice [46]. A mutation in the *R*-gene in white wheat is required for low tannin content (higher flour quality) but is also responsible for lower levels of dormancy (low PHS resistance). However, grain dormancy is a complex trait that relies on numerous genes, and other QTLs contributing to dormancy that are not linked to seed color have been identified and are being considered in breeding for PHS tolerance in white wheat [47].

In addition to classical breeding techniques, elucidation of the mechanisms involved in the control of dormancy may open other possibilities for manipulation of dormancy through genetic engineering techniques. Cereal species like barley and sorghum have served as model systems to study the mechanisms behind dormancy with the objective to understand the hormonal metabolic and signaling steps involved in the repression of germination (*Barley*: 31, 30, 34, 48; *Sorghum*: 37, 38, 39).

Dormancy in Natural Seed Banks and Its Implications in Weed Control

Dormancy is a common attribute of many weed species, and is probably the most important of a series of processes that determine the seasonal annual pattern of weed emergence from soil seed banks under field conditions [49, 50]. Although there are many environmental factors that can be affecting the dormancy status of seeds composing the soil bank (i.e., alternating temperature, soil water status, light, etc.), seasonal dormancy changes are mainly regulated by soil temperature [4, 51]. For example, in summer annual species, dormancy relief is produced by the low temperatures experienced during winter, while high temperatures enhance their dormancy level during summer. Most winter annual species show the reverse dormancy pattern. Hence, high temperatures during summer produce dormancy relief, while low temperatures during winter induce secondary dormancy. Although much experimental data support the main role of soil temperature as regulator of seed dormancy, there is evidence indicating that the effect of temperature on dormancy may be modulated by soil moisture conditions [16, 52].

Changes in dormancy status of weed seed populations are associated with changes in the range of temperatures and water potentials permissive for seed germination [15]. As dormancy is relieved, the range of temperatures and water potentials permissive for germination widens until it is maximal; on the contrary, as dormancy is induced, the range of temperatures and water potentials over which germination can proceed narrows until germination is no longer possible at any temperature or water potential. Germination in the field is therefore restricted to the period when the field temperature and soil water potential,

and the temperature and water potential range over which germination can proceed, overlap [14, 53].

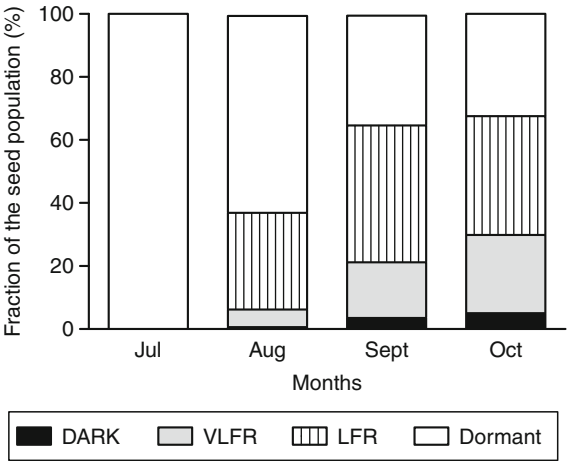
However, in many weed species, once environmental temperature and water potential are within the permissive range, dormancy must be terminated by the effect of additional environmental factors for germination to proceed. In these cases, changes in the degree of dormancy not only comprise changes in temperature and water potential requirements for germination, but also in the sensitivity of the seed population to the effect of those dormancy terminating factors [51]. Fluctuating temperatures and light are two critical environmental factors that can trigger dormancy termination in seeds of many weed species. An ecological interpretation of this requirement to complete exit from dormancy has been related to the possibility of detecting canopy gaps as well as depth of burial under field situations [51, 54, 55].

Agronomic practices can alter the physical environment to which weed seed banks are exposed, and thus, their dormancy status. Knowledge about the ways in which agronomic practices affect the dormancy status of weed seeds could be used to develop and improve weed management strategies [56, 57]. For example, many weed seeds require light to terminate dormancy and give way to the germination process; consequently, the light environment could be managed in order to impede seed germination. Light signals are perceived by seeds through the phytochrome system. Generally, low red/far-red wavelengths ratios inhibit seed germination, while high red/far-red ratios promote seed germination. Light filtered by green leaves are rich in far-red wavelengths and explains the low red/far-red ratios measured under plant canopies [58]. Therefore, plant cover could be managed to reduce some weed problems. For example, changing plant architecture, crop-sowing densities, and crop plant spacing may have a high potential for improving weed management by preventing the exit of weed seeds from dormancy and reducing germination under field situations [59].

The brief light pulse received during soil cultivation can promote seed germination of buried weed seeds. Many weed seeds buried in the soil acquire an extremely high light sensitivity that permits them to detect submilliseconds of sunlight when the soil is disturbed. This is reflected in the high weed emergence rates usually observed following tillage operations, and suggests that germination of light-requiring seeds would be impeded if a non-tillage crop production system is implemented or if cultivation is performed at night [60]. Indeed, Scopel et al. [61] observed a significant reduction in weed emergence (between 70% and 400%) when plots were cultivated at night in comparison to emergence levels obtained under daytime cultivation.

Other environmental factor that usually terminates dormancy in many weed seeds under field conditions is temperature fluctuation. Generally, large temperature fluctuation regimes determine dormancy breakage of a higher proportion of the seed bank population than that observed under small temperature fluctuation regimes. Thus, changing the amplitude of temperature fluctuation regimes in the field environment through management of plant cover or crop residues would lead to a reduction in weed emergence [57, 59]. This is the case of Johnson grass (*Sorghum halepense*), in which seeds do not germinate if they are under a dense plant cover due to the lack of temperature fluctuations stimuli required to terminate seed dormancy [54].

Weed emergence models that predict the proportion and timing of seed bank emergence would be useful tools for determining the most suitable time for seedling control and, consequently, should result in a higher efficacy of controls methods. In the last decade, many models able to predict seed dormancy changes in relation to environmental factors for different weed species have been developed [14, 15]. For example, Batlla et al. [62] and Batlla and Benech-Arnold [63] developed models to account for the effect of fluctuating temperatures and light on *Polygonum aviculare* (a worldwide distributed spring emerging weed) seed dormancy level based on a stratification thermal time index (S_{tt}). This index allow the prediction of changes in seeds sensitivity to increasing doses of cycles of fluctuating temperatures or different light treatments for seeds stratified at different temperatures in relation to the accumulation of degree days units below a ceiling temperature of 17° C. Using these models, for example, the progressive increase in *P. aviculare* seed bank sensitivity to light during winter and early spring can be predicted based on soil temperature records (Fig. 2). Due to the fact that, for example, the acquisition of a very low fluence response (VLFR) phytochrome action mode would permit buried seeds to germinate



Seed Dormancy and Agriculture, Physiology. Figure 2 Simulated proportions of a *Polygonum aviculare* seed bank showing different action modes of phytochrome (low fluence response [*LFR*] and very low fluence response [*VLF*]), seeds not requiring light for germination (darkness) and the non-germinating fraction (dormant) at the beginning of different months. Simulations were done using year 2000 soil temperature and equations published in [63] (Redrawn from [14])

in response to the light flash perceived during soil disturbance [64], this model could be used to predict the proportion of the seed bank that will germinate in response to tillage operations after the accumulation of a certain amount of S_{tt} during winter burial [63].

Another example of how knowledge in relation to regulation of seed bank dormancy by environmental factors could be used to forecast weed emergence is the WeedCast model developed by Forcella [65] at the US Department of Agriculture. This model can be used to predict the "emergence potential" of several summer annual weeds. In the model, the "emergence potential" indicates which fraction of the weed seed bank would be nondormant and available for germination at a given time during the season. The possibility of predicting the "emergence potential" of the different weed species allows farmers to make more rationale decisions regarding the degree and type of weed management required [65].

With increased pressure to reduce pesticide inputs in agricultural systems, optimal timing and rates of chemical products as well as finding sustainable nonchemical options for weed control will be of paramount importance. A better understanding of how environmental factors and agronomic practices affect the dormancy status of weed seed banks could be used to develop and improve weed control strategies in order to meet this challenge.

Future Directions

Seed dormancy has enormous implications in agriculture. Its importance is related both to its persistence and to its lack. Dormancy persistence affects crop establishment as well as grain industrialization whenever this process requires seed germination (i.e., malting), but also the design of weed management practices. The lack of dormancy can lead to untimely germination in the mother plant (i.e., preharvest sprouting) with severe consequences to grain quality. The possibility of assessing the very many aspects of the agricultural activity that could be interfered by the presence or absence of dormancy requires a thorough knowledge of the phenomenon, not only at a fundamental level (i.e., physiological and/or molecular) but also in terms of its control by environmental factors. In this entry, the attempt was to illustrate with examples the way in which these different aspects can be assessed. Only a detailed knowledge of how the dormancy mechanisms are controlled at a physiological, molecular, and environmental level would eventually lead to attain our ultimate aim: to adjust the timing of dormancy release to our convenience from an agricultural point of view.

Bibliography

1. Baskin CC, Baskin JM (1998) Seeds: ecology biogeography and evolution of dormancy and germination. Academic, San Diego
2. Nikolaeva MG (1967) Physiology of deep dormancy in seeds. Leningrad, Russia: Izdatel'stvo 'Nauka' (in Russian). [Trans from Shapiro Z (1969), National Science Foundation, Washington, DC, p 219]
3. Baskin JM, Baskin CC (2004) A classification system for dormancy. Seed Sci Res 14:1–16
4. Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. New Phytol 171:501–523
5. Hilhorst HWM (1995) A critical update on seed dormancy. I. Primary dormancy. Seed Sci Res 5:61–73
6. Benech-Arnold RL (2004) Inception, maintenance and termination of dormancy in grain crops. Physiology, genetics and environmental control. In: Benech-Arnold R, Sánchez RA (eds) Handbook of seed physiology: applications to agriculture. Food Product Press, New York, pp 169–198

7. Walker-Simmons MK, Sesing J (1990) Temperature effects on embryonic abscisic acid levels during development of wheat grain dormancy. J Plant Growth Regul 9:51–56
8. Fenner M (1991) The effects of the parent environment on seed germinability. Seed Sci Res 1:75–84
9. Benech-Arnold RL, Fenner M, Edwards PJ (1991) Changes in germinability, ABA levels and ABA embryonic sensitivity in developing seeds of *Sorghum bicolor* induced by water stress during grain filling. New Phytol 118:339–347
10. Benech-Arnold RL, Fenner M, Edwards PJ (1995) Influence of potassium nutrition on germinability, ABA content and embryonic sensitivity to ABA of developing seeds of *Sorghum bicolor* (L.) Moench. New Phytol 130:207–216
11. Gate P (1995) Ecophysiologie de la germination sur pied. Perspect Agri 204:22–29
12. Fenner M, Thompson K (2005) The ecology of seeds. Cambridge University Press, Cambridge, pp 250
13. Allen PS, Benech-Arnold RL, Batlla D, Bradford KJ (2007) Modeling of seed dormancy. In: Bradford K, Nonogaki H (eds) Seed development, dormancy and germination, vol 27. Blackwell, Oxford, pp 72–112
14. Batlla D, Benech-Arnold RL (2007) Predicting changes in dormancy level in weed seed soil banks: implications for weed management. Crop Prot 26:189–197
15. Batlla D, Benech-Arnold RL (2010) Predicting changes in dormancy level in natural seed soil banks. Plant Mol Biol 73:3–13
16. Batlla D, Benech-Arnold RL (2006) The role of fluctuations in soil water content on the regulation of dormancy changes in buried seeds of *Polygonum aviculare* L. Seed Sci Res 16:47–59
17. Corbineau F, Bagniol S, Côme D (1990) Sunflower (*Helianthus annuus* L.) seed dormancy and its regulation by ethylene. Israel J Bot 39:313–325
18. Corbineau F, Côme D (1987) Regulation de las semences de tournesol par l'éthylene. In: Annales ANPP (ed) 2ème Colloque sur les substances de croissance et leurs utilisations en agriculture, vol 1. Association Nationale de Protection des Plantes, Paris, pp 271–282
19. Cseresnyes Z (1979) Studies on the duration of dormancy and methods of determining the germination of dormant seeds of *Helianthus annuus*. Seed Sci Technol 7:179–188
20. Corbineau F (1987) La germination des semences de tournesol et sa regulation par l´éthylene. CR Acad Sci Agr Fr 266:477–479
21. Le Page-Degivry MT, Barthe P, Garello G (1990) Involvement of endogenous abscisic acid in onset and release of *Helianthus annuus* embryo dormancy. Plant Physiol 92:1164–1168
22. Le Page-Degivry MT, Garello G (1992) In situ abscisic acid synthesis. A requirement for induction of embryo dormancy in *Helianthus annuus*. Plant Physiol 98:1386–1390
23. Oracz K, El-Maarouf BH, Farrant JM, Cooper K, Belghazi M, Job C, Job D, Corbineau F, Bailly C (2007) ROS production and protein oxidation as a novel mechanism of seed dormancy alleviation. Plant J 50:452–465
24. Abeles FB (1986) Role of ethylene in *Lactuca sativa* cv. Grand Rapids seed germination. Plant Physiol 81:780–787
25. Ketring DL (1977) Ethylene and seed germination. In: Khan AA (ed) The physiology and biochemistry of seed dormancy and germination. Elsevier/North Holland Biomedical Press, Amsterdam, pp 157–178
26. Srivastava AK, Dey SC (1982) Physiology of seed dormancy in sunflower. Acta Agron Hung 31:70–80
27. Bagniol S (1987) Mise en évidence de l´intervention de l'ethylene dans la germination et la dormance des semences de tournesol (*Helianthus annuus* L.). Diplôme d'Ëtudes Approfondies, Université Pierre et Marie Curie, Paris
28. Seiler GJ (1998) Seed maturity, storage time and temperature, and media treatment effects on germination of two wild sunflowers. Agron J 90:221–226
29. Oracz K, El Maarouf-Bouteau H, Bogatek R, Corbineau F, Bailly C (2008) Release of sunflower seed dormancy by cyanide: crosstalk with ethylene signaling pathway. J Exp Bot 59:2241–2251
30. Benech-Arnold RL, Giallorenzi MC, Frank J, Rodriguez V (1999) Termination of hull-imposed dormancy in barley is correlated with changes in embryonic ABA content and sensitivity. Seed Sci Res 9:39–47
31. Lenoir C, Corbineau F, Come D (1986) Barley (*Hordeum vulgare*) seed dormancy as related to glumella characteristics. Physiol Plant 68:301–307
32. Corbineau F, Poljakoff-Mayber A, Côme D (1991) Responsiveness to abscisic acid of embryos of dormant oat (*Avena sativa*) seeds. Involvement of ABA-inducible proteins. Physiol Plant 83:1–6
33. Wang M, van der Meulen RM, Visser K, Van Schaik H-P, Van Duijn B, de Boer AH (1998) Effects of dormancy-breaking chemicals on ABA levels in barley grain embryos. Seed Sci Res 8:129–137
34. Benech-Arnold RL, Gualano NA, Leymarie J, Come D, Corbineau F (2006) Hypoxia interferes with ABA metabolism and increases ABA sensitivity in embryos of dormant barley grains. J Exp Bot 57:1423–1430
35. Mendiondo GM, Leymarie J, Farrant J, Corbineau F, Benech-Arnold RL (2010) Differential expression of abscisic acid metabolism and signaling genes induced by seed covering structures or hypoxia in barley (*Hordeum vulgare* L.) grains. Seed Sci Res 20:69–77
36. Biddulph TB, Plummer JA, Setter TL, Mares DJ (2008) Seasonal conditions influence dormancy and preharvest sprouting tolerance of wheat (*Triticum aestivum* L.) in the field. Field Crop Res 107:116–128
37. Steinbach HS, Benech-Arnold RL, Kristof G, Sánchez RA, Marcucci-Poltri S (1995) Physiological basis of pre-harvest sprouting resistance in *Sorghum bicolor* (L.) Moench. ABA levels and sensitivity in developing embryos of sprouting-resistant and sprouting-susceptible varieties. J Exp Bot 46:701–709
38. Steinbach HS, Benech-Arnold RL, Sánchez RA (1997) Hormonal regulation of dormancy in developing sorghum seeds. Plant Physiol 113:149–154

39. Rodríguez MV, Mendiondo GM, Maskin L, Gudesblat GE, Iusem ND, Benech-Arnold RL (2009) Expression of ABA signalling genes and ABI5 protein levels in imbibed *Sorghum bicolor* caryopses with contrasting dormancy and at different developmental stages. Ann Bot 104:975–985
40. Gao FY, Ren GJ, Lu XJ, Sun SX, Li HJ, Gao YM, Luo H, Yan WG, Zhang YZ (2008) QTL analysis for resistance to preharvest sprouting in rice (*Oryza sativa*). Plant Breed 127:268–273
41. Kumar A, Kumar J, Singh E, Garg T, Chhuneja P, Balyan HS, Gupta PK (2009) QTL analysis for grain colour and pre-harvest sprouting in bread wheat. Plant Sci 177:114–122
42. Lohwasser U, Roder MS, Borner A (2005) QTL mapping of the domestication traits pre-harvest sprouting and dormancy in wheat (*Triticum aestivum* L.). Euphytica 143:247–249
43. Zanetti S, Winzeler M, Keller M, Keller B, Messmer M (2000) Genetic analysis of pre-harvest sprouting resistance in a wheat x spelt cross. Crop Sci 40:1406–1417
44. Del Fueyo P, Sánchez RA, Benech-Arnold RL (2003) Seed longevity in two sorghum varieties with contrasting dormancy level prior to harvest. Seed Sci Technol 31:639–650
45. Finkelstein RR, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. Annu Rev Plant Biol 59:387–415
46. Ji HS, Chu SH, Jiang W, Cho YI, Hahn JH, Eun MY, McCouch SR, Koh HJ (2006) Characterization and mapping of a shattering mutant in rice that corresponds to a block of domestication genes. Genetics 173:995–1005
47. Mares DJ, Mrva K, Cheong J et al (2005) A QTL located on chromosome 4A associated with dormancy in white- and red-grained wheats of diverse origin. Theor Appl Genet 111:1357–1364
48. Barrero JM, Talbot MJ, White RG, Jacobsen JV, Gubler F (2009) Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. Plant Physiol 150:1006–1021
49. Buhler DD, Hartzler RG, Forcella F (1997) Implications of weed seedbank dynamics to weed management. Weed Sci 45:329–336
50. Forcella F, Benech-Arnold RL, Sánchez RA, Ghersa CM (2000) Modelling seedling emergence. Field Crop Res 67:123–139
51. Benech-Arnold RL, Sánchez RA, Forcella F, Kruk BC, Ghersa CM (2000) Environmental control of dormancy in weed seed banks in soil. Field Crop Res 67:105–122
52. Bair NB, Meyer SE, Allen PS (2006) A hydrothermal after-ripening time model for seed dormancy loss in *Bromus tectorum* L. Seed Sci Res 16:17–28
53. Karssen CM (1982) Seasonal patterns of dormancy in weed seeds. In: Khan AA (ed) The physiology and biochemistry of seed development, dormancy and germination. Elsevier, Amsterdam, pp 243–270
54. Benech-Arnold R, Ghersa C, Sánchez R, García Fernandez A (1988) The role of fluctuating temperatures in the germination and establishment of *Sorghum halepense* (L.) Pers. Regulation of germination under leaf canopies. Funct Ecol 2:311–318
55. Ghersa CM, Benech-Arnold RL, Martinez Ghersa MA (1992) The role of fluctuating temperatures in germination and establishment of *Sorghum halepense* (L.) Pers. II. Regulation of germination at increasing depths. Funct Ecol 6:460–468
56. Dyer WE (1995) Exploiting weed seed dormancy and germination requirements through agronomic practices. Weed Sci 43:498–503
57. Ghersa CM, Martinez-Ghersa MA, Benech-Arnold RL (1997) The use of seed dormancy to improve grain production. J Prod Agric 10:111–117
58. Smith H (1982) Light quality, photoperception, and plant strategy. Annu Rev Plant Phys 33:481–518
59. Kruk B, Insausti P, Razul A, Benech-Arnold RL (2006) Light and thermal environments as modified by a wheat crop: effects on weed seed germination. J Appl Ecol 43:227–236
60. Juroszek P, Gerhards R (2004) Photocontrol of weeds. J Agron Crop Sci 190:402–415
61. Scopel AL, Ballaré CL, Sánchez RA (1991) Induction of extreme light sensitivity in buried weed seeds and its role in the perception of soil cultivations. Plant Cell Environ 14:501–508
62. Batlla D, Verges V, Benech-Arnold RL (2003) A quantitative analysis of seed responses to cycle-doses of fluctuating temperatures in relation to dormancy level. Development of a thermal-time model for *Polygonum aviculare* L. seeds. Seed Sci Res 13:197–207
63. Batlla D, Benech-Arnold RL (2005) Changes in the light sensitivity of buried *Polygonum aviculare* seeds in relation to cold-induced dormancy loss: development of a predictive model. New Phytol 165:445–452
64. Casal JJ, Sánchez RA (1998) Phytochromes and seed germination. Seed Sci Res 8:317–329
65. Forcella F (1998) Real-time assessment of seed dormancy and seedling growth for weed management. Seed Sci Res 8:201–209

Shellfish Aquaculture, Methods of Sustainable

Francis X. O'Beirn[1], Christopher W. McKindsey[2], Thomas Landry[3], Barry A. Costa-Pierce[4]
[1]Marine Institute, Galway, Ireland
[2]Ocean and Environmental Sciences Division, Fisheries and Oceans Canada, Institut Maurice-Lamontagne Fisheries and Oceans Canada, Mont-Joli, Québec, Canada
[3]Fisheries and Oceans Canada, Gulf Fisheries Centre, Moncton, NB, Canada
[4]University of New England, Marine Science Center, Biddeford, ME, USA

Article Outline

Glossary

Azoic conditions Conditions that prevail when no organisms or their remains are found in a system as a consequence of stress on a system.

Biodeposition Organic matter deriving from (shellfish) species that falls to the seafloor. The matter can take the form of feces, pseudofeces, or the shellfish themselves.

Far-field effects The effects of impacts of activities measured at a predefined distance or time from the location of the pressure. The effects may or may not be distinguishable from other effects in the system leading to cumulative impacts.

Ecological aquaculture The implementation of aquaculture practices whose design and implementation result in economically viable and socially responsible aquaculture systems.

Integrated coastal zone management (ICZM) The management of activities in marine environments in a coherent and practical fashion so as to result in the most efficient use of resources and avoid conflicting claims on space and missed opportunities for more sustainable coastal development.

Performance standards Defined expectations represented by some measurable variable which reflects the impact an activity will have on the marine environment.

Best management practices A series of operating procedures, schedules of activities, and other management practices that aquaculture operations can use to prevent or reduce impact on the marine environment while retaining an economically viable operation.

Definition of the Subject

Worldwide aquaculture production of finfish and shellfish species is an ever-increasing sector of food production and now represents nearly half (48%) of all aquatic species intended for human consumption [1]. It is thought that the increased aquaculture production is driven primarily by the vacuum created as a consequence of the static (or declining) status of wild capture fisheries allied to an overall greater demand for fishfood products [1]. This generalization is broadly accepted as the primary driver for increased aquaculture production; however, more specific drivers may be, increased profit as a consequence of targeted marketing allied with development of new species in developing countries and as a means of providing more self-sufficient mechanisms to grow fishfood and provide a regular income in developing countries. Typically, aquaculture production has been dominated by the culture of finfish species; however, shellfish production has shown a steady increase in production over the last number of decades [1] and represents 27% by weight and 15% by value of worldwide aquaculture production. The culture of shellfish in aquaculture is comprised primarily of species of crustaceans (e.g., shrimp and crab) and molluscs (e.g., oysters, clams, mussels). A major distinction

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

between the crustaceans and molluscs is that crustaceans, as omnivores, typically require the input into their culture system of feed, usually derived from external sources (e.g., fish protein or oils). Consequently, issues surrounding the sustainability of crustacean culture as activities are more akin to those encountered with finfish aquaculture. Molluscs, particularly those identified above, are filter feeders. Filter-feeding organisms, for the most part, feed at the lowest trophic level, usually relying primarily on ingestion of phytoplankton. The process in extractive in that it does not rely on the input of feedstuffs in order to produce growth.

The steady increase in world aquaculture production over the last number of decades allied with greater environmental awareness has resulted in an increased level of scrutiny of these activities in relation to their interactions with the environment. The increased focus on environmental issues in the marine environment (driven by consumer demands and/or regulatory requirements) has resulted in a concomitant increase in efforts to identify methods to culture shellfish that are considered sustainable. Consequently, there is a move toward rearing aquatic shellfish species in the marine environment such that negative interactions are minimized. In addition, it has also become apparent that there are considerable marketing benefits that will accrue as a consequence of food being reared in an environmentally acceptable fashion. These efforts (to identify and activate more sustainable method of culture) have been driven both by industry and environmental nongovernmental organisations (e.g., Pacific Shellfish Growers Association and World Wildlife Fund) as well as regulatory drivers (e.g., Natura 2000 Legislation in the European Union). Notwithstanding the efforts to carry out shellfish aquaculture activities in a more sustainable fashion, there is still some confusion relating to the definition of sustainability and how an activity might be carried out in a sustainable fashion. At first, identifying if an activity and its consequences in the marine environment is acceptable or not will help define whether the activity is sustainable. Clarifying or clearing defining some of the terms utilized in this subject area will lend itself to a clearer and understandable of how the culture of shellfish can be managed to achieve sustainability goals.

Introduction

Worldwide aquaculture production has increased steadily since 1980 [1]. Production of aquatic products in 1980 accounted for 7% of food fish supply (five million tons), in 2007 this quantity had risen to 50 million tons. Based upon current trends, it is apparent that production of shellfish will continue to increase at a stable rate (6.5% per annum since 2002). This increase is fueled by an increase in market demand, adoption of more efficient and effective culture methods, and the financial rewards associated with the production of value-added products to higher end markets. Notwithstanding the important differences between finfish production and the majority of shellfish production methods, i.e., the introduction of feedstuffs into the environment, which has led to well-documented resource demands and impacts on the marine environment, shellfish aquaculture also has the potential to impact on the marine environment in a negative fashion if not carried out in a responsible manner. This is underpinned by a background of greater environmental awareness and increased legislative drivers toward maintaining biodiversity and minimizing negative interactions between a range of conservation goals and development activities. Some authors [2] correctly highlight that aquaculture cannot be considered as one single "monolith," which can be tarnished with the single level of criticism, as it reflects a diverse array of species, methods, and potential interactions. In addition to the differences highlighted above between those culture methods reliant on input of feedstuffs necessary for the culture of some species (finfish and crustaceans), the degree of structural input and environmental alteration is also highly varied among the different species cultured and the method employed. In short, this entry will identify some of the issues associated with sustainability of shellfish aquaculture and whether or not the efforts directed to date are considered to be on a trajectory toward sustainability [3].

Interactions of Shellfish Culture

Early incarnations of shellfish culture are considered not far removed from wild fisheries in that the activities were broadly extensive with only small-scale

manipulation of stocks (e.g., movement of wild seed to production areas; an activity still practiced today with on-bottom mussel and oyster culture). Over time, the industry has evolved with the development of more contained systems. With such intensification, the risk of detrimental environmental interactions increased, e.g., disease risks or greater deposition of organic matter beneath structures, as a consequence of higher holding densities of culture organisms. Notwithstanding density-related issues, in many instances, it is not the presence of the culture organisms that result in largely negative interactions but the activities associated with the culture mechanisms, e.g., dredging extensive culture systems, pesticides, or chemotheraputents in pond systems.

The culture of shellfish (bivalve molluscs) as distinct from finfish and crustaceans, for the most part, requires no input of feed to the culture process. Given this difference, this entry will focus primarily on the culture of molluscan shellfish (primarily bivalves). Under certain circumstances, e.g., hatchery production of shellfish, the input of feed in the form of phytoplankton is required to produce seed [4]. Thereafter, for the majority of their life cycle, shellfish consume at the lowest trophic level, feeding largely as herbivores and relying on ambient seston [5]. This distinction is important in that it highlights the fact the culture of shellfish, upon harvest, is considered a process that facilitates the net export of carbon (and other nutrients) from marine systems. While this may be considered an exploitation of a resource and detrimental to the system, there are situations where this is also inherently beneficial to the ecosystem. The impacts of shellfish culture have been well documented in research literature where specific interactions are described and quantified (Table 1). In addition, this topic has been a subject of numerous reviews highlighting similarities and differences inherent in culture methods and considers the factors governing any differences observed.

The extent of the interactions between shellfish culture and the environment are primarily a function of the type of species being cultured, the system of culture, and the properties of the receiving environment. Table 1 provides a summary of the interactions identified between shellfish culture practices and the environment. The interactions are summarised as the mechanism which acts on the system. For example, the dredging associated with the collection of mussel seed for aquaculture practices can have the effect of physically disturbing the seafloor and the organisms therein (the impact indicator). It has been demonstrated empirically that this activity can have an impact at the community level of marine ecosystems. The culture of shellfish species using structures presents a number of likely interactions. The use of structures, i.e., bags and trestles, longlines with droppers will increase the density of culture organisms above the seafloor, thus influencing the flux of material and nutrients to the seafloor and into suspension. This higher density can also modify water flow in and around the culture system. These and other more general interactions are discussed below.

Nutrients

Bivalve shellfish can function in the ecosystem by filtering seston and releasing nutrients in solid or dissolved forms. They are responsible for deposition onto the seafloor of particulate matter (as either feces or pseudofeces), thus influencing benthic-pelagic coupling of organic matter and nutrients. The deposition of organic matter by molluscs has been demonstrated to impact on the infauna organisms and communities found in sedimentary environments. The changes have broadly reflected the Pearson–Rosenberg [6] pattern of community development whereby moderate increases in organic matter stimulate species' richness and abundance. Further increases in organic matter could result in a reduction in species richness and abundance such that excesses result in azoic conditions as a consequence of protracted anoxic conditions. The effects of biodeposition are exacerbated by the increase in density of culture organisms above the seafloor. The use of bags on trestles (e.g., oyster culture) and longlines (with mussels) will increase the density of culture organisms over a particular point and increase the risk of impact due to biodeposition. While effects of organic loading have been demonstrated with shellfish culture activities, the impacts are considered relatively small when compared with other culture systems where externally derived organic matter, i.e., food, is inputted directly to the system, e.g., finfish culture. A number of factors mediate the level of impact on the seafloor.

Shellfish Aquaculture, Methods of Sustainable. Table 1 Interactions between shellfish culture methods and the environment identifying the interaction route and indicator

Culture type (species)	Interactions	Indicator	References
*Off-bottom – Suspended culture (*e.g.*, mussels, oysters) using longlines, rafts, floating bags*	Water flow alteration	Sediment particle size analysis (PSA) – increase in fine sediment composition due increased sediment deposition or increase in coarse sediment complement due to scouring	[7, 79–85, 163]
		Benthic infauna – adjustment in species composition and abundance; community composition	[86–89]
	Depostion of organic matter (feces and pseudofeces)	Increase in sulfide reduction, Decrease in REDOX depth; Sediment biogeochemistry changes	[84, 88, 90–93]
		Benthic infauna	[81, 86, 87, 94, 95]
	Shading	Condition of light-sensitive species (macroalgae, maerl, eel grass)	[96, 97]
	Habitat creation/fouling	Secondary production on culture organisms or structures. Increased nekton species	[22, 98–103]
	Seston filtration	Alteration of phytoplankton communities, impact on production/ecological carrying capacity; changes in zooplankton assemblages	[80, 104–107]
	Nutrient exchange	Ammonium, DIN – increased primary production, N2 removal via harvest or denitrification	[93, 108–111]
	Introduction of exotic species with culture organisms	Presence of non-endemic or exotic species	[22, 112, 151]
On-bottom (Mussels, oysters, clams)	Physical alteration, dredging, intertidal picking	Benthic infauna	[86, 114, 115]
	Monoculture	Epifuana community alteration: PSA alteration	[52, 86, 89, 91, 96, 115–118]
	Depostion of organic matter (feces and pseudofeces)	Increase sulfide reduction, Decrease in REDOX depth; sediment biogeochemistry changes	[119–126]
		Benthic infauna	[8, 127]
	Shading	Condition of light-sensitive species (macroalgae, maerl, eel grass)	[11]
	Habitat creation/fouling	Secondary production on culture organisms or structures	[78, 79, 89, 115, 127–129]
	Seston filtration	Alteration of phytoplankton communities, impact on production/ecological carrying capacity; increased light penetration	[11, 91, 106, 107, 109, 130–141]
	Nutrient exchange	Increased primary production, N_2 removal via harvest or denitrification; alteration of N:P ratios	[109, 110, 142–150]
	Introduction of exotic species with culture organisms	Presence of non-endemic of exotic species	[112, 113]

In addition to density of culture organisms, the hydrography of the system including residence time, tidal range, and residual flow will all dictate the likely influence the extent of an impact on the seafloor. The greater the residual flow and/or tidal regime, the risk of accumulation of organic material is reduced [7, 8] due to the dispersive regime. Similarly, the high density of structures can result in the impediment of water flow (baffling effect), slow it down, and cause localized deposition of suspended material on the seafloor. Depending on the extent of the structures, the effect can be localized or extensive [9] with concomitant impacts on sedimentary infauna.

Organic deposition by shellfish on the seabed can also influence the remineralization of nutrients in marine systems. This process as well as normal excretion (of ammonium NH_4^+) demonstrates that shellfish in both their natural state and in culture can influence nutrient dynamics in marine systems. In fact, in a coastal bay in France it has been estimated that between 15% and 40% of nitrogen in the system is derived from oysters in culture [10]. Notwithstanding the factors that govern extent of impacts on systems, molluscs are considered net consumers of particulate and dissolved nutrients, and, by virtue of the movement of product to market the nutrients are exported from the system. The area required to assimilate material is generally confined to the production area and as such the area required to assimilate material is less with culture than without.

Filtration

Bivalve shellfish (oysters and mussels) have a high filtration capacity and can respond rapidly to changes in phytoplankton abundance (as a result of eutrophication) in marine systems. In nearshore marine environments, the presence of large numbers of bivalve shellfish has provided the system with the ability to buffer the effects of large phytoplankton blooms. This phenomenon applies equally to shellfish in culture which likewise provides the system with resilience against natural or anthropogenically derived fluctuations in phytoplankton numbers (blooms) [11–14]. As a consequence of this phenomenon, the subsequent removal of natural bivalve populations from marine systems (e.g., by fishing) has resulted in well-documented ecological shifts in system processes. The dramatic reduction of oyster numbers in the Chesapeake Bay (USA) has coincided with a deterioration in water quality of the Bay; this situation was exacerbated by increased athropogenic pressures [15–17]. Shellfish in culture while having demonstrable localized effects do also appear to have the ability to moderate the effects of nutrients more broadly in marine systems [9, 18].

Exotic Species

The importance of aquaculture as a vector for the introduction and spread of exotic species has been well documented [19–23]. There are two broad classes of introductions that may result from bivalve aquaculture.

First, there is the establishment and spread of non-endemic species that have been intentionally introduced into an area for aquaculture purposes, the "target" species. Classic examples of this include the establishment of the Pacific oyster (*Crassostrea gigas*) on the Pacific coast of North America [24] and in various countries throughout Europe [25–27] and of the Mediterranean mussel (*Mytilus galloprovincialis*) in South Africa [28]. More recently, the large expansion of *C. gigas* in the Oosterschelde and the Dutch and German Wadden Sea have been a cause of concern in both countries from a fisheries, ecological, and human health perspective. Wild populations of the Pacific oysters have expanded from 15 ha in 1980 to 750 ha in 2005 in the Oosterschelde [29]. They have become a competitor (for space and food) with the commercially important mussel industry [25, 30]. In addition, they have become an increasing health risk associated with human encounters given the sharp nature of the shell [31]. Efforts to remove wild Pacific oysters from the Oosterschelde in the Netherlands are ongoing (Aad Smaal, IMARES, NL personnel communication). It is important to note that in the Netherlands, Pacific oysters were first introduced by broadcast spreading in an uncontained fashion on the seabed under the assumption that the summer temperatures were such that they would not successfully complete gametogenesis, spawn, and most importantly recruit.

Second, there is the establishment and spread of species that are associated with the introduced bivalves [32, 33]. These species may include both "hitchhiking"

species – animals, plants that grow associated with the bivalves and diseases or parasites that may cause outbreaks in the same or other species [34]. This acts at two spatial scales: at an interregional or international scale with respect to the initial introduction of hitchhiking species and also at a regional scale, where the transfer of stock among sites may be very important to the spread of established exotic species locally [35]. The provision of novel habitat by the species being cultured may also allow for the establishment or amplification of exotic species that may be introduced through other vectors or of native species that thrive in the novel habitat [36–38].

Introductions of the *C. gigas*, and to a lesser extent *C. virginica* and other oyster species, outside of their native range for aquaculture have been suggested to be one of the greatest single modes of introduction of exotic species worldwide [19, 39]. For example, transfer of organisms with bivalves has been suggested to be the most important source of exotic species in northern Europe [17, 40] and among the most important vectors elsewhere in that continent [17, 41, 42]. In the northeast Pacific, some authors suggest that oyster (*C. gigas*) introductions have even been the major source of introduction of exotic molluscs [14] and invertebrates in general [43], historically contributing at least as many of the exotic species in that area as has international shipping.

The slipper limpet (*Crepidula fornicata*), originally introduced into England with *C. virginica*, has had great impacts on some benthic communities in Europe, particularly in France [44] and the UK [45]. It has displaced important commercial bivalves, such as the great scallop (*Pecten maximus*) in some areas and native oysters beds in Normandy [46] and the south coast of England [39]; however, in other areas where it has not proliferated as much, it appears to have had little effect on overall macrobenthic community diversity [47].

Introductions have not just been confined to macrofaunal species. Oyster introductions have also been strongly implicated in the introduction of parasitic organisms and macroalgal species into novel regions [48–50]. Notwithstanding the records of aquaculture-mediated introductions of nonnative species into marine systems, there still appears to be a paucity of information and experimental evidence quantifying the impacts of nonnative oyster introductions on the receiving environment [13]. The structures associated with shellfish culture (e.g., ropes, bags, floats) in some areas provide novel habitat for the colonization and proliferation of exotic species [18]. In addition to the likely effects on system function, the colonization of structures associated with shellfish culture also presents practical problems for the aquaculturists from a husbandry perspective.

Biodiversity

The physical presence of large numbers of shellfish in culture can result in a monoculture which has a finite period of time in the system as a consequence of husbandry practices, e.g., thinning or harvesting. Given this constraint, the development of communities associated with the cultured shellfish will be restricted and hence biodiversity is likely to be reduced. This is particularly true if the culture period is short (i.e., approximately 1 year). The activities associated with shellfish culture can also be impacting. For example, dredging associated with on-bottom culture of mussels and oysters can cause damage to seabed (and organisms therein) and cause sediment plumes to be distributed beyond the culture environs [51, 52].

Structures

The culture of shellfish species using structures presents a number of likely interactions with the environment. The use of structures, i.e., bags and trestles, longlines with droppers will increase the density of culture organisms above the seafloor, thus influencing the flux of materials and nutrients to the seafloor and into suspension. The physical presence of the structures can also modify water flow in and around the culture system resulting in increased deposition of material or scouring in areas of higher flow. Either way there is a potential to impact on the sediment structure and associated communities. As stated above, shellfish aquaculture can also provide structures that can provide for the proliferation of individual (fouling) organisms in a system. Some of which might be new to the system. Finally, the physical presence of the structures and culture animals can have the effect of shading the seafloor and thus potentially impacting on species reliant on light (e.g., maerl or seagrasses).

The interaction of shellfish culture activities with the marine environment can be considered from the perspective of near-field and far-field impacts. While near-field effects are more easily measured (using many standard near-field impact indicators identified in Table 1) and mitigated, the measurement of far-field effects is more difficult to achieve, although some of the impacts might be lessened if near-field activities are reduced or mitigated. Other far-field effects may not be as easily measured and assigned to a specific causative activity (as there may be multiple causative factors) yet they all must be considered when managing marine systems. To this end, a number of authors (e.g., Tucker and Hargraves [163]; Costa-Pierce and Page [3]) have provided a good contextual presentation on the issues surrounding sustainability of aquaculture activities. They acknowledge that sustainability solutions can extend from ecological, technical, and socioeconomic and cover very small spatial scales to adjusting broader societal values.

It is important to appreciate that in order to manage activities in marine systems, it is imperative that there is a good understanding linking the culture practice to a specific environmental response. These interactions are important in order to coordinate management responses between regulatory agents and aquaculture operators, in order to minimize negative environmental effects while maintaining production returns and hence, profits.

Evolution of Sustainability for Shellfish Culture

It is widely accepted that most human activities in the marine environment will have some effect on marine species and habitats. The scale of these impacts depends on the nature of the activity, its intensity, and the sensitivity of the receiving environment. The degree of change that is considered permissible depends on a number of factors, not the least of which is public perception. Empirical data demonstrating change or impact is the most obvious basis on which to justify management actions or inactions. Equally important is linking the change observed directly to the process under consideration (e.g., bivalve molluscan mariculture). Best management practices (BMPs) and performance standards have been adopted as means of mitigating against unacceptable environmental interactions. The major categories of practices and standards include the following:

- Regulatory standards governing molluscan mariculture
- BMPs (or design standards or specifications) for bivalve growers, mariculture regulators, and managers
- Certification standards for molluscan products (e.g., organic, sustainable, fair trade, domestically or even locally grown)
- Other innovations, Integrated Multi-Trophic Aquaculture (IMTA)

Legislative Drivers Toward Sustainable Practices

As in many countries there is much national and internationally derived legislation governing the production and placement of aquaculture-derived food products on the market. These regulations consider the product primarily from a food safety perspective with the goal of protecting the consumer. In European Union member states, Regulation (EC) No 854/2004 [53] of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption was implemented to ensure that consumers of shellfish products are not exposed to toxins that might have accumulated in shellfish flesh as a consequence of filtering phytoplankton species responsible for producing these toxins. Shellfish are effective bioaccumulators. Such regulation requires continual monitoring of shellfish products derived from marine waters. In the event of an excess of defined thresholds, the product is not placed on the market and monitoring continues. The broader environmental benefits of this legislation are that it can identify areas of risk (for harmful algal blooms) and has spawned research to identify factors governing the causes of bloom events including anthropogenic sources. How this impacts on sustainability of shellfish aquaculture? The persistence of HAB and toxic events can dictate the feasibility of locating or developing shellfish culture these areas. If areas are subject to prolonged closures, the product is generally restricted from being removed and can result in overload and subsequent impact on system processes.

The Water Framework Directive [54] is a legislative driver designed to improve surface and groundwater quality throughout the European Union. Allied with a full-risk assessment, each waterbody will be assessed for its condition in terms of ecological quality elements. This monitoring program focuses on a range of ecological quality elements (e.g., benthic invertebrates, phytoplankton, macroalgae, fish in transitional waters) for which a series of standards have been developed. The waterbodies included in the monitoring program were selected on the basis of no obvious pressures and site for which some pressure has been identified, e.g., aquaculture activities. The goal of the WFD is to ensure that all waterbodies achieve good ecological status by 2015.

The Habitats and Birds Directive [55] in EU member states is considered the cornerstone of Europe's nature conservation policy. The Directive requires that certain areas are designated as conservation sites (Natura sites) and that the conservation features therein managed such that they are preserved in a natural state. In many EU member states, licensed aquaculture activities take place in Natura sites. The designation does not preclude licensing activities in Natura sites but the licensing process must ensure that the proposed activities do not pose a significant risk to the conservation objectives of the site. In this regard, the licensing authority must be seen to carry out and appropriate assessment on the likelihood of these activities significantly impacting on the conservation features. If the activity is considered impacting, then the licensing authority or the applicant can mitigate with a view to reducing the significance of the impacts and still fulfill both conservation and aquaculture objectives. Similarly, in the USA, the Magnuson-Stevenson Fishery Conservation and Management Act, provides for the protection of essential fish habitat and the restoration of coastal habitats. Both objectives can cause the relocation of shellfish aquaculture operations to less ecologically sensitive areas (e.g., away from seagrass beds). However, the act also provides some opportunity for shellfish aquaculturists from the perspective of the benefits shellfish aquaculture can provide specifically as it relates to habitat restoration goals. The worldwide depletion of natural populations of shellfish in nearshore coastal areas has been well documented (reviewed in National Research Council [18]). The ability of aquaculture to fill the ecological niche previously provided by native populations has been postulated. At a minimum aquaculture has provided stock for the implementation of restoration projects in Chesapeake Bay in Virginia and Maryland [56].

The International Council Exploration of the Sea Code of Practice on the Introductions and Transfers of Marine Organisms [57] is not legislation per se; however, it is cited in legislation and is considered a good example of a guide/code of practice developed in response to particular pressures or risks identified with human activities including, *inter alia*, shellfish aquaculture. The ICES Code of Practice recommends a series of protocols with a view to mitigating any negative risks associated with intentional introductions and transfers of marine organisms and is targeted at individuals or organization that engages in such activities. As an example of specific legislation citing the ICES Code is the European Union regulation concerning use of alien and locally absent species in aquaculture (708/2007/EC). Specifically, the legislation is designed to avoid alterations to ecosystems, prevent negative biological interactions (including genetic change) with indigenous populations, and restrict the spread of nontarget species and detrimental impacts on natural habitats. This legislation directs member states to ensure that a full-risk assessment is carried out prior to the introduction of a nonnative species for aquaculture purposes. In order to facilitate any introduction, the regulator must ensure that the operator mitigate fully any negative interactions identified as a consequence of the risk assessment. The protocols identified in the ICES Code can provide an avenue toward this mitigation.

The Code is aimed at a broad audience since it applies to both public (commercial and governmental) and private (including scientific) interests. In short, any persons engaged in activities that could lead to the intentional or accidental release of exotic species should be aware of the procedures covered by the Code of Practice.

Notwithstanding the potential for regulation to positively guide the development and practice of shellfish aquaculture activities, there is also the risk that regulation can result in constraint of bivalve aquaculture development. This has been demonstrated in the

USA where a range of local, state, and federal ordinances govern the licensing of activities in nearshore waters (i.e., areas conducive to bivalve aquaculture). For example, while some coordination has occurred between state and federal agencies in the USA, it still requires up to 30 permits to establish a shellfish culture operation (National Research Council [18]). The complexity can result in a protracted and expensive application process on the part of the aquaculturist that can end with the permit application being refused. The conflict inherent in the regulatory processes is also reflected by conflicts of users in the coastal areas, e.g., wild fishery and aquaculture interests. As a solution to such conflicts, some jurisdictions have implemented zoning of activities where certain activities are only permitted. For example, in an effort to mitigate the conflicts between shellfish culturists and fisherman, some states (Massachusetts and North Carolina) allow shellfish culture in areas where bivalves do not naturally grow. While this offers a somewhat artificial solution to user conflicts, it would appear to favor fishermen, as shellfish typically grow best where they are found naturally in the wild and the availability of approved growing areas might be hard to locate if shellfish are ubiquitous.

Industry Solutions Toward Sustainable Practices

There have been a number of industry-led initiatives that have directly or indirectly led to the implementation of more sustainable practices relating to shellfish culture. As already stated, an important realization among promoters of aquaculture and/or regulatory bodies is that a good understanding of the likely interactions between culture practices and environmental concerns is paramount in order to structure management responses. From the industry perspective, these responses are structured with a view to minimizing the negative environmental interactions while maximizing profits.

Integrated Multi-Trophic Aquaculture

Integrated Multi-Trophic Aquaculture (IMTA) is based upon the principle that the coculture of aquaculture products in carried out in sequence and that one species production is dependent upon the outputs of another [58]. Integration at larger scales may address the optimization of shared resources among various aquaculture users (e.g., shellfish or seaweed culture near fish farms), but assumes that the integrated components (species) are situated within the influence of the system component upon which it directly depends for waste/energy transfer and utilization. In a well-balanced system this relationship provides the environmental benefits associated with polyculture, and is the basis of definitions such as sustainable, or ecological, aquaculture.

Initiatives on the east coast of Canada (New Brunswick) have recently evaluated the performance of mussels (*Mytilus trossulus*) and large macrophytes (*Laminaria*) cultured within the infrastructure of an open net-cage salmon (*Salmo salar*) aquaculture facility (see IMTA chapter) [58]. To date, there have been mixed results in terms of performance of secondary species, i.e., shellfish species in demonstration projects for IMTA; however, there are a number of other perceived benefits of location shellfish operations in the influence of finfish farms. Most notably is the effect of the shellfish on diseases and parasites of the fish species. Concern has been raised about the ability of the shellfish species of retaining and/or transmitting disease-causing organisms to fish species. The counterargument to these concerns is that the shellfish species is likely endemic to the area and may form part of fouling community on structures such that the risk is not magnified by the presence of the species in culture. In addition, a demonstrated benefit is that the blue mussel (*Mytilus edulis*) has been shown to destroy the virus for infectious salmon anemia (ISA) [59]. Furthermore, the blue mussel has also been demonstrated to eat copepodids (the larval stage of the sea louse, a parasite of salmon) [60] and represents a potential alternative control mechanism to sea lice to chemical treatment which have limited efficacy due to a buildup of resistance in the louse.

The environmental benefits of MTA are not constrained solely to the direct assimilation of waste constituents among the cocultured species, but will also be achieved indirectly through the physical design/configuration and orientation of such a system with respect to adjacent, and potentially sensitive marine habitats. Furthermore, there are a number of perceived social benefits associated with the development of marine integrated aquaculture including: (1) optimizing culture opportunities where space is constrained; (2) the provision of development opportunities in remote coastal regions; and/or (3) improving public

awareness of aquaculture or aquaculture subsequent environmental accountability. To this end, in the European Union, the Common Agriculture Policy and Common Fishery Policy requires primary users of the natural resources (e.g., agriculturists, fishermen, aquaculturists) to implement an ecosystem approach in the management and conservation of the environment and landscape. It considers polyculture (MTA) as a viable utilization approach for these areas that could provide restoration at a lower cost for society.

Certification Schemes

The increasing demand from consumers for information pertaining to the products they are consuming has been the primary driver for the development of aquaculture certification schemes. The schemes operate on the principal that aquaculture products are produced in fashion that considers a range of factors including, *inter alia*:

- Social responsibility and comply with laws such that they are produced in a legal, safe, and fair manner
- Environmentally responsible manner such that any negative impacts on the system are minimized
- That all food safety and quality standards are complied with in the member states
- That all animal health issues are considered and managed consistent with legislative requirements

To date, the most prominent schemes are overseen by the Global Aquaculture Alliance (GAA) [61], which is responsible for the development of best aquaculture standards for mostly "fed" aquaculture products, including shrimp hatchery and farm standards. It would appear that there are currently no plans for the GAA to develop standards for bivalve shellfish culture. The Aquaculture Stewardship Council is an association founded jointly by the WWF and Dutch Sustainable Trade Initiative with a view to ensuring aquaculture is carried out in an environmentally and socially sustainable fashion. The ASC has developed standards for the culture of bivalve shellfish products (on foot of the Bivalve Aquaculture Dialogues [62]). The standards are governed by a range of broad principles for addressing the environmental and social issues associated with bivalve aquaculture. These principles are consistent with those identified above. The principles provided the framework for developing the criteria, indicators, and standards applicable to bivalve farming. The standards attempt to provide quantitative performance levels that determine whether a principle is achieved or not. The ultimate goal of ASC is to have comprehensive participation in the certification scheme by shellfish aquaculturists, which is planned to be certified by a third party. In addition to bivalves (clams, oysters, mussels, and scallops) standards have also been established for abalone. In summary, certification standards have been developed by buyers, public agencies, nongovernmental organizations, or marketing groups as a means of providing consumers with information about a product. The ultimate goal of certification schemes is to persuade growers to modify culture practices by influencing consumer choice and market forces. However, pursuit of certification is voluntary for growers.

Best Management Practices

The origin of Best Management Practices (BMPs) dealing specifically with environmental interactions is primarily from broader land-based agricultural practices used to mitigate the effects of soil erosion and nutrient loading. Similarly, mariculture BMPs have been developed with a view to minimizing effects resulting from aquaculture culture practices (see Table 2). Best management practices often are developed or strongly supported by the industry group (e.g., oyster growers) to which they apply and as such, adoption of and adherence to these codes is usually voluntary. As with other practices (cited above) the adoption of BMPs tend to have multiple objectives including, for example, reducing the likelihood that shellfish farming will have unacceptable ecological effects. These effects relate primarily to changes in the ecology of the system and interactions with other stakeholders in the system. As highlighted in Table 2, some examples of BMPs relating to shellfish culture consist of outreach brochures on husbandry techniques (e.g., Washington Sea Grant, 2002; Alaska Sea Grant, 2009; University of Maryland, 2009) to identify the optimal ways to culture bivalve molluscs. While these publications focus on methodologies to maximize production, they can still be considered as rudimentary BMPs, in that they require, of the culturists or advisors,

Shellfish Aquaculture, Methods of Sustainable. Table 2 Examples of best management practices and environmental standards for the farming of bivalve molluscs produced by a range of organizations, demonstrating the range of topics and the variety of subjects covered. Ecosystem Concepts for Sustainable Bivalve Mariculture by Committee on Best Practices for Shellfish Mariculture and the Effects of Commercial Activities in Drakes Estero, Pt. Reyes National Seashore, California Reproduced with permission of National Academies Press

Author	Affiliation	Scope	Scale	References
U.S. Agency for International Development	Regulator, nongovernmental organization, and academia	Generic guidelines and environmental interactions	International	[151]
World Wildlife Fund	Nongovernmental organization	Environmental interactions	International	[72]
U.S. Department of Agriculture	Regulator	Policy on organic certification and environmental interactions	National	[152]
State of Virginia	Advisory agency, regulator, and industry	Environmental interactions and permitting	State	[153]
Pacific Coast Shellfish Growers Association	Industry	Policy and environmental interactions	Regional	[154]
Seafish (UK)	Regulator, industry, and advisory agency	Alien species interactions	National	[155]
State of Massachusetts	Industry, regulatory, and advisory agency	Environmental interactions, permitting, and husbandry	State (local)	[156]
Maryland Aquaculture Coordinating Council	Industry, regulatory, and advisory agency	Environmental interactions, permitting, and husbandry advice	State	[157]
Ireland	Industry and advisory agency	Generic environmental interactions	National	[158]
Florida Department of Agriculture and Consumer Services	Regulator	Permitting and environmental interactions	State	[159]
International Council for the Exploration of the Sea	International convention	Alien species Interactions	International	[56]
Maine Aquaculture Association	Industry	Environmental interactions and permitting	State	[160]
National Oceanic and Atmospheric Administration	Regulatory	Environmental interactions and policy	National	[161]
Creswell and McNevin (2008)	Academia	Generic guidelines, environmental interactions, and husbandry	International	[162]

Source: From National Research Council [18]

a good understanding of the likely interactions between the specific culture methods and the environment (e.g., performance in light of productivity).

The development of BMPs with a view to managing environmental interactions should be considered a fluid or transitory process that should allow for feedback and subsequent modification of culture practices to mitigate negative effects associated with the shellfish culture.

Some BMPs are usually nontechnical wherein they identify a range of issues and present a framework of

general principles and solutions and as such, are unlikely to address the range of detailed and local in aspect, issues that present to regulatory agencies and the producers. Other BMPs do deal with more regional issues, and yet others specifically consider the day-to-day operations at farms and their interactions. These guidelines can provide important advice on pertinent laws and ordinances and focus on important local issues (e.g., environmental interactions, stakeholder interactions, community relationships). In addition, they can identify solutions that range from farm- or small-scale measures to broader societal solutions focusing upon competing uses and values. Toward this end, there are a number of general principles that have applied to the development of BMPs that have directed the practice of shellfish aquaculture toward producing molluscs under the broader umbrella of sustainability. These principles can be broadly categorized under the following headings [50, 63]:

1. Promote a good understanding of environmental and ecological processes, with the broader view of generating carrying capacity models for production areas. This can be further expanded including important socioeconomic considerations also (i.e., ecological and socioeconomic sustainability).
2. Utilize the BMP as a means of promoting the product, i.e., a marketing tool to target a niche market and/or premium price (i.e., economic sustainability).
3. Allow a staged increase in production and reduce uncertainty in terms of environmental interactions (i.e., ecological sustainability).
4. Provide an important communication tool to highlight issues and benefits of shellfish culture to relevant regulatory authorities as well other stakeholders (e.g., nongovernmental organizations) (i.e., social sustainability).

While a goal of implementing BMPs will undoubtedly be the improvement of environmental conditions or mitigation of negative effects caused by the aquaculture activity, there still remains a question of how the effects of the BMP are measured. There can be a sharp distinction between BMPs and performance standards. On the face of it, both schemes are broadly designed to limit risk of undesirable environmental impacts. Some authors [15, 64] correctly note, however, that BMPs are not a proxy for performance and that they may have little or no impact in terms of measurable environmental improvements. Typically there are no measures associated with BMPs to validate the claims about mitigating environmental effects. BMPs, in effect, are akin to design standards, whereby procedures and practices are strictly defined (e.g., number of longlines per hectare) which are relatively easily verified. BMPs, therefore, will have a lower administrative burden for demonstrating compliance by identifying easily measured metrics, but have the drawback that there is no guarantee of environmental benefit. Performance standards, on the other hand, measure specific environmental objectives, i.e., the effect on the activity on some aspect of the environment (e.g., free sulfide concentration in sediments). Performance standards relate more specifically to objectives focusing upon ecological integrity of a system [15]. However, performance standards have resource implications in terms of monitoring and enforcement; they are likely more expensive to administer and implement that BMPs.

Notwithstanding with distinction highlighted between BMPs and performance standards, it is still possible for managers to align husbandry practices with some measure of performance. For example, it is possible for managers to identify the carrying capacity of a system and subsequently advise on the design of husbandry systems. The process must be fluid and allow for changes in practices and standards as more information on effects is gathered and uncertainty is reduced. It also should allow for stakeholder participation to inform management decisions. In this regard, the science underpinning management actions is communicated concisely to all with an interest in the subject matter and that the views and experience of stakeholders are brought to bear on the process.

Measuring Sustainability: The Development of Indicators

Indicators are important management and communication tools used in order to

1. Inform and raise public awareness on environmental issues
2. Provide information linking driving forces and impacts

3. Develop policy responses. To this end, the indicators can be used:
   a. To supply information on environmental problems in order to enable policy makers to value their seriousness
   b. To support policy development and priority setting by identifying key factors that cause pressure on the environment
   c. To monitor the effects of policy decisions

Communication is a primary function of indicators – they should enable or promote information exchange regarding the issue they address. Communication, however, demands simplicity and therefore indicators should always simplify complex realities. Sustainability indicators are different from "impact" indicators in that they tend to be more inclusive and consider not only environmental issues but also social and economic features [3]. Based upon these broad criteria, sustainability indicators represent an amalgam of the Integrated Coastal Area Management (ICAM) guidance of the United Nations Educational, Scientific, and Cultural Organisation (UNESCO) recommends three basic categories of indicators:

1. Environmental: Reflect trends in the state of the environment; are descriptive in nature; and become performance indicators if they compare actual conditions to desired conditions expressed in terms of environmental targets.
2. Socioeconomic: Represent the demographics of humans in the coastal zone and measure quality of life issues.
3. Governance: Measure the performance of the state of implementation, measuring the progress and quality of interventions of the governance process in relation to program goals set at the outset.

To this end, sustainability indices (SIs) are needed by aquaculture resource managers who must sort through large amounts of information and make numerous decisions relating to environmental management and policy implementation. SIs, therefore, offer a means to prioritize those aquaculture systems most in need of immediate management attention and allow scarce management assets to be applied in the most cost-effective manner. The development and subsequent utilization of SIs are also valuable to owners of seafood businesses who wish to procure or develop "sustainable seafoods," i.e., as a marketing tool.

Well-developed and scientifically credible SIs are important tools which can make a range of ventures including monitoring, baseline data collection, research enterprises, and communications efforts better organized and targeted, and thereby, more cost effective. Therefore, indicators must be selected or developed and cover the three components of sustainability. It has been argued that indicators should be as quantitative as possible. While this is a practical goal, some descriptors do not lend themselves as easily to quantitative measurements and therefore qualitative descriptions may be more appropriate for describing, say, for example, the social aspects of sustainability [3].

Indicators must be measurable objects that can be simplified by aggregation and calculation. Outcomes from theoretical models cannot be considered as indicators. Nevertheless, models may help to indicate the most relevant factors to be monitored. Ideally, indicators might address the following issues:

- Continuity of supply (environmental, economic, and social services)
- Social, economic, and environmental costs to provide this continuity of supply
- Long-term aspects
- Financial viability
- Social and ecological impacts
- Global efficiency

Environmental Indicators

While there is considerable effort directed toward the development of indicators for coastal areas throughout the world, the focus primarily remains on environmental indicators that link specific pressures with impacts. The development in European Union Member States of indicators relating to the implementation of the Water Framework Directive [52, 65] is a clear example of one such endeavor. Indicators developed in response to this legislation, for the most part, are responsive to a range of pressures. The development of sustainability indicators (particularly those targeted at shellfish aquaculture) is at a more nascent stage. It is broadly accepted that sustainability indicators for shellfish aquaculture

(and aquaculture generally) should encompass the criteria outlined previously, i.e., track aquaculture's (positive and negative) impacts on the environment and be able to monitor economic, social, and cultural externalities, as well as evaluate governance impacts of policies and regulatory measures on aquaculture. Once accepted as practical tools, SIs need to be ultimately included in codes of best practices, decision support systems, and should be used by managers to steer aquaculture development and how it might interact with other activities and features in the marine environment.

Composite Sustainability Indices

While it is possible to assess sustainability with several indicators [66], it may sometimes be difficult to make decisions and comparisons among sectors, production systems, or companies based on a large number of performance measurements. To help decision makers in this respect, it may be useful to use composite sustainable development index, linking many sustainability issues and so reducing the number of decision-making criteria that need to be considered.

In recent years, research has focused on the development of composite indices mostly for cross-national comparisons of economic, societal, environmental, and/or sustainable progress of nations in a quantitative fashion [67]. Care must be taken in the development of composite indicators as, for example, weighting of constituent indicators can be applied subjectively without any clear quantitative basis. However, this may be corrected by the application of sensitivity analysis whereby weightings are adjusted to reflect the true influences of indicators (and by association, pressures). In addition, the selection of the indicators to be used in a composite indicator is crucial to the utility of a sustainability indicator. A solution to this is it requires a transparent and inclusive process to determine how and who will select the most appropriate indicators [50]. Such a process should avoid risks associated with too much complexity as well as concerns about the costs associated with monitoring multiple indicators that could be irrelevant to managers and the public. Usable indicators must be more than just a description of state and should have diagnostic properties that lead to some insights into processes taking place (i.e., provide a specific link to the pressure under consideration).

As previously stated, SIs for environmental and aquaculture management are still in the early stages of development. However, some investigators [68] have recommended a simple set of easily quantifiable indicators for sustainability in aquaculture (generally) and which broadly reflect the criteria outlined previously describing sustainability indicators:

- Biological: domestication, trophic level, nutrient/energy conversion
- Ecological: footprint, emissions, escapes
- Intersectoral: water sharing, diversity, cycling, stability, and capacity

The development of tool-box approach toward assessing sustainability for aquaculture has been proposed [60]. This approach has been taken further with the utilization of indicators specifically to shellfish culture operations [69] with the production of guidance for effective site selection and targeted approach to conducting environmental impact assessments and establishing monitoring standards. Furthermore, the incorporation of a stakeholder consultation process in order to identify the most pertinent indicators has also been incorporated into a variety of initiatives.

Notwithstanding the relatively slow progress toward the development of specific indicators of sustainability for shellfish aquaculture, there are still a number of clear criteria proposed that can help define and direct future development efforts. These are best elucidated by the International Council for the Exploration of the Sea [70] which has addressed the issue of sustainability in aquaculture over the last number of years. They offer some general principles on sustainability indices and how they relate to aquaculture activities (including shellfish culture). The basic points below summarize much of the previous discussion and are as follows:

1. It is important to discern the differences between "sustainability" indicators and "impact" indicators which are narrow in their focus. Sustainability indicators should be able to track more than aquaculture's impacts on the environment and be able to monitor economic, social, and cultural externalities, as well as evaluate governance impacts (e.g., policies and regulatory measures) on aquaculture and the environment broadly.

2. Once accepted, SIs should be ultimately included in codes of best practices (BMPs), decision support systems, and should be used to inform aquaculture development by the authorities (e.g., integrated coastal zone management systems, see below).
3. Sustainability indices must be of the highest scientific credibility and be accepted only after peer review of the chosen index, and analyses of precision, accuracy, reliability, and consistency are completed. It may be contrary to the criteria outlined above but the SIs must also be cost-effective and be applicable across a range of environments, habitat types, and activities.
4. SIs must be flexible enough to be adapted to the local environment in which they will be used. There is no chance that a single set of "generic" indicators may be universally applicable and used in all the situations in the aquaculture sector. In addition, SIs may be of use to address the interactions with other users of marine resources, locally or internationally because of the opening of global markets.
5. In the development of SIs for aquaculture it is important to develop them collaboratively and consider SIs from a managers' and other stakeholders' perspective. Managers are a critical link between the science community and the public. Collaborations between managers, scientists, and the public should be formalized to facilitate rapid decision-making and communication. It is important that levels that exceed those which are accepted by the society are scientifically credible so that managers can determine causative agents and what remediation actions are necessary.
6. SIs must be able to detect the linkages between the 3Ps – people, profit, planet – which suggests a development of a "sustainability indicator matrix." Such a matrix approach could allow flexibility and be able to address the sustainability factors more comprehensively for any given situation. Regular evaluation of SIs may be necessary among the various actors assessing aquaculture developments, e.g., producers, policy makers, consumers, NGOs, suppliers, since aquaculture technologies and site locations are constantly evolving.
7. To this end it is important to emphasize that SIs must be sustainable themselves. The production of information must be practicable at a low cost for the government, public, and the aquaculture sectors. Data from SIs must provide meaningful long-term data series. These time series will need to be housed in data management frameworks at the institutional level, but be universally accessible.

Progressing Sustainability in Shellfish Aquaculture

Heretofore, the difficulty in developing a single (composite) indicator relating to shellfish aquaculture remains and one has yet to be definitively proposed. This is borne out by the general conclusion that the majority of exercises examining the sustainability of aquaculture operations recommend only specific and localized "sustainability" (or impact) indicators, without fully addressing the sustainability of aquaculture from an interactive perspective. It is generally accepted that the main goal of these programs is to determine an acceptable aquaculture production capacity for a defined area. It is also accepted that aquaculture activities should be managed while fully cognizant of other activities in a particular area. Consequently, the likely impacts of aquaculture will, therefore, have to be assessed individually, cumulatively (with other aquaculture operations), and in combination with other activities, on the environment, ecosystem, and function of a system. The interactions and impacts of aquaculture have been well documented; it has been this information that has provided the framework for many monitoring programs for aquaculture operations. The notion of acceptability is critical to fully determining the sustainability of an activity in the marine environment. The term "acceptable" is governed primarily by social values, starting from a global to local perspectives. The social carrying capacity of aquaculture should be the basis of a sustainability program to assess the sum of activities, such as aquaculture, within a defined area. The principles of these programs could flow from global vision, with regionally based criteria. For example, no net loss or biodiversity may be a global vision.

Considering the goal of ensuring sustainable levels of activities in the marine environment, the application of Integrated Coastal Zone Management (ICZM) is likely to be an important tool toward achieving this objective. As already highlighted, the importance of

linking social, economic, and environmental aspects into the management of marine systems is critical as well as the need to have broad sectoral cooperation and input into the development of these practices.

ICZM facilitates a shift from management and regulation of activities in the marine environment in isolation to a system where all activities can be considered and the resource use is optimized with a view to maintaining the health and productivity of coastal ecosystems so that they can continue to supply resources that sustain different forms of activity, including mariculture. While these goals are lofty, the implementation will be challenging. Benthic and pelagic effects of shellfish culture are well documented in the literature (from both modeled and empirical studies). The risk posed by practices associated with aquaculture (both finfish and shellfish) have also been well documented and evaluated in the areas of disease transmission and introductions of exotic species to areas. The *in-depth* knowledge relating to these impacts and interactions have placed the pressures posed by mariculture to the forefront in terms of public awareness and criticism. Ironically the lack of information pertaining to other pressures may be a reason they have not been the focus of scrutiny or criticism (e.g., static gear fisheries, sewage effluent discharges over broad spatial scales). ICZM can be supported by the development of appropriate decision support systems (DSS), i.e., in the form of conceptual models allied with the presentation of geospatial data in geographic information systems (GIS) can be used to identify what would seem to be the most appropriate use of marine ecosystems. The term "use" also includes nonexploitive activities particularly in areas that have high intrinsic natural value or have some protection conferred by legislation (e.g., national parks).

The social science dimension is an important component on the issue of ICZM [50, 65]. Application of social science principles will facilitate a better understanding of the expectations of different stakeholders competing for space and resources in coastal areas and help establish a consensus among relevant users. More specifically, a recent initiative in France [71] called EVAD (ÉVAluation de la Durabilité des systemés aquacole) addressed the issue of aquaculture sustainability by examining the contribution that aquaculture systems make to the sustainability at regional levels. The first component of this approach much merit and could be an important component into an Integrated Coastal Zone Management program. Similarly an ecosystem approach to aquaculture management that is comprehensive and based on the best available scientific knowledge of the ecosystem and its dynamics is also proposed by numerous fora [72, 73]. Actions are designed to be taken on the influences of aquaculture developments that are critical to the health of ecosystems, thereby achieving sustainable uses of ecosystem goods and services and maintenance of ecosystem integrity. In these instances, the aim is to assess the sustainability of all the activities in an area where aquaculture is fully and fairly integrated rather than the sustainability of aquaculture in isolation. At that point, indicators can be developed, negotiated, and used to evaluate the effect or impact (positive or negative) of aquaculture and other activities (drivers) on the sustainable utilization of resources in a region, based upon the global principals and the regional-territorial criteria. Sustainability would thereby no longer be assessed by indicators of impacts, but mainly criteria based on objectives instead of thresholds. The sustainability of all activities within a region would thereby be evaluated on the merit of criteria developed under an Integrated Coastal Zone Management (ICZM) approach. It is important to note that assessment of sustainability at the activity level can lead to the establishment of thresholds that can be ineffective or even detrimental to the activity which may only contribute marginally to one or several criterion. In addition, smaller aquaculture operations would not be constrained by the responsibility of monitoring the sustainability of an area, because they are the newer addition to the coastal zone territory, already affected by numerous players.

Future Directions

Sustainability by definition and in application must include consideration of planning for multiple impacts and identifying the likely challenges posed by existing and future development and conservation (general sense) needs. To this end, sustainability therefore refers to the ability of a society to continue functioning in the future without being forced into decline through exhaustion or overloading of key resources upon which society's systems rely [74]. Proponents of

shellfish aquaculture [75] propose that the accelerated development of aquaculture in a sustainable fashion is a realistic goal going forward. Consistent with the broad view offered above, the focus for aquaculture development should be to manage existing activities and proposed expansions in coincidence with other activities in marine systems such that the management actions are fully integrated, and that any impacts will be at a minimum, neutral on both natural and social ecosystems [76]. It is appreciated that some codes of conduct and guidelines for certifying sustainability are sometimes too complex and narrowly focused. Therefore, there would be an imperative on the part of managers to see to it that inclusive yet simple and scientifically credible sustainability indices for mariculture activities are developed and are considered a cost-effective complement to any codes employed. This would be driven by the need of regulatory or licensing agents to have systems with which to take defendable decisions relating to management of marine issues. Furthermore, the need to communicate in a transparent, concise, and credible fashion the likely issues relating to shellfish culture activities to consumers is another important justification toward the development of sustainability indices for shellfish culture.

In summary, a SI applicable to shellfish aquaculture should be able to incorporate all information in a system, identify what the goals (global vision) for the system are, and evaluate both positive and negative aspects of any proposed development. It should be able to determine if the positive aspects outweigh the negative ones such that a decision to permit the activity can be taken. In other words, will the mitigation of eutrophication effects afforded by the filtration of shellfish outweigh the increased biodeposition beneath the culture structures? While these considerations apply generally to all activities and how they interact with the natural environment (both terrestrial and aquatic), they also provide a framework with which to focus more clearly on specific activities in the marine environment and how these activities interact with features therein. However, it has become clear that the application of these concepts have not been fully implemented because it is apparent that such generalities apply at the level of the (eco)system and are difficult to apply at the level of specific activities and/or environmental features. It is apparent that in order to fully implement these definitions and take a total view of sustainability in the marine environment, it is the job of managers to apply a system-wide view of sustainability. Such an initiative would have to take into account a broad range of pressures and would have to define clearly what might be permissible and acceptable (i.e., social carrying capacity guided by legislative or policy drivers). Such an initiative would have to consider both spatial and temporal considerations of pressures (activities) and sensitivities. Of considerable importance, however, is that the development of management systems must be informed by a clear notion of an acceptable endpoint that would be either defined as the level that a particularly activity (or range of activities) might be carried out in the marine environment or the maintenance of a specific condition of an environmental feature (environmental standard). It is important to point out that, to date, sustainability has been a much used word with abundant pedagogy but little practice [77] (this article is a case in point). It would appear that progression toward truly sustainable practices is hampered when there is a clear need for interdisciplinary actions but there is little scientific knowledge to inform these actions. This contribution highlights, we hope, that the mechanism toward identifying sustainable activities in the marine environment will be progressed when clear policies are elucidated and an inclusive approach is adopted reflecting fully legislative requirements and the views of all stakeholders.

Bibliography

Primary Literature

1. Food and Agriculture Organization of the United Nation (2009) The state of world fisheries and aquaculture 2008. Food and Agriculture Organization of the United Nation, Rome
2. Tucker CS, Hargreaves JA, Boyd CE (2008) Aquaculture and the environment in the United States. In: Tucker CS, Hargreaves JA (eds) Environmental best management practices for aquaculture. Blackwell, Ames, pp 3–54
3. Costa-Pierce BA, Page GG (2011) Sustainability science in aquaculture. In: Meyers RA (ed) Encyclopedia of sustainability science and technology. Springer, New York
4. Helm M, Bourne N (2004) Hatchery culture of bivalves: a practical manual. Food and Agriculture Organization of the United Nations, Rome
5. Duarte CM, Holmer M, Olsen Y, Soto D, Marba N, Guiu J, Black J, Karakassis I (2009) Will the oceans help feed humanity? Bioscience 59:967–976

6. Pearson T, Rosenberg R (1978) Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. Oceanogr Mar Biol Ann Rev 16:229–311
7. Chamberlain J, Fernandes TF, Read P, Nickell TD, Davies IM (2001) Impacts of biodeposits from suspended mussel (*Mytilus edulis* L.) culture on the surrounding surficial sediments. ICES J Mar Sci 58:411–416
8. Weise AM, Cromey CJ, Callier MD, Archambault P, Chamberlain J, McKindsey CW (2009) Shellfish-DEPOMOD: modelling the biodeposition from suspended shellfish aquaculture and assessing benthic effects. Aquaculture 288: 239–253
9. Nugues MM, Kaiser MJ, Spencer BE, Edwards DB (1996) Benthic community changes associated with intertidal oyster cultivation. Aquac Res 27:913–924
10. Dame RF (1996) Ecology of marine bivalves: an ecosystem approach. CRC Press, Boca Raton
11. Jackson JBD, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA, Hughes TP, Kidwell S, Lange CB, Lenihan HS, Pandolfi JM, Peterson CH, Steneck RS, Tegner MJ, Warner RR (2001) Historical overfishing and the recent collapse of coastal ecosystems. Science 295:629–637
12. Newell RIE, Koch EW (2004) Modeling seagrass density and distribution in response to changes to turbidity stemming from bivalve filtration and seagrass sediment stabilization. Estuaries 27:793–806
13. DeAngelis DL, Post WM, Travis CC (1986) Positive feedback in natural systems. Springer, Berlin
14. Lotze HK, Lenihan HS, Bourque BJ, Bradbury RH, Cooke RG, Kay MC, Kidwell SM, Kirby MX, Peterson CH, Jackson JBC (2006) Depletion, degradation, and recovery potential of estuaries and coastal seas. Science 312:1806–1809
15. Newell RIE, Fisher TR, Holyoke RR, Cornwell JC (2005) Influence of eastern oysters on N and P regeneration in Chesapeake Bay, USA. In: Dame R, Olenin S (eds) The comparative roles of suspension-feeders in ecosystems: proceedings of the NATO advanced research workshop on the comparative roles of suspension-feeders in ecosystems, Nida, 4–9 Oct 2003. Springer, Dordrecht
16. Newell RIE, Kemp WM, Hagy JD, Cerco CF, Testa JM, Boynton WR (2007) Top-down control of phytoplankton by oysters in Chesapeake Bay, USA: comment on Pomeroy et al. (2006). Mar Ecol Prog Ser 341:293–298
17. Pomeroy LR, D'Elia CF, Schaffner LC (2006) Limits to top-down control of phytoplankton by oysters in Chesapeake Bay. Mar Ecol Prog Ser 325:301–309
18. National Research Council (2010) Ecosystems concepts for sustainable bivalve culture. Academic, Washington, DC
19. Carlton JT (1992) Introduced marine and estuarine mollusks of North America: an end-of-the-20th-century perspective. J Shellfish Res 11:489–505
20. Carlton JT (1992) Dispersal of living organisms into aquatic ecosystems as mediated by aquaculture and fisheries activities. In: Rosenfield A, Mann R (eds) Dispersal of living organisms into aquatic ecosystems. Maryland Sea Grant, College Park, pp 13–46
21. Naylor RL, Williams SL, Strong DR (2001) Aquaculture – a gateway for exotic species. Science 294:1655–1656
22. Streftaris N, Zenetos A, Papathanassiou E (2005) Globalisation in marine ecosystems: the story of non-indigenous marine species across European seas. Oceanogr Mar Biol Ann Rev 43:419–453
23. McKindsey CW, Landry T, O'Beirn FX, Davies IM (2007) Bivalve aquaculture and exotic species: a review of ecological considerations and management issues. J Shellfish Res 26:281–294
24. Ruesink JL, Lenihan HS, Trimble AC, Heiman KW, Micheli F, Byers JE, Kay MC (2005) Introduction of non-native oysters: ecosystem effects and restoration implications. Annu Rev Ecol Syst 36:643–689
25. Grizel H, Heral M (1991) Introductions into France of the Japanese oyster (*Crassostrea gigas*). ICES J Mar Sci 47:339–403
26. Reise K (1998) Pacific oysters invade mussel beds in the European Wadden. Senckenbergiana maritima 28:167–175
27. Drinkwaard AC (1999) Introductions and developments of oysters in the North Sea area: a review. Helgolander Meeresun 52:301–308
28. Branch GM, Steffani CN (2004) Can we predict the effects of alien species? A case-history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck). J Exp Mar Biol Ecol 300:189–215
29. Smaal AC, van Stralen M, Craeymeersch J (2005) Does the introduction of the pacific oyster Crassostrea gigas lead to species shifts in the Wadden sea? pp 277-289. In: Dame R, Olenin S (eds). The comparative roles of suspension-feeders in ecosystems. Proceedings NATO ARW, Nida. Kluwer Academic, Dordrecht, NL
30. Diederich S (2005) Differential recruitment of introduced Pacific oysters and native mussels at the North Sea coast: coexistence possible? J Sea Res 53:269–281
31. Smaal A, Wijsman J, Poelman M (2006) Proliferation of the pacific oyster, *Crassostrea gigas* in Dutch coastal waters and its consequence for human use. In: ICES annual science conference, Maastricht
32. Carlton JT (1989) Man's role in changing the face of the ocean: biological invasions and implications for conservation of nearshore environments. Conserv Biol 3:265–273
33. Carlton JT (1999) Molluscan invasions in marine and estuarine communities. Malacologia 41:439–454
34. Barber BJ (1996) Impacts of Shellfish introductions on local communities. In: Pedersen J (ed) Exotic species workshop: issues relating to aquaculture and biodiversity. MIT Sea Grant College Program (MITSG 96–15), Cambridge, pp 18–21
35. Bourque D, Landry T, Davidson J, McNair N (2003) Impact of an invasive tunicate in Atlantic Canada: recruitment and competition. J Shellfish Res 22:320
36. Carver CE, Chisholm A, Mallet A (2003) Strategies to mitigate the impact of *Ciona intestinalis* (L.) biofouling on shellfish production. J Shellfish Res 22:621–631

37. Rodriguez LF (2005) Novel habitats created by non-indigenous species: the role of oysters and ascidians as biological substrata for fouling communities. In: Carman M (ed) International invasive sea squirt conference, Woods Hole Oceanographic Institution, 21–22 Apr 2005, pp 17/59
38. Locke A, Hanson JM, Ellis KM, Thompson J, Rochette R (2007) Invasion of the southern Gulf of St. Lawrence by the clubbed tunicate (*Styela clava* Herdman): potential mechanisms for invasions of Prince Edward Island estuaries. J Exp Mar Biol Ecol 342(1):69–77
39. Wasson K, Zabin CJ, Bedinger L, Diaz MC, Pearse JS (2001) Biological invasions of estuaries without international shipping: the importance of intraregional transport. Biol Conserv 102:143–153
40. Minchin D (1996) Management of the introduction and transfer of marine molluscs. Aquat Conserv Mar Freshwater Ecosyst 4:229–244
41. Ribera-Siguan MA (2003) Pathways of biological invasions of marine plants. In: Ruiz GM, Carlton JT (eds) Invasive species: vectors and management strategies. Island Press, Washington, DC, pp 183–226
42. Gollasch S (2006) Overview on introduced aquatic species in European navigational and adjacent waters. Helgol Mar Res 60:84–89
43. Wonham MJ, Carlton JT (2005) Trends in marine biological invasions at local and regional scales: the Northeast Pacific Ocean as a model system. Biol Invasions 7:369–392
44. Goulletquer P, Bachelet G, Sauriau PG, Noel P (2002) Open Atlantic coasts of Europe – a century of introduced species into French waters. In: Leppäkoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe: distribution, impacts and management. Kluwer, Dordrecht, pp 276–290
45. Barton E, Heard J (2005) Alien, non-native and invasive marine species. The marine biological association of the United Kingdom and the marine life information network for Britain and Ireland. http://www.marlin.ac.uk/PDF/MLTN_alien_non_natives.pdfs. Accessed Aug 2011
46. Blanchard M (1997) Spread of the slipper limpet *Crepidula fornicata* (L.1758) in Europe. Current state and consequences. Sci Mar 61(Suppl 9):109–118
47. De Montaudouin X, Labarraque D, Giraud K, Bachelet G (2001) Why does the introduced gastropod *Crepidula fornicata* fail to invade Arcachon Bay (France)? J Mar Biol Assoc UK 81:97–104
48. Critchley AT, Dijkema R (1984) On the presence of the introduced brown alga *Sargassum muticum*, attached to commercially imported *Ostrea edulis* in the S. W. Netherlands. Bot Mar 27:211–216
49. Maggs CA, Stegenga H (1999) Red algal exotics on North Sea coasts. Helgolander Meeresun 52:243–258
50. Verlaque M (2001) Checklist of the macroalgae of Thau Lagoon (Herault, France), a hot spot of marine species introduction in Europe. Oceanol Acta 24:29–49
51. Beadman HA, Kaiser MJ, Galanidi M, Shucksmith R, Willows RI (2004) Changes n species richness with stocking density of marine bivalves. J Appl Ecol 41:464–475
52. Kaiser MJ (2001) Ecological effects of shellfish cultivation. In: Black KD (ed) Environmental impacts of aquaculture. CRC Press, Boca Raton
53. Regulation 854/2004/EC of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32004R0854:EN:NOT
54. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. http://ec.europa.eu/environment/water/water-framework/index_en.html. Accessed Aug 2011
55. Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. online: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31992L0043:EN:NOT. Accessed Oct 2011
56. Luckenbach MW, Mann R, Wesson JA (eds) (1999) Oyster reef habitat restoration. A synopsis and synthesis of approaches. Virginia Institute of Marine Science, Gloucester Point, p 358
57. ICES (2005) International council for the exploration of the sea code of practice on the introductions and transfers of marine organisms, 30 pp
58. Chopin T (2011) Integrated multi-trophic aquaculture (IMTA). In: Meyers RA (ed) Encyclopedia of sustainability science and technology. Springer, New York
59. Skar CK, Mortensen S (2007) Fate of infectious salmon anaemia virus (ISAV) in experimentally challenged blue mussels *Mytilus edulis*. Dis Aquat Organ 74:1–6
60. Molloy SD, Pietrak MR, Bouchard DA, Bricknell I (2011) Ingestion of *Lepeophtheirus salmonis* by the blue mussel *Mytilus edulis*. Aquaculture 311:61–64
61. Global Aquaculture Alliance (2011) Best Aquaculture Practices Program. online http://www.gaalliance.org/bap/. Accessed Oct 2011
62. World Wildlife Fund (2010) Bivalve aquaculture dialogue standards, 50 pp. http://www.worldwildlife.org/what/globalmarkets/aquaculture/WWFBinaryitem17872.pdf. Accessed Aug 2011
63. McKindsey CW, Thetmeyer H, Landry T, Silvert W (2006) Review of recent carrying capacity models for bivalve culture and recommendations for research and management. Aquaculture 261(2):451–462
64. Clay JW (2008) The role of better management practices in environmental management. In: Tucker CS, Hargreaves JA (eds) Environmental best management practices for aquaculture. Wiley, Hoboken
65. Carletti A, Heiskanen A-S (eds) (2009) Water framework directive intercalibration technical report part 3: coastal and transitional waters. JRC scientific and technical reports no. 51341, 244 pp. http://publications.jrc.ec.europa.eu/repository/bitstream/111111111/10473/1/3010_08-volumecoast.pdf. Accessed Oct 2011
66. Caffey RH, Kazmierczak RF, Avault Jr JW (2001) Developing consensus indicators for sustainability for southeastern United

States aquaculture. LSU Agriculture Center Bulletin Number 879, Baton Rouge
67. Krajnc D, Glavic P (2005) A model for integrated assessment of sustainable development. Resour Conserv Recy 43:189–208
68. Pullin R, Froese R, Pauly D (2001) Indicators for the sustainability of aquaculture. In: Bert T (ed) Ecological and genetic implications of aquaculture activities. Kluwer, Dordrecht
69. Scottish Association for Marine Science, Oban, Scoltland (2004) ECASA- An Ecosystem Approach for Sustainable aquaculture. EU Framework 6 RTD project. (www.ecasa.org.uk). Accessed Oct 2011
70. ICES (2005) Report of the Working Group on Environmental Interactions of Mariculture (WGEIM), 11–15 Apr 2005, Ottawa. CM 2005/F:04, 112 pp
71. Rey-Valette H, Clément O, Aubin J, Mathé S, Chia E, Legendre M, Caruso D, Mikolasek O, Blancheton J-P, Slembrouck J, Baruthio A, René F, Levang P, Morissens P, Lazard J (2008) Guide to the co-construction of sustainable development indicators in aquaculture, 144 pp. © Cirad, Ifremer, Inra, IRD, Université Montpellier 1; Diffusion: Cirad-Montpellier
72. ICES (2009) Report of the Working Group for Marine Shellfish Culture (WGMASC). 7–9 Apr 2009, Bremerhaven, ICES CM 2009/MCC:02
73. Soto D, Aguilar-Manjarrez J, Bermúdez J, Brugère C, Angel D, Bailey C, Black K, Edwards P, Costa-Pierce B, Chopin T, Deudero S, Freeman S, Hambrey J, Hishamunda N, Knowler D, Silvert W, Marba N, Mathe S, Norambuena R, Simard F, Tett P, Troell M, Wainberg A (2008) Applying an ecosystem-based approach to aquaculture: principles, scales and some management measures. In: Soto D, Hishamunda N (eds) Building an ecosystem approach to aquaculture. Food and Agriculture Organization of the United Nations, Rome
74. Frankic A, Hershner C (2003) Sustainable aquaculture: developing the promise of aquaculture. Aquacult Int 11: 517–530
75. Costa-Pierce BA (2002) Ecological aquaculture. Blackwell, Oxford, UK
76. Byron C, Bengtson D, Costa-Pierce B, Calanni J (2011) (2011) Integrating science into management: ecological carrying capacity of bivalve shellfish aquaculture. Marine Policy 35:363–370
77. Ecological Aquaculture (2011) online: http://ecologicalaquaculture.org/welcome/the-future-of-the-blue-revolution/sustainable-aquatic-foods. Accessed Oct 2011
78. Mallet AL, Carver CE, Landry T (2006) Impact of suspended and off-bottom Eastern oyster culture on the benthic environment in eastern Canada. Aquaculture 255:362–373
79. Mallet AL, Carver CE, Hardy M (2009) The effect of floating bag management strategies on biofouling, oyster growth and biodeposition levels. Aquaculture 287:315–323
80. Rodhouse PG, Roden CM (1987) Carbon budget for a coastal inlet in relation to intensive cultivation of suspension-feeding bivalve molluscs. Mar Ecol Prog Ser 36:225–236
81. Christensen PB, Glud RN, Dalsgaard T, Gillespie P (2003) Impacts of longline mussel farming on oxygen and nitrogen dynamics and biological communities of coastal sediments. Aquaculture 218:567–588
82. Mesnage V, Ogier S, Bally G, Disnar JR, Lottier N, Dedieu K, Rabouille C, Copard Y (2007) Nutrient dynamics at the sediment–water interface in a Mediterranean lagoon (Thau, France): influence of biodeposition by shellfish farming activities. Mar Environ Res 63:257–277
83. Richard M, Archambault P, Thozeau G, McKindsey CW, Desrosiers G (2007) Influence of suspended scallop cages and mussel lines on pelagic and benthic biogeochemical fluxes in havre-aux-Maisons lagoon, Iles-de-la-Madelaine (Quebec, Canada). Can J Fish Aquat Sci 64:1491–1505
84. Hargrave BT, Doucette LI, Cranford PJ, Law BA, Milligan TG (2008) Influence of mussel aquaculture on sediment organic enrichment in a nutrient-rich coastal embayment. Mar Ecol Prog Ser 365:137–149
85. De Grave S, Moore SJ, Burnell G (1998) Changes in benthic macrofauna associated with intertidal oyster, *Crassostrea gigas* (Thunberg) culture. J Shellfish Res 17:1137–1142
86. Ysebaert T, Hart M, Herman PMJ (2009) Impacts of bottom and suspended cultures of mussels *Mytilus* spp. on the surrounding sedimentary environment and macrobenthic biodiversity. Helgoland Mar Res 63:59–74
87. Callier MD, McKindsey CW, Desrosiers G (2008) Evaluation of indicators used to detect mussel farm influence on the benthos: two case studies in the Magdalen Islands, Eastern Canada. Aquaculture 278:77–88
88. Grant J, Hatcher A, Scott DB, Pocklington P, Schafer CT, Winters GV (1995) A multidisciplinary approach to evaluating impacts of shellfish aquaculture on benthic communities. Estuaries 18:124–144
89. Murray LG, Newell CR, Seed R (2007) Changes in the biodiversity of mussel assemblages induced by two methods of cultivation. J Shellfish Res 26:153–162
90. Dahlbäck B, Gunnarsson LAH (1981) Sedimentation and sulfate reduction under mussel culture. Mar Biol 63: 269–275
91. Tenore KR, Boyer LF, Cal RM, Corral J, García-Fernández C, González N, González-Gurriaran E, Hanson RB, Iglesias J, Krom M, López-Jamar E, McClain J, Pamatmat MM, Pérez A, Rhoads DC, deSantiago G, Tietjen J, Westrich J, Windom HL (1982) Coastal upwelling in the Rias Bajas, N.W. Spain: contrasting the benthic regimes of the Rias de Arosa and de Muros. J Mar Res 40:701–772
92. Kautsky N, Evans S (1987) Role of biodeposition by *Mytilus edulis* in the circulation of matter and nutrients in a Baltic coastal ecosystem. Mar Ecol Prog Ser 38:201–212
93. Giles H, Pilditch CA (2006) Effects of mussel (*Perna canaliculus*) biodeposit decomposition on benthic respiration and nutrient fluxes. Mar Biol 150:261–271
94. Mattsson J, Linden O (1983) Benthic macrofauna succession under mussels, *Mytilus edulis* L. (Bivalvia), cultured on hanging long-lines. Sarsia 68:97–102

95. Hartstein ND, Rowden AA (2004) Effect of biodeposits from mussel culture on macroinvertebrate assemblages at sites of different hydrodynamic regime. Mar Environ Res 57:339–357
96. Dumbauld BR, Ruesink JL, Rumrill SS (2009) The ecological role of bivalve shellfish aquaculture in the estuarine environment: a review with application to oyster and clam culture in West Coast (USA) estuaries. Aquaculture 290:96–223
97. Tallis HM, Ruesink JL, Dumbauld BR, Hacker SD, Wisehart LM (2009) Oysters and aquaculture practices affect eelgrass density and productivity in a Pacific Northwest estuary. J Shellfish Res 28:251–261
98. Brooks KM (2004) The fouling community found in association with the intensive raft culture of *Mytilus edulis galloprovincialis* in Totten Inlet, Washington. Report to the national oceanic and atmospheric administration, Seattle
99. Switzer SE, Barnes PA, McKinley RS (2008) Invertebrate fouling community composition and succession associated with Pacific oyster *Crassostrea gigas* deep-water suspended tray culture. Abstract. J Shellfish Res 27:1055
100. McKindsey CW, Lecuona M, Huot M, Weise AM (2009) Biodeposit production and benthic loading by farmed mussels and associated tunicate epifauna in Prince Edward Island. Aquaculture 295:44–51
101. Lambert G (2007) Invasive sea squirts: a growing global problem. J Exp Mar Biol Ecol 342:3–4
102. Grant J, Stenton-Dozey JME, Monteiro P, Pitcher G, Heasman K (1998) Shellfish culture in the Benguela System: a carbon budget of Saldanha Bay for raft culture of *Mytilus galloprovincialis*. J Shellfish Res 17:41–49
103. Ramsay A, Davidson J, Landry T, Arsenault G (2008) Process of invasiveness among exotic tunicates in Prince Edward Island. Canada. Biol Invasions 10:1311–1316
104. Comeau LA, Drapeau A, Landry T, Davidson J (2008) Development of longline mussel farming and the influence of sleeve spacing in Prince Edward Island Canada. Aquaculture 281:56–62
105. Heral M (1993) Why carrying capacity models are useful tools for management of bivalve molluscs culture. In: Dame RF (ed) Bivalve filter feeders in estuarine and coastal ecosystem processes. Springer, Berlin/Heidelberg, pp 455–477
106. Lam-Hoai T, Rougier C (2001) Zooplankton assemblages and biomass during a 4-period survey in a northern Mediterranean coastal lagoon. Water Res 35:271–283
107. Lam Hoai T, Rougier C, Lasserre G (1997) Tintinnids and rotifers in a northern Mediterranean coastal lagoon. Structural diversity and function through biomass estimations. Mar Ecol Prog Ser 152:13–25
108. Lindahl O, Hart R, Hernroth B, Kollberg SS, Lars-Ove L, Olrog L, Rehnstrom-Holm A, Svensson J, Svensson S, Syversen U (2005) Improving marine water quality by mussel farming: a profitable solution for Swedish society. Ambio 34:131–138
109. Newell RIE (2004) Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. J Shellfish Res 23:51–62
110. Kaspar HF, Gillespie PA, Boyer IC, MacKenzie AL (1985) Effects of mussel aquaculture on the nitrogen cycle and benthic communities in Kenerupu Sound, Marlborough Sounds, New Zealand. Mar Biol 85:127–136
111. Hatcher A, Grant J, Schofield B (1994) Effects of suspended mussel culture (*Mytilus* spp.) on sedimentation, benthic respiration and sediment nutrient dynamics in a coastal bay. Mar Ecol Prog Ser 115:219–235
112. Minchin D (2007) Aquaculture and transport in a changing environment: overlap and links in the spread of alien biota. Mar Pollut Bull 55:302–313
113. Carlton JT, Mann R (1996) Transfers and world-wide introductions. In: Kennedy VS, Newell RIE, Eble AF (eds) The eastern oyster *Crassostrea virginica*. Maryland Sea Grant, College Park, pp 691–706
114. Kaiser MJ, Laing I, Utting SD, Burnell GM (1998) Environmental impacts of bivalve mariculture. J Shellfish Res 17:59–66
115. Dolmer P (2002) Mussel dredging: impact on epifauna in Limfjorden, Denmark. J Shellfish Res 21:529–537
116. Dittmann S (1990) Mussel beds – amensalism or amelioration for intertidal fauna? Helgoländer Meeresun 44:335–352
117. Commito JA, Boncavage EM (1989) Suspension-feeders and co-existing infauna: an enhancement example. J Exp Mar Biol Ecol 125:33–42
118. Coen LD, Luckenbach MW, Breitberg DL (1999) The role of oyster reefs as essential fish habitat: a review of current knowledge and some new perspectives. In: Benaka LR (ed) Fish habitat: essential fish habitat and rehabilitation. American Fisheries Society, Bethesda, pp 438–454
119. Graf G, Rosenberg R (1997) Bioresuspension and biodeposition: a review. J Mar Syst 11:269–278
120. Norkko A, Hewitt JE, Thrush SF, Funnell GA (2001) Benthic–pelagic coupling and suspension-feeding bivalves: linking site specific sediment flux and biodeposition to benthic community structure. Limnol Oceanogr 46:2067–2072
121. Sorokin II, Giovanardi O, Pranovi F, Sorokin PI (1999) Need for restricting bivalve culture in the southern basin of the Lagoon of Venice. Hydrobiologia 400:141–148
122. Stenton-Dozey JME, Probyn T, Busby AJ (2001) Impact of mussel (*Mytilus galloprovincialis*) raft-culture on benthic macrofauna, in-situ oxygen uptake, and nutrient fluxes in Saldanha Bay, South Africa. Can J Fish Aquat Sci 58: 1021–1031
123. Holmer M, Wildish D, Hargrave BT (2005) Organic enrichment from marine finfish aquaculture and effects on sediment processes. Environmental effects of marine finfish aquaculture, Chapter 9. In: The handbook of environmental chemistry, vol 5. Springer, Berlin, pp 181–206
124. Vinther HF, Holmer M (2008) Experimental test of biodeposition and ammonium excretion from blue mussels (*Mytilus edulis*) on eelgrass (*Zostera marina*) performance. J Exp Mar Biol Ecol 364:72–79
125. Vinther HF, Laursen JS, Holmer M (2008) Negative effects of blue mussel (*Mytilus edulis*) presence in eelgrass

(*Zostera marina*) beds in Flensborg fjord, Denmark Estuarine. Coast Shelf Sci 77:91–103
126. Castel J, Labourg PJ, Escaravage V, Auby I, Garcia ME (1989) Influence of seagrass beds and oyster parks on the abundance and biomass patterns of meiobenthos and macrobenthos in tidal flats. Estuar Coast Shelf Sci 28:71–85
127. Gutierrez JL, Jones CG, Strayer DL, Iribarne OO (2003) Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. Oikos 1001:79–90
128. Asmus H (1987) Secondary production of an intertidal mussel bed community related to its storage and turnover compartments. Mar Ecol Prog Ser 39:251–266
129. Crooks JA (2002) Characterizing ecosystem-level consequences of biological invasions: the role of ecosystem engineers. Oikos 97:153–166
130. Souchu P, Vaquer A, Collos Y, Landrein S, Deslous-Paoli JM, Bibent B (2001) Influence of shellfish farming activities on the biogeochemical composition of the water column in Thau lagoon. Mar Ecol Prog Ser 218:141–152
131. Prins TC, Escaravage V, Smaal AC, Peeters JCH (1995) Nutrient cycling and phytoplankton dynamics in relation to mussel grazing in a mesocosm experiment. Ophelia 41: 289–315
132. Prins TC, Smaal AC, Dame RF (1998) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquat Ecol 31:349–359
133. Cranford PJ, Anderson R, Archambault P, Balch T, Bates SS, Bugden G, Callier MD, Carver C, Comeau L, Hargrave B, Harrison WG, Horne E, Kepkay PE, Li W, Mallet A, Ouellette M, Strain P (2006) Indicators and thresholds for use in assessing shellfish aquaculture impacts on fish habitat. Can Sci Advis Sec Res Doc 2006/034, Can Dept Fish Oceans, Ottawa, 126 pp
134. Bougrier S, Hawkins AJS, Heral M (1997) Preingestive selection of differential microalgal mixtures in *Crassostrea gigas* and *Mytilus edulis*, analysed by flow cytometry. Aquaculture 150:123–134
135. Cranford PJ, Hill PS (1999) Seasonal variation in food utilization by the suspension-feeding bivalve molluscs *Mytilus edulis* and *Placopecten magellanicus*. Mar Ecol Prog Ser 190:223–239
136. Wetz MS, Lewitus AJ, Koepfler ET, Hayes KC (2002) Impact of the Eastern oyster *Crassostrea virginica* on microbial community structure in a salt marsh estuary. Aquat Microb Ecol 28:87–97
137. Sfriso A, Pavoni B (1994) Macroalgae and phytoplankton competition in the central Venice lagoon. Environ Technol 15:1–14
138. Phelps HL (1994) The Asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, DC. Estuaries 17:614–621
139. Wall CC, Peterson BJ, Gobler CJ (2008) Facilitation of seagrass *Zostera marina* productivity by suspension-feeding bivalves. Mar Ecol Prog Ser 357:165–174
140. Ulanowicz RE, Tuttle JH (1992) The trophic consequences of oyster stock rehabilitation in Chesapeake Bay. Estuaries 15:298–306
141. Miller DC, Geider RJ, Macintyre HL (1996) Micro-phytobenthos: The ecological role of the "secret garden" of unvegetated, shallow-water marine habitats. II. Role in sediment stability and shallow-water food webs. Estuaries 19:202–212
142. Reusch TBH, Chapman ARO, Groger JP (1994) Blue mussels *Mytilus edulis* do not interfere with eelgrass *Zostera marina* but fertilize shoot growth through biodeposition. Mar Ecol Prog Ser 108:265–282
143. Reusch TBH, Williams SL (1998) Variable responses of native eelgrass *Zostera marina* to a non-indigenous bivalve *Musculista senhousia*. Oecologia 113:428–441
144. Peterson BJ, Heck KL Jr (1999) The potential for suspension feeding bivalves to increase seagrass productivity. J Exp Mar Biol Ecol 240:37–52
145. Peterson BJ, Heck KL Jr (2001) An experimental test of the mechanism by which suspension feeding bivalves elevate seagrass productivity. Mar Ecol Prog Ser 218:115–125
146. Peterson BJ, Heck KL Jr (2001) Positive interactions between suspension-feeding bivalves and seagrass – a facultative mutualism. Mar Ecol Prog Ser 213:143–155
147. Swanberg IL (1991) The influence of the filter-feeding bivalve *Cerastoderme edule* L. on microphytobenthos, a laboratory study. J Exp Mar Biol Ecol 151:93–111
148. Mazouni N (2004) Influence of suspended oyster cultures on nitrogen regeneration in a coastal lagoon (Thau, France). Mar Ecol Prog Ser 276:103–113
149. Asmus H, Asmus RM (2005) Significance of suspension-feeders systems on different spatial scales. In: Dame RF, Olenin S (eds) The comparative roles of suspension- feeders in ecosystems. Springer, Berlin, pp 199–219
150. Sara G (2007) A meta-analysis on the ecological effects of aquaculture on the water column: dissolved nutrients. Mar Environ Res 63:390–408
151. Boyd CE, Lim C, Queiroz J, Salie K, de Wet L, McNevin A (2008) Best management practices for responsible aquaculture [Online]. http://pdacrsp.oregonstate.edu/pubs/featured_titles/boyd.pdf
152. Belle S, Bullis RA, Elston R, Goldburg R, Hardy RW, Hargreaves JA, Lockwood GS, Mayo RA, Nelson CL, Reid B, Tacon AGJ, Quagrainie KK (2008) Revisions to the supplemental to interim final report (Bivalve molluscs) of the Aquaculture Working Group. In: Response to public comments. National Organic Program, U.S. Department of Agriculture, Washington, DC
153. Oesterling M, Luckenbach M (2008) Best management practices for the Virginia Shellfish culture industry [Online]. http://web.vims.edu/adv/aqua/MRR%202008_10.pdf?svr=www
154. Pacific Coast Shellfish Growers Association (2005) *Shellfish production on the West Coast* [Online] http://www.pcsga.org/pub/uploads/production.pdf. Accessed Aug 2009

155. Syvret M, Fitzgerald A, Hoare P (2008) Development of a pacific oyster aquaculture protocol for the UK—technical report. Sea Fish Industry Authority, Edinburgh
156. Leavitt DF (ed) (2004) Best management practices for the shellfish culture industry in Southeastern Massachusetts [Online]. http://www.mass.gov/agr/aquaculture/docs/Shellfish_BMPs_v09-04a.pdf. Accessed Sep 2009
157. Maryland Aquaculture Coordinating Council (2007) Best management practices: a manual for Maryland aquaculture [Online]. http://www.marylandseafood.org/pdf/best_management_practices_manual.pdf. Accessed Sep 2009
158. Irish Sea Fisheries Board (2003) ECOPACT: environmental code of practice for irish aquaculture companies and traders. Irish Sea Fisheries Board, Dublin
159. Bronson C (2007) Aquaculture best management practices rule. Division of Aquaculture, Florida Department of Agriculture and Consumer Affairs, Tallahassee
160. Maine Aquaculture Association (2006) Recommended code of practice for aquaculture in maine. In: Howell L, Belle S (eds) Maine aquaculture association. Hallowell, Maine
161. National Oceanic and Atmospheric Administration (1998) NOAA's aquaculture policy. National Oceanic and Atmospheric Administration, Silver Spring
162. Creswell RL, McNevin AA (2008) Better management practices for bivalve molluscan aquaculture. In: Tucker CS, Hargreaves JA (eds) Environmental best management practices for aquaculture. Blackwell, Oxford, UK
163. Tucker CS, Hargreaves JA (eds) (2008) Environmental best management practices for aquaculture. Blackwell, Oxford, p 592

Books and Reviews

Costa-Pierce BA (2010) Sustainable ecological aquaculture systems: the need for a new social contract for aquaculture development. Mar Technol Soc J 44(3):88–112

Soto D, Hishamunda N (eds) (2008) Building an ecosystem approach to aquaculture. Food and Agriculture Organization of the United Nations, Rome, p 231

Simulation Models as Tools for Crop Management

Herman van Keulen
Chairgroup Plant Production Systems, Wageningen University, Wageningen, The Netherlands
Business Unit Agrosystems Research, Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands

Article Outline

Glossary

Strategic crop management Decisions with respect to agro-ecosystems on the basis of the expected long-term performance of these systems, as influenced by the natural resource base and the socioeconomic boundary conditions, independent of the current state of the system.

Tactical crop management Seasonal decision-making in reaction to the variable and unpredictable environment, with a time horizon of the order of 1 year, independent of the current state of the system.

Operational crop management Decisions concerned with daily activities of the farm, in response to the current state of the system and the (anticipated) environment.

Simulation Building a model and studying its dynamic behavior.

Model Simplified description of a system.

System A limited part of reality with well-defined boundaries, containing related elements.

Decision support system (DSS) A class of information systems (including but not limited to computerized systems) that support business and organizational decision-making activities. A properly designed DSS is an interactive software-based system, intended to help decision makers compile useful information from a combination of raw data, documents, personal knowledge, or business models to identify and solve problems and make decisions.

Farming system The particular mix of agricultural activities in which a farm household engages.

Definition of the Subject

Agricultural production can be defined as the transformation of sun energy in useful organic material in the form of food, feed, and fiber. The transformation requires in principle only limited resources: a piece of land, some seeds from a wanted plant species, some sun and rain, and some human labor. However, the transformation takes place under erratic and unpredictable conditions, as especially the availability and timing of the sun and the rain are extremely difficult, if not impossible to predict, while their effects are modified by the qualities of the land and the interventions of the farmer. Any methodology that would improve the predictability of the availability of the resources and their impact on the performance of the production system could in principle improve that performance and reduce the level of uncertainty. Crop growth simulation models that were developed from the late 1960s onward and that allowed exploration of the performance of plant production systems as governed by the properties of the plant species/variety in interaction with the environmental conditions, promised to be excellent tools for the reduction of this uncertainty. Following 40 years of experience in this realm of the application of crop growth simulation models in support of the management of plant production systems, the current situation is still highly ambiguous. On the one hand, some of the modelers still advocate the use of these tools in crop management, while many others have become disenchanted and emphasize their limited impact on crop performance. Although definitely the last word has not been said about their advantages and disadvantages, in this contribution an attempt will be made to summarize the past experiences and the current state of affairs, and to make an effort to identify their possible contribution in the future. As mentioned, there appear large differences among agricultural and systems-analytical specialists in judging the contribution and the

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

possibilities for application of crop growth models in support of crop management, and thus this contribution will not lead to unequivocal judgments and recommendations, but it should serve as a starting point for those interested in the subject.

Introduction

Crop Management

Performance of agricultural production systems at any given location is determined on the one hand by the agro-technical possibilities and constraints and on the other by the socio-economic conditions under which the farmer and his customers operate. The agro-technical possibilities are largely determined by the quality of the natural resources, most importantly, soil characteristics and the prevailing climatic conditions. Agricultural systems that have developed in various regions are therefore strongly linked to these characteristics of the environment. However, within comparable natural environments, the viability of various agricultural systems is dependent on the socio-economic environment, which can broadly be characterized by the prevailing price ratios of crop products and inputs, modified by other factors, such as the risk that can be accepted, access to credit facilities, and the relative importance attached to other objectives than purely economic, for example, environmental considerations. In agricultural production systems, decision making can be schematically subdivided in three hierarchical levels [1]. The first level is that of *strategic management*, that is, strategic decisions with respect to agro-ecosystems, made on the basis of expected long-term performance of these systems, determined by the (quality of the) natural resource base and the socio-economic boundary conditions, but independent of the current state of the system and its expected short-term performance. Strategic management is mainly oriented toward long-term planning and deals with the selection of farming systems, that is, the particular mix of agricultural activities in which the farm household engages (e.g., arable farming, horticulture, animal husbandry), and the associated investment decisions. The time horizon for such decisions varies therefore from more than 1 year up to 10 or 20 years, that is, the time it takes, for instance, for machinery to become technically obsolete, or for farmers to become acquainted with new production techniques.

Once the strategic decisions have led to the selection of a certain cropping system, the farmer, in actual practice, is faced with the task of implementing a certain number of crop systems. A conspicuous characteristic of agricultural production systems is that they operate in highly variable and hardly predictable natural environments [2]. Although climate for a given region may be characterized on the basis of long-term records, that hardly helps the farmer in his actual decision-making, as year-to-year variability is generally large and the actual performance of the system is dependent on weather conditions rather than on climate. Therefore, in most agricultural production systems, the quality of the *tactical decision-making process*, that is, the reaction to this variable and unpredictable environment, is a major factor in determining their success. The time horizon of tactical management, that is, seasonal decision making, is therefore in the order of 1 year, dealing with such issues as the selection of a specific crop (and/or variety) mix to be cultivated and the allocation of land, both in terms of how much and where, to the various crops.

Finally, decisions at the *operational level* are mainly concerned with daily activities on the farm, such as selection of sowing dates, water (irrigation), nutrient (fertilizer), and pest and disease management, and time horizons varying from days to, sometimes, hours in relation to decisions on spraying and harvesting.

Crop Modeling

Crop modeling as a scientific activity has been in existence now for almost 50 years, following among others, the pioneering work of De Wit (cf. [3–5]). Its history and its position within current agricultural research have been dealt with extensively in the last two decades from various angles [6–10]. An interesting analysis is that of Sinclair and Seligman [6], who draw parallels between the growth and development of crop simulation models and human development. Their overview ranges from *birth* and *infancy* of the discipline with the appearance of mainframe computers in the 1960s, via a *juvenile* stage in the 1970s, and an *adolescent* stage in the 1980s, to a *maturity* stage in the 1990s. The *infancy* and *juvenile* stages were characterized by the

expectation that crop modeling would provide the answers to questions in many areas of crop science, which led to the development of detailed "comprehensive" models, aiming mainly at increasing understanding of the interactions between the crop and its environment.

These development stages of the models were subsequently translated into functional categories [9], that is, in the 1960s and 1970s, the main aim of these modeling activities was to generate understanding at the crop scale, on the basis of the underlying processes and to test hypotheses on crop-physiological functioning, as illustrated among others in the series Simulation Monographs (cf. [11]). In the 1980s, major efforts were directed to obtaining full understanding of crop performance under a wide range of environmental conditions. In the 1990s, the focus shifted toward applications in agronomic practice and policy making. In the 1990s, crop models also found their application in studies at higher levels of integration, that is, farm and regional scale. In these studies, crop models were used to quantify a broad range of land use systems; subsequently, these land use systems were aggregated to farm or regional scale using various techniques (e.g., linear programing) or procedures. Studies on designing environmentally friendly systems for arable, dairy, and flower bulb farms were conducted, also enabling analysis of trade-offs between economic and environmental objectives.

Land use studies were carried out with a focus on interactive exploration of different strategies for the European Union, Mali, Costa Rica, and Southeast Asia. Finally, crop models were used to explore limits for food production capabilities at global scale.

In the beginning of the twenty-first century, all of the functions of crop growth models continued to be performed and improved, but the development efforts shifted to reuse of models and to their applications for policy support [12].

Crop simulation models were thus originally developed as research tools and have had their greatest usefulness and impact so far as part of the research process. The advantages of integrating simulation modeling approaches into a research program have adequately been summarized [13]: (1) identification of knowledge gaps; (2) generation and testing of hypotheses, as an aid to the design of experiments; (3) determination of the most influential parameters of a system (sensitivity analysis); (4) provision of a medium for better communication between researchers in different disciplines; and (5) bringing researchers, experimenters, and producers together to solve common problems.

In addition to their use as tools in research, almost from the beginning of their development, crop models have been advocated as tools to help in decision-making processes of practitioners, with the development of so-called decision support systems (DSSs) for crop management. DSSs have evolved over the years from rudimentary single-decision rules to multiple-criteria optimization software. In its simplest form, a DSS can be a pest management threshold, calculated using empirical relations and field data on a calculator. In a sophisticated form, it can be an interactive computer system that utilizes simulation models, databases, and decision algorithms in an integrative manner. DSSs typically have quantitative output (as opposed to an expert system with qualitative reasoning) and place emphasis on the end user for final problem solving and decision-making. There is often no clear-cut boundary between models used in research and those used in DSS, that is, in the literature, models used in research are promoted in terms of their potential to aid in decision-making, although there is not always evidence that they have been or are being used for this purpose!

Simulation Models in Support of Crop Management

Crop modeling has helped in finding improved ways to manage crops, fields, and farms in practice, but the pathway has been problematic. Early expectations of computerized decision support systems as the connecting vehicle between crop models and management practice have, to a large extent, been unrealized. Nonetheless, some lessons have emerged from the attempts. It has been argued that the most significant contribution of these attempts at decision support has not been the actual production of decision support systems, but rather the bringing together of researchers and farmers to improve farm management [14]. Alternatively, it has been argued that for successful development and adoption of simulation approaches: (1) issues to be addressed must be neither trivial nor

obvious, (2) a modeling approach must reduce complexity rather than proliferate choices in order to aid the decision-making process, and (3) the cropping systems must be sufficiently flexible to allow management interventions based on insights gained from models [15]. Despite the limited application of operational decision support systems in actual commercial farming at the moment, a wide variety of such systems have been described. Without trying to be exhaustive, a number of illustrative examples are given here, following the classification of strategic, tactical, and operational decisions, as explained earlier.

Strategic Management

Strategic management support does not aim at generating answers for management to practitioners, but focuses on facilitating and supporting the dialogue with various stakeholders. This has led to the development of "discussion" support software [16], systems designed to facilitate dialogue about management practice that is relevant and significant to the decision maker. There exist significant opportunities for current capabilities in cropping systems analysis and modeling to contribute better to discussions about long-term economic and ecological issues associated with agricultural production systems. A number of these opportunities have been explored during the 1990s and cropping system simulation tools, such as APSIM (Agricultural Production system SIMulator; [17, 18]), CropSyst [19, 20], STICS [21], and DSSAT (Decision Support System for Agrotechnology Transfer; [22]), were designed with these targets in mind.

Increasingly over the last decade, crop models have been used to explore and design options for farm and regional land use systems. Studies at farm and regional scale require proper coverage of the system's physical conditions and the agroecological production options. Once sufficient information on soils and climate of an agricultural production system is available, crop models are useful tools for assessing their performance, that is, quantifying the inputs and outputs of (new) production systems. Two ways of using crop-soil models for farm and regional scale studies have been developed and elaborated. In the first method, crop yields are simulated for a range of well-defined (by soil and climate) points in a region and a geographical information system (GIS) is used to interpolate input data and aggregate results for the entire region. This method is generally suited to analyze or evaluate a limited number of land uses with little or no spatial interaction, but if necessary, with significant physiological detail. Examples of such approaches are an agroecological zonation for potato production at global scale [23] and an assessment of potential global food supply for a number of scenarios with respect to food demand, using low and high external input production systems [24]. In the second approach, crop models, supplemented with empirical relations and expert knowledge, are incorporated in so-called technical coefficient generators (TCGs) for production activities (e.g., [25, 26]). In this approach, crop models are used to quantify a broad range of land use systems in terms of inputs and outputs, which are subsequently aggregated to farm or regional scale using bio-economic optimization models (e.g., [27–34]). Examples of such TCGs are PASTOR and LUCTOR [35] and TechnoGIN [36]. The use of TCGs in combination with optimization models is particularly helpful in situations where many alternative crops and production technologies must be evaluated concurrently, and where spatial or temporal interactions are limited or less relevant for the type of questions to be addressed. The approach has proven to facilitate effective cooperation between agro-ecological and economic disciplines, and enables analysis of trade-offs between economic and environmental objectives at farm and regional scales.

Tactical Management

Tactical management basically refers to the medium term and deals with decisions that are taken once during the growing season, with respect to such issues as crop/variety selection, allocation of land to various crops, and identification of the most appropriate sowing or planting date.

An early example of a tactical decision support system is the program TACT, targeted at farm advisers and consultants but as the authors assure, readily useable by farmers [37]. It includes a simulation model of wheat performance (development, biomass accumulation, and yield) with a user-friendly interface. It calculates probability distributions of yield and gross

margin, using data on location, soil type, wheat variety, sowing time, and weather conditions to date, and historical rainfall records. In addition to calculating expected crop performance, TACT can also be used for climatological analyses which have an impact on 'break of season' decisions, using criteria defined by the program user in combination with historical daily rainfall and temperature records to investigate, for example, the incidence of frost. Other suggested applications at the time were calculating the probabilities of sowing opportunities, given a sowing rule and the probability of a dry period after a given date. This kind of information aids tactical decisions by estimating the risks of replanting if crops are sown early and the penalties for missing an early sowing opportunity. Most of the analyses generate a distribution of expected outcomes, for example, yield, which is used to derive probabilities.

TACT could be tailored to individual circumstances in two ways. Users could select from a wide range of parameters from TACT program menus, such as location, soil type, and sowing rule. These parameters tailor either the simulation model or climatic analyses to the user's specifications. Users could also adopt a probability level in the output, consistent with their attitude to risk.

In the last decade, computer-aided support for tactical decision making has received substantial attention, especially since it appeared possible to generate a characterization of in-season rainfall in (semi-)arid regions on the basis of, for instance, the El Niño/Southern Oscillation (ENSO) phenomenon [38]. Especially in (northeast) Australia this procedure has been relatively successful, because of three factors: a highly variable environment, which heightened riskiness of crop production decisions [39]; some ability to anticipate risks by utilizing seasonal climate forecasting [40, 41]; and a research culture that fostered close linkages between researchers and decision makers [42]. To facilitate crop management discussions in farmer-driven workshops, using analyses of management options based on crop simulation and seasonal climate forecasts, the Whopper Cropper software tool was designed [43]. As an example of using Whopper Cropper in relation to an issue involving seasonal climate forecasting, consider farmers in the Callide valley of Central Queensland, Australia, planning summer cropping options. For this scenario, it is November and the SOI phase [40] for September–October was positive. The farmers have heard that a positive SOI at this time of year means an improved chance of a better-than-usual season in terms of rainfall, and are considering sowing sorghum earlier than usual. The clay soil on properties in this area has a plant available water-holding capacity (PAWC) of about 170 mm, and is two-thirds full of moisture after good spring rain. To start with, it was assumed that a medium-maturing hybrid of sorghum was sown at a density of 10 plants m^{-2} on November 15 and that total nitrogen available to the crop (the amount in the soil plus the amount applied) is 100 kg ha^{-1}. By conducting a simulation of this scenario with the 100-year historical climate record for the location, a 100-year time series of yield outcomes was generated, with each year differing only in the seasonal climate realized. Time series analysis showed the year-to-year variability in yield for this scenario, compared to the long-term median, and then compared to those years in which the September–October SOI phase was positive. For this scenario, a positive SOI phase is often, but not always, associated with higher-than-median yields. Similar graphs can be produced for each SOI phase. The value of seasonal climate forecasting based on SOI phases in crop management stems from the shift in the expected yield distribution associated with each of the five phases [44]. For the sorghum cropping scenario, the distribution of yield associated with years having a positive SOI phase at the end of September–October has higher median yield and reduced risk of low yield compared to the distributions associated with years in the other phase categories. Products such as Whopper Cropper can be used for discussions about tactical decision-making, but can also be used to encourage debate on the design of planned and flexible rotations.

The El Niño-Southern Oscillation (ENSO) also contributes to the vulnerability of crop production to climate variability in the Pampas region of Argentina. Predictability of regional climate anomalies associated with ENSO provides opportunities to tailor decisions to expected climate, either to mitigate expected adverse conditions or to take advantage of favorable conditions. Model analysis was used to explore the potential for tailoring land allocation among crops to ENSO phases at the farm scale in two subregions of the Pampas [45]. The model identifies, as a function of risk preferences and initial wealth, the crop mix that

maximizes expected utility of wealth at the end of a 1-year decision period, based on current costs and prices, and crop yields simulated for each year of historical weather. The model reproduced recent land allocation patterns at the district scale under moderate risk aversion, and predicted increasing diversification with increasing risk aversion. Differences in land allocation among ENSO phases were consistent with known climate response to ENSO, and crop response to water availability. The relationship between the potential value of ENSO information and risk aversion was not monotonic, and differed between locations. Crop mix and information value also varied with crop prices and initial soil moisture. There are potential financial benefits of applying this approach to tailoring decisions to ENSO phases.

It was also shown that a similar procedure yielded relevant results for Kenya [46], thus increasing confidence in the use of seasonal weather forecasts for tactical crop management.

Water Management Crop growth simulation models have been used extensively to analyze the effects of tactical crop water (irrigation) management. In most instances, various water management regimes are being tested under local conditions, sometimes with the aim of exploring the scope for improvements in actual management practices, sometimes with the aim of contributing to the discussion on water management. Without trying to be exhaustive, a few typical examples are given.

The ORYZA W model [47] was used [48] to evaluate alternative management options for rice production in the light of farmers' attitudes toward risk. The model was used to generate probability distributions of rainfed lowland rice yields under different management scenarios which included water management (bund height, puddled soil depth, planting density, seedling age at transplanting). Stochastic dominance analysis was applied to identify risk-efficient management options. It appeared not possible to evaluate to what extent farmers (or extension officers) actually (have) apply(ied) the methodology in decision-making in commercial farming.

The interactive computer program WIRROPT7, a modification of the CERES-Wheat model, was applied [49] to explore the possibilities for the intra-seasonal irrigation regime that maximizes total gross margin for particular soil, weather, and crop management combinations, within the constraints of land and water availability. The use of the system was demonstrated for two soils near Harare in Zimbabwe and for two strategies – maximizing gross margin per unit area with a frequent irrigation schedule, or maximizing overall profits by reducing the application per unit area by irrigating less frequently but growing a larger area. As the variability of yields and gross margins are higher with the second strategy, the farmer's attitude to risk will determine which strategy he or she adopts. The authors recommend that the model should be seen only as a guide to irrigation management due to its limitations such as lack of routines describing nutrients and pests and the need to use mean historical weather data. The actual irrigation strategy should take into account real-time estimates of water use as well. It is not stated if the system is actually being used by farmers or extension staff.

The Environmental Policy Integrated Climate (EPIC) model was used to evaluate its application as a decision support tool for irrigation management of cotton and maize under South Texas conditions [50]. Simulation of the model was performed to determine crop yield, crop water use, and the relationships between the yield and crop water use parameters such as crop evapotranspiration (ETc) and water use efficiency (WUE). On-farm simulation results in this study demonstrated that the EPIC model can be used as a decision support tool for crops under full and deficit irrigation conditions in South Texas. EPIC specifically appears to be effective in long-term (strategic) and preseason (tactical) decision making for irrigation management of crops. However, more studies are needed to employ the model as a decision support tool addressing other irrigation issues such as operational management, dealing with irrigation allocation and scheduling.

Within the Australian cotton industry, the imperative to reduce water use and optimize irrigation management through the understanding of risk, using information generated by computerized decision aids was identified and subsequently developed into the HydroLOGIC irrigation management software [51]. On-farm experiments throughout the development period allowed the validation of internal software

logic, irrigator decision processes, and the OZCOT cotton growth model [52]. The software demonstrated the ability to improve yield and water use efficiency by optimizing strategic and tactical irrigation decisions in the Australian furrow irrigation cotton production system. In 7 of the 11 on-farm experiments conducted, the use of HydroLOGIC helped improve overall field water use efficiency by optimizing the timing of irrigation events or by indicating further irrigations would not provide yield or maturity benefits. Using concise reports, users can easily assess the risk of different irrigation management options at any crop stage, from land preparation through to postharvest benchmarking. HydroLOGIC may also be used within an integrated whole farm water management planning process to provide information on the impact of water allocations and subsequent evaluation of water security, water cost, and risk.

Nutrient Management As a basis for formulation of crop nitrogen requirements for rice, the ORYZA 0 model was developed and tested [53, 54], a "parsimonious" model based on incident solar radiation, bulk leaf nitrogen, and a site calibration factor. The model was subsequently applied [55] to classify some irrigated rice soils in India into those in which the soil N supply was sufficient to meet crop demand up to the onset of flowering and those that required a basal dressing of N fertilizer. The results were then used to generate fertilizer N recommendation curves that identified different optimal timing of N application for the different soil N supply regimes. The model was also used in a similar exercise in China, where it was found that significantly higher yields were obtained by following the recommendations produced by the model compared to the local recommendations [56]. Whether the model is able to make significant improvements over local recommendations in a wider range of environments remains to be seen. A limitation of ORYZA 0 is that it must be calibrated for each site, in this case by measuring the seasonal pattern of crop N uptake, which, because of year-to-year variation, makes it difficult to use the model in a predictive way. Also, no account is taken of the ability of the soil to act as a reservoir or "bank" of nitrogen – that is, nitrogen applied earlier in the season can remain in the soil for uptake by the crop later, so that it is not critical when the fertilizer is applied. The model only indicates how much nitrogen must have been applied by a specific crop age, and not when and how (i.e., as a single dressing or as split doses) it should be applied.

In a study for Walbundrie in Southeast Australia, the Agricultural Production System Simulator (APSIM) was used to simulate crop production and drainage passing the crop root zone of wheat and canola crops in response to nitrogen application rates from 0 to 300 kg N ha^{-1} $year^{-1}$, using historical climate records from 1889 to 2002 and variable values of stored soil water at sowing time (SSMS) [57]. The capability of the Southern Oscillation Index (SOI) phases to forecast growing season rainfall and crop yield was analyzed. The results showed that the optimal N rates were 100, 150, and 200 kg N ha^{-1} $year^{-1}$ in the years when April/May SOI phases were falling/negative, near zero, and rising/positive, respectively. Combining April/May SOI phases and SSMS to manage N application increased wheat gross margin, and reduced deep drainage for wheat, compared with N management based on historical climate data only. Similar results were obtained for a continuous canola cropping system.

Pest Management InfoCrop, a generic crop growth simulation model [58] was applied to simulate rice planthopper damage on Pusa Basmati 1 rice [59]. The model was used to simulate economic injury levels (EILs) of planthoppers and iso-loss curves, depicting combinations of crop age and planthopper populations that resulted in similar yield losses. Combinations of EILs and iso-loss curves can be used in monitoring planthopper populations and promoting judicious pesticide applications to avoid unwarranted control expenditure and environmental contamination. The simulation models, based on detailed crop ecological and physiological processes and pest damage mechanism can thus aid in the development of location-specific decision support tools and ensure precision in pest management decisions.

Operational Management

Operational management refers to day-to-day management of agricultural production systems, and deals with decisions by individual farmers on timing and intensity of interventions in their crops. From the

very beginning of the development of crop growth simulation models, their capabilities for support in operational management have been recognized. In hindsight, it must be concluded that in actual practice, at the moment, only a very limited number of such operational support systems are actually being applied at large scale. The scope for the use of crop growth simulation models as part of operational decision support systems has been expanded in the last decade through integration of mechanistic crop growth simulation models with earth observation techniques. Both, simulation modeling and remote sensing have been shown to be valuable tools in separate applications in agriculture. Integration of both techniques can lead to improvements in the dynamic simulation of the crop-soil system and thus contribute to improvements in management decision support systems for environmentally sound agricultural production [60–63].

In this section, a number of the decision support systems for operational management are illustrated that have been proposed, subdivided in the categories derived from the production situations [25], that is, water management (including irrigation), nutrient management (including fertilizer application), and crop protection management (including weed control and pest and disease control).

Water Management In many agricultural production systems, availability of water is the yield-determining factor, and therefore water management is an important aspect of crop management. The objective generally is to supply supplementary water to the crop in such a way that the water constraint is alleviated, the applied water is used as efficiently as possible, and, in some cases, specific water quality criteria are met.

Irrigation scheduling is an area in which models have been used extensively as decision support systems. It has been claimed [64] that there are at least 140 models based on the use of the water production functions developed by FAO [65]. However, he cautions that such models do not correctly forecast the effect of water stress on crop growth, as they do not take into account the dynamic processes. In mechanistic crop growth models such as CERES–Maize [66], EPIC [67], and CROPSYST [20], the effects of soil water depletion in the course of the crop growth cycle are simulated. They can, therefore, be used as effective tools for forecasting the water content of the soil and the crop response to it [64].

As an early example, the use of the real-time crop growth model EPIC-PHASE is illustrated [68], functioning at a daily time step, as a new method that might support the irrigator in answering questions such as "how much" and "when" to irrigate. Their results showed the potential progress that the irrigator could make by scheduling irrigation according to the predictions of water stress intensities. However, the efficiency of this schedule is dependent on the accuracy of weather forecasts. The study noted that the simulations gave a large difference in the choice of operational irrigation, depending on whether weather forecasts or actual weather data were used. From an agronomic standpoint, it is necessary to test other methods such as updating the weather forecasts every 24 h, or determining, in terms of risk on yields, the weight of possible climatic errors according to the crop phase, thus allowing the irrigator to take his decision to irrigate or not with a full knowledge of the facts.

A pilot irrigation scheduling project was established in Northern Zululand in South Africa on a commercial estate [69]. Meteorological variables were measured with an Automatic Weather Station (AWS) and the data transmitted electronically to the experimental station every week. A model was used to estimate the soil water content on a daily basis. A report on the current soil water status was then generated and advice on when next to irrigate was sent to the irrigator.

In Denmark, irrigation scheduling based on local experience and rules of thumb proved inadequate to deal with the increasing complexity of water management. A PC-based DSS (MARKVAND) was developed in response to a need to find more efficient forms of irrigation due to increasing water demands in different sectors of society in Denmark [70]. It is being used to give daily information on the timing, amount, and economic net return of irrigation for a wide group of agricultural crops. The model includes conceptual and empirical sub-models for crop development, water balance, and crop yield.

In the United Kingdom, Irrigation Management Services (IMS) provided a consultancy service that gave farmers weekly advice on which fields to irrigate, when and how, based on the results of computer

simulation [71, 72]. IMS employs a simple water balance model with crop evapotranspiration estimated on the basis of soil, crop, and weather factors. It was used between 1984 and 1989 to provide an irrigation scheduling service to farmers and growers in eastern England in conjunction with in-field monitoring of soil water and crop cover [72]. When the service began in 1984, it operated on a bureau basis with communication by phone combined with farm visits. Farmers saw this personal touch as beneficial, since it gave them confidence in the service and enabled them to discuss broader issues with the adviser. However, by 1990, the use of microcomputers had become more common and farmers began to demand the scheduling packages themselves as this was greatly cheaper than paying for a consultancy service [72]. In time, some farmers eventually felt experienced enough in estimating when to irrigate such that they no longer needed to use the model. Despite the technological advancements in irrigation scheduling, most irrigators do not use the real-time procedures that have been developed by scientists. However, it has been argued [73] that if the efficiency of irrigation is to improve in order to help meet the increased demands for water from all sectors of society, such tools must be adopted. The challenge to researchers is to develop economically viable technology that is readily adaptable for use by farmers. This requires more interactive communication between researchers, extension staff, and farmers for improving the transferability and applicability of irrigation scheduling techniques [73].

Recently [74], an approach was presented to explore water management options in irrigated agriculture, combining remote sensing, crop growth modeling, and optimization, taking into account the constraints of water availability and the heterogeneity of irrigation system properties. The method contains two components: (i) system characterization using a stochastic data assimilation procedure where the irrigation system properties and operational management practices are estimated using remote sensing (RS) data; and (ii) water management optimization, exploring water management options under various levels of water availability. A soil–water–atmosphere–plant model (SWAP) is applied in a deterministic–stochastic mode for regional modeling. The distributed data, for example, sowing dates, irrigation practices, soil properties, depth to groundwater, and water quality, required as inputs for the regional modeling is estimated by minimizing the residuals between the distributions of field-scale evapotranspiration (ET) simulated by the regional application of SWAP, and a surface energy balance algorithm for land (SEBAL; [75]) using two Landsat7 ETM+ images. The derived distributed data were used as inputs in exploring water management options. A generic algorithm was used in data assimilation and water management optimizations.

The results showed that regional crop productivity can be improved by considering water and crop management practices as one, not as independent entities under limited water conditions. Adjusting sowing dates and their distributions in the irrigated area were found to impact positively the regional yield. This management option could complement the practice of deficit irrigation. On average, the farmers could allow their crops to experience water stress of about 27% before irrigation, with the current conditions in the study area. This could result in an increase of about 8.5% in the expected regional wheat yield and a regional water productivity of 1.6 kg m^{-3}. When water supply is very limited, high equity in water distribution could result in better performance of the irrigation system, and this should be also complemented by an earlier date of sowing in the growing season with wider distribution. This is also true when water is non-limiting but the farmers have higher degrees of freedom in their planting activities. There is an optimum point where the benefit would justify additional use of water for irrigation, beyond this point, water should be saved.

In a more proactive mode, if a seasonal climate forecast is available, the approach can be applied to explore water management decisions before the wheat-growing season [76].

Nutrient Management In the Netherlands, in an interdisciplinary project, nitrogen-fertilizer recommendations were formulated on the basis of results of a simulation model [77]. The model was initialized in early spring on the basis of field measurements of crop biomass and soil mineral nitrogen contents. Anticipated wheat yields were calculated on a weekly basis using measured weather data until the day of simulation and historical weather data for a 20-year period

prior to the season of prediction. Expected yield and its range were then calculated from the simulated yields for the 20-year period. Fertilizer recommendations were derived from these values on the basis of simulated N deficiencies in the crop. The results, following 3 years of testing on a number of experimental and commercial fields in the Netherlands, were rather disappointing, as final yield appeared strongly dependent on the actual weather conditions (temperature, determining the length of the grain filling period and radiation, determining the rate of grain growth) after flowering of the wheat crop. At that point in the crop's phenology, total crop nitrogen content can hardly be changed anymore (uptake takes place essentially before flowering), so that no adjustment was possible. The recommendations were therefore not better than those based on measured mineral nitrogen content in the soil profile in early spring, the common method at that time in the Netherlands.

AmaizeN [78] is a decision support system to help maize growers schedule nitrogen (N)-fertilizer applications for site-specific maize crops. It forecasts crop yields and N-fertilizer application rates for potential yield and best economic returns, and predicts the consequences of user management decisions. It takes into account both crop production and environmental impact. In an evaluation study with 16 field trials, covering a wide range of weather and soil conditions, AmaizeN-predicted maize yields (for both silage and grain) matched field measurements well, and gave a reasonably good indication of silage crude protein content and silage harvest date. The system was also capable of estimating N-leaching during the cropping season and predicting residual soil mineral-N at the end of the season, but more effort is needed to improve the accuracy of some predictions. In all instances, the AmaizeN-recommended N-fertilizer strategy was more efficient than the growers' practice. Recommended N-fertilizer rates were on average 85 kg ha^{-1} less than conventional application rates across ten crops, with no yield reduction. Its recommended higher-than-conventional application rate at another crop brought about a significant yield increase. System development was guided by an industry user group who requested the decision support system interface to be 'simple and easy to use'. To ensure user adoption of the system, some compromises in system prediction accuracy were required. Local agricultural production conditions were also incorporated.

Pest and Disease Management Because of the complex relationships between crops and their pests and diseases, and because populations of pests and diseases are dynamic in nature, a systems analysis approach is required to understand how pest and disease problems arise and how they may be tackled. In the case of pest and disease populations themselves, the approach bridges the gap between knowledge at the individual level and understanding at the population level.

The EPIPRE (EPIdemic PREvention) system was developed between 1977 and 1981 in the Netherlands as a system for supervised control of diseases and pests in winter wheat [79] and was intended to reduce the use of chemical crop protection agents. A major advantage of the system was that the farmers, recruited from Wheat Study Clubs, were able to learn as they went along and were always aware of how the system worked. They were trained to recognize disease symptoms, carrying out their own disease and pest monitoring, and sending their field observations to a central team who entered them on a daily basis into a computerized data bank. The system produced recommendations for treatment by optimizing financial returns for crop protection. There were three major decision options: (1) treat, (2) do not treat, and (3) make another field observation. When the season was over, every farmer received a record of his actions and the recommendations, as well as a financial account of the crop protection activities. The results were discussed with farmers in regional meetings. The farmers listened critically and often expressed appreciation of their improved expertise. Eighty-five percent of farmers participating in 1 year participated in the next. Complete adherence varied between 20% and 80%. Partial adherence was far greater than full adherence [79]. In 1981, 6% of Dutch winter wheat was covered by the system [80] saving on average £15 ha^{-1} [79]. In Belgium, the system functioned under the auspices of the National Soil Service, and in 1992, EPIPRE was used to advise on disease control in 500 fields in Belgium and northern France [81]. The research in Sweden (from 1982 to 1985) found that although EPIPRE was an interesting and useful system, it would require alteration if it were to

be introduced to farmers for routine use. There was a need to incorporate meteorological parameters into the model and to adjust the plant growth model to suit Swedish conditions, as the model was still recommending unnecessary spraying [82]. Experience in Switzerland found that EPIPRE-treated fields yielded 3% less than traditionally treated fields (0.5% with corrected gross return), but that farmers were able to reduce the spray frequency by 20–100%. The conclusion was that although the farmers did not make more profit, the new practice was ecologically beneficial and helped to reduce selection pressure for pesticide resistance [83]. It was calculated that in 1981, if all Dutch winter wheat would have been treated with the EPIPRE recommendations, the pollution load would have been reduced by 12 Mg [79]. Characteristic for the EPIPRE system was that there was an initial steady increase in membership, which resulted in an improvement in pest management. However, subsequently membership declined, as the growers felt that they had learned what the model would predict and therefore did not need it any more [6]. The system was successful in that it contributed to improved pest management by developing farmers' understanding and by helping them to interpret their own field observations more effectively.

A comparable system was developed in Australia, that is, SIRATAC, a dial-up crop management system to assist cotton growers in making good tactical decisions about the use of insecticides in irrigated cotton on a day-to-day basis [84]. It was run by SIRATAC Ltd., a nonprofit commercial company formed in 1981 to market the system to the cotton industry. SIRATAC aimed to reduce the risk associated with pesticide use by adopting Integrated Pest Management principles. It consisted of several simulation models and a decision model helping the grower decide whether or not to spray pesticide and which pesticide. The area managed using SIRATAC increased steadily during the early 1980s. In 1981–1982, there was demand for a tenfold expansion [14]. However, by 1985, it had reached a ceiling in adoption at 25% (by area) of the industry and its use declined after 1987 [85]. In 1989, SIRATAC Ltd. went into voluntary liquidation due to a predicted declining market share and cash flow problems, despite the fact that the area managed by SIRATAC was at a historical high [14]. It was replaced by an informal user group (SUG) that continued the program for a few more seasons, but by 1993 the group had ceased operation and the field-support system was no longer available. A moratorium on SIRATAC development meant that only minor changes were made to the program. Attempts to achieve similar functionality on a microcomputer failed. An in-depth participatory analysis found that many of the reasons for the poor adoption of SIRATAC were based on organizational issues rather than on problems with the model itself [84]. The important themes that emerged from their survey were:

- Widely held negative views against SIRATAC and SIRATAC Ltd. by nonusers.
- Many growers did not consider SIRATAC's intangible benefits to be worth paying for. They wanted to see tangible cost-saving benefits.
- Many people based their opinions on hearsay and did not really know what SIRATAC was or what it could do.
- Many felt that they were often overriding the system and so abandoned it. They felt uncomfortable with the recommendations of the system (e.g., expensive sprays when cheaper ones would do, not enough spraying – they felt it may put crops at risk).
- Practical limitations: no office or computer or reliable telephone service.
- Growers felt threatened – they saw it as a man versus machine issue and felt that their own experience and knowledge was being undervalued.

Hence, in both, the SIRATAC and EPIPRE cases, there was an initial steady increase in membership, which resulted in an improvement in pest management. However, both organizations then experienced a decline in membership as the growers felt that they had learned what the models would predict and therefore did not need them any more [6]. Both systems were successful in that they contributed to improved pest management by developing farmers' understanding and by helping them to interpret their own field observations more effectively.

Future Directions

Since the beginning of the development of crop growth simulation models, attention has been paid to application of these models in crop and farm management.

The possibility to quantitatively address, in an integrated fashion, many different aspects of decision-making and the expected consequences on crop and agricultural system performance, made such models attractive tools for such analyses.

With respect to *strategic management*, substantial progress has been made in methodological development in the last decade, and tools have been developed that (can) play a role as discussion and decision support systems in such areas as agricultural land use planning [86–88]. However, in a recent review on quantitative assessment of agricultural production systems, the remark was made: "The probably biggest challenge is to transfer the methodologies developed in land use studies to the unruly practice of land use policy formulation and implementation" [89]. "That requires close cooperation with the ultimate users of the methodologies, the various stakeholders, through the development of discussion and negotiation platforms" [90].

In the same sphere, it was concluded that the combination of the boundary arrangement perspective with critical leverage points presents a basis to design an institutional pathway for enhancing impact of modeling research in the policy sphere [91]. For researchers functioning in a science-domain-oriented environment, more options appear available than the frequently proposed 'more participation' for increasing the likelihood that their policy-oriented work is used. These include establishing contacts with research groups or institutes that are in a position to function as "stepping stones," or engaging with others to develop social networks in the policy sphere.

Following a critical evaluation of development and application of the Land Use Planning and Analysis System (LUPAS), it was concluded that its successful use is only possible if there is sufficient, shared awareness of problems and the need for solutions among the stakeholders [88]. Once a prototype of LUPAS was operational (preferably with a user-friendly interface) and stakeholders had the proper level of comprehension, LUPAS served as a vehicle to demonstrate potential conflicts, reveal strategic resource use options, and stimulate discussion among different stakeholder groups. However, presumably the project would have benefited from a more complete identification of problems and information needs as perceived by stakeholders and policy makers. A better contextualization (cf. [92]) would have been an asset in bridging the gap between the information supply of LUPAS and the needs of the stakeholders (cf. [93, 94]).

All of these experiences thus point in the direction of the fact that cooperation with the stakeholders, that is to say with the clients of the information, is critical in creating impact of the modeling work in the policy arena. This interaction with stakeholders should start at the beginning of the modeling work, so that problem identification and the associated design of the modeling tool should also be carried out jointly. The transition from the research field to the application field thus requires more attention than at the moment in general is being accorded.

In the area of *tactical management*, an extensive analysis of a long-term effort to develop in a multi-stakeholder setting an operational system was analyzed by the FARMSCAPE project team in Australia [95]. Their self-evaluation, supported by external evaluations, shows mixed feelings with respect to their success. Near the end of the first round of training and accrediting commercial agronomists in the application of FARMSCAPE tools, with the main focus on facilitating the agronomists involved in the application of these new skills and tools within their business systems, the team states "their success is our primary goal and our evaluation of how system monitoring and simulation is perceived and adopted within agribusiness and their clientele will continue as a key learning objective."

Looking ahead, they state that they are in serious negotiations with a consortium of agribusiness companies who wish to invest in a national delivery system for APSIM simulations targeted at farmer clients. This initiative was first mooted and is now being designed, promoted, and will be financed and implemented by the agribusiness consortium. APSRU (Agricultural Production System Research Unit)'s role will be to provide and support the APSIM software and train accredited users. The commercial system being proposed is well aligned with the FARMSCAPE approach to decision support, with emphasis on the need for simulations to be contextualized to the requests and circumstances of individual farmers. If established, this commercial delivery system could be an important market test for the applicability of computerized decision support to the industry.

While the operational focus is on facilitating development of delivery systems through commercial agronomic service providers, research continues to focus on learning about the market for decision support and its viable delivery, through (i) continued efforts at assessing the internet as a delivery vehicle [96] and (ii) addressing a market barrier to wide adoption of decision support. As pointed out [97], a major challenge to the delivery of DSSs is gaining the attention of farmers beyond "Innovator"/"Early Adopter" types to those who comprise the majority. Accordingly, research has been initiated aimed at penetrating the "Early Majority" market for improved risk management, by creating reference groups of satisfied adopters among the pragmatist category of farmers who are crucial to the diffusion process required for viable market volume. This new project is to be based on case studies built around farmer groups involving both innovative and pragmatist farmers, consultant agronomists, and key researchers.

FARMSCAPE has developed into a successful approach to systems research, specifically in the exploration of a role for computerized decision support. Its focus has been on dryland farming systems of northeast Australia with participant interest concentrating on decisions that impact on the economics of alternative cropping options. There is now interest, both within APSRU and from collaborating researchers, to expand and replicate the FARMSCAPE approach in other regions, and to address a broader range of issues. Research projects implementing the FARMSCAPE approach have already commenced elsewhere in Australia, as well as internationally. Collaborative projects have commenced to assess the role for APSIM in improving management practices in smallholder agriculture, such as a WifAD session successfully held with smallholder farmers in Zimbabwe [98]. Expanding the systems view to incorporate natural resource management, weed management and agroforestry systems are all new project initiatives being undertaken by the FARMSCAPE team.

When leaning backward, and trying to identify systems that at the moment actually are used in *operational farm management*, the results are on the whole disappointing.

Although crop-soil simulation models dealing with soil water balances and crop water used have proliferated and in general, the relevant processes are well understood, there are very few examples where these models are being applied at the individual plot level to guide irrigation scheduling. One of the reasons is no doubt the fact that there is always a substantial degree of uncertainty, because rainfall cannot be predicted with great accuracy, neither in timing nor in amount, an uncertainty that propagates into predictions of future irrigation requirements. An additional constraint on the use of these models is that individual farmers are often dependent on irrigation system management, that determines when and how much water is available.

Application of operational nutrient management systems (fertilizer recommendations) is hampered by a number of difficulties. As mentioned in the description of the wheat nitrogen-fertilizer recommendation system in the Netherlands, the "final" demand for nitrogen, governed by the realized yield level, is determined at a point in the crop's life cycle when uptake by the crop cannot any more be affected by management. An additional problem is that the fertilizer is added to the soil and that it is impossible to predict when and how much of the fertilizer nutrient is actually being taken up by the crop, because that depends, among others, again on the soil and crop water balance, almost impossible to accurately predict. The uncertainty associated with simulation of the nutrient supply from natural sources, particularly that from soil organic matter, in general the largest source, adds uncertainty to the accuracy of estimating fertilizer requirements, both in timing and in rate.

Crop protection systems in the field of pest and disease management have had substantial success in the past, in a combination of farmers' monitoring and simulation models. However, these systems have largely disappeared again, mainly as farmers indicated, because the learning associated with application of these systems made them redundant. Farmers felt that after a number of years, interpretation of the monitoring data did not require model results, but could be done by the farmers themselves. Whether crop growth simulation models can really provide added value in this area is difficult to foresee, although they could play a role in quantitative assessment of damage levels on the basis of famer-supplied incidence levels at different stages of crop development, with or without control measures. The farmer could then decide, taking into

account the level of risk that he is prepared to take, whether to implement control measures or not, very much in line with the original EPIPRE and SIRATAC decision support systems.

At present, there is an increasing demand for weed management systems with reduced use and reliance on herbicides, and the ecophysiological competition models have great potential for contributing to the design of such alternative weed management systems. In the near future, more attention should be given in these models to the accuracy with which growth, morphological development, and seed production of weed species are being simulated, as the long-term development of weed populations becomes increasingly important. In this way, a functional link can be established between crop-weed competition research and research on weed population dynamics. Continuous interaction between modeling and experimental research should support determination of the required level of detail in crop-weed competition models and will provide leads for focusing future weed ecological research.

In conclusion, the great expectations that originally existed with respect to the use of crop growth simulation models in crop management (cf. [6]) unfortunately have not materialized. There are a number of reasons for this disappointing development. One is doubtless the fact that crop production takes place in an uncertain environment and that meteorological sciences have as yet, not been able to predict weather conditions in the near future with any great accuracy. The performance of crops is to a large extent governed by these weather conditions and thus it remains difficult to recommend actual actions when the future weather conditions are uncertain. Another problem is that most simulation models have been developed, tested, and validated against data from experimental stations and commercial crops often perform differently from those in experimental stations. When sufficient time and capacity is available, this problem can to a large extent be overcome, as reported for the Australian situation [99], but such extended programs for the development of crop management systems are seldom if ever available.

In contrast, there appears to be ample scope for application of crop growth simulation models in strategic and tactical management, in which such models are part of so-called discussion support systems that can be used by farmers to explore "what-if" questions, based on explicit quantitative information [100]. It has been shown convincingly that the success of such discussion support systems is greatly enhanced when the relevant stakeholders ("clients") are from the very beginning involved in the design and development of these systems, so that the relevant questions and the available information can be dovetailed in the most efficient way [101].

Therefore, although substantial progress has been made in the last 3 years, the situation has not basically changed and that in order to effectively make use of crop growth simulation models in crop management, the biggest challenge is to transfer the methodologies developed in land use studies to the unruly practice of land use policy formulation and implementation. That requires close cooperation with the ultimate users of the methodologies, the various stakeholders, through the development of discussion and negotiation platforms [89, 91, 101].

Bibliography

Primary Literature

1. Lentz W (1998) Model applications in horticulture: a review. Sci Hortic 74:151–174
2. Van Keulen H (1987) Forecasting and estimating effects of weather on yield. In: Wisiol K, Hesketh D (eds) Plant growth modeling for resource management. CRC Press, Boca Raton, pp 105–124
3. Brouwer R, De Wit CT (1968) A simulation model of plant growth, with special attention to root growth and its consequences. In: Whittington WJ (ed) Root growth. Butterworths, London, pp 224–244
4. De Wit CT (1970) Dynamic concepts in biology. In: Setlik I (ed) Prediction and measurement of photosynthetic productivity. Proceedings of IBP/PP Technical Meeting, Trebon, Pudoc, Wageningen, The Netherlands, pp 17–23
5. De Wit CT, Brouwer R, Penning de Vries FWT (1970) The simulation of photosynthetic systems. In: Setlik I (ed) Prediction and measurement of photosynthetic productivity. Proceedings of IBP/PP Technical Meeting, Trebon, Pudoc, Wageningen, The Netherlands, pp 47–70
6. Sinclair TR, Seligman NG (1996) Crop modelling: from infancy to maturity. Agron J 88:698–703
7. Bouman BAM, Van Keulen H, Van Laar HH, Rabbinge R (1996) The 'School of de Wit' crop growth simulation models: a pedigree and historical overview. Agr Syst 52:171–198
8. Boote KJ, Jones JW, Pickering NB (1996) Potential uses and limitations of crop models. Agron J 88:704–716
9. Van Ittersum MK, Leffelaar PA, Van Keulen H, Kropff MJ, Bastiaans L, Goudriaan J (2003) On approaches and

applications of the Wageningen crop models. Eur J Agron 18:201–234
10. Hammer GL, Kropff MJ, Sinclair TR, Porter JR (2002) Future contributions of crop modelling-from heuristics and supporting decision making to understanding genetic regulation and aiding crop improvement. Eur J Agron 18: 15–31
11. Rabbinge R, Goudriaan J, Van Keulen H, de Vries Penning FWT, Van Laar HH (eds) (1990) Theoretical production ecology: reflection and prospects. Simulation Monographs. Pudoc, Wageningen, The Netherlands
12. Van Ittersum MK, Ewert F, Heckelei T, Wery J, Alkan Olsson J, Andersen E, Bezlepkina I, Brouwer F, Donatelli M, Flichman G, Olsson L, Rizzoli AE, Van der Wal T, Wien JE, Wolf J (2007) Integrated assessment of agricultural systems – a component-based framework for the European Union (SEAMLESS). Agr Syst 96:150–165
13. Seligman NG (1990) The crop model record: promise or poor show? In: Rabbinge R, Goudriaan J, Van Keulen H, Penning de Vries FWT, Van Laar HH (eds) Theoretical production ecology: reflection and prospects. Simulation Monographs. Pudoc, Wageningen, The Netherlands, pp 249–263
14. Cox P (1996) Some issues in the design of agricultural decision support systems. Agr Syst 52:355–381
15. Meinke H, Baethgen WE, Carberry PS, Donatelli M, Hammer GL, Selvaraju R, Stőckle CO (2001) Increasing profits and reducing risks in crop production using participatory systems simulation approaches. Agr Syst 70:493–513
16. Nelson RA, Holzworth DP, Hammer GL, Hayman PT (2003) Infusing the use of seasonal climate forecasting into crop management in North East Australia using discussion support software. Agr Syst 74:393–414
17. McCown RL, Hammer GL, Hargreaves JNG, Holzworth DP, Freebairn DM (1996) APSIM: a novel software system for model development, model testing and simulation in agricultural systems research. Agr Syst 50:255–271
18. Keating BA, Carberry PS, Hammer GL, Probert ME, Robertson MJ, Holzworth D, Huth NI, Hargreaves JNG, Meinke H, Hochman Z, McLean G, Verburg K, Snow V, Dimes JP, Silburn M, Wang E, Brown D, Bristow KL, Asseng S, Chapman S, McCown RL, Freebairn DM, Smith CJ (2003) An overview of APSIM, a model designed for farming analysis simulation. Eur J Agron 18:267–288
19. Stöckle CO, Donatelli M (1997) The CropSyst model: a brief description. In: Plentinger MC, Penning de Vries FWT (eds) Rotation models for ecological farming, pp 35–43 (Quantitative Approaches in Systems Analysis No. 10, AB-DLO). Wageningen, The Netherlands
20. Stöckle CO, Donatelli M, Nelson R (2003) CropSyst, a cropping systems simulation model. Eur J Agron 18:289–307
21. Brisson N, Gary C, Justes E, Roche R, Mary B, Ripoche D, Zimmer D, Sierra J, Bertuzzi P, Burger P, Bussière F, Cabidoche YM, Cellier P, Debaeke P, Gaudillère JP, Hénault C, Maraux F, Seguin B, Sinoquet H (2003) An overview of the crop model STICS. Eur J Agron 18:309–332
22. Jones JW, Hoogenboom G, Porter CH, Boote KJ, Batchelor WD, Hunt WA, Wilkens PW, Singh U, Gijsman AJ, Ritchie JT (2003) The DSSAT cropping system model. Eur J Agron 18:235–265
23. Van Keulen H, Stol W (1995) Agro-ecological zonation for potato production. In: Haverkort AJ, MacKerron DKL (eds) Potato ecology and modelling of crops under conditions limiting growth. Kluwer, Dordrecht, The Netherlands, pp 357–371
24. Penning de Vries FWT, Van Keulen H, Rabbinge R (1995) Natural resources and limits of food production in 2040. In: Bouma J, Kuyvenhoven A, Bouman BAM, Luyten J, Zandstra HG (eds) Eco-regional approaches for sustainable land use and food production. Kluwer, Dordrecht, The Netherlands, pp 65–87
25. Van Ittersum MK, Rabbinge R (1997) Concepts in production ecology for analysis and quantification of agricultural input-output combinations. Field Crops Res 52:197–208
26. Hengsdijk H, Van Ittersum MK (2002) A goal-oriented approach to identify and engineer land use systems. Agr Syst 71:231–247
27. Rabbinge R, Van Latesteijn HC (1992) Long term options for land use in the European Community. Agr Syst 40:195–210
28. Ten Berge HFM, Van Ittersum MK, Rossing WAH, Van de Ven GWJ, Schans J, Van de Sanden PACM (2000) Farming options for the Netherlands explored by multi-objective modelling. Eur J Agron 13:263–277
29. Roetter R, Van Keulen H, Van Laar HH (2000) Synthesis of methodology development and case studies, vol 3, Sysnet Research Paper Series. International Rice Research Institute, Los Banos, Philippines, p 94
30. Dogliotti S, Rossing WAH, Van Ittersum MK (2004) Systematic design and evaluation of crop rotations enhancing soil conservation, soil fertility and farm income: a case study for vegetable farms in South Uruguay. Agr Syst 80:277–302
31. Dogliotti S, Van Ittersum MK, Rossing WAH (2005) A method for exploring sustainable development options at farm scale: a case study for vegetable farms in South Uruguay. Agr Syst 86:29–51
32. Van de Ven GWJ, Van Keulen H (2007) A mathematical approach to comparing environmental and economic goals in dairy farming: identifying strategic development options. Agr Syst 94:231–246
33. Hengsdijk H, Bouman BAM, Nieuwenhuyse A, Jansen HGP (1999) Quantification of land use systems using technical coefficient generators: a case study for the northern Atlantic zone of Costa Rica. Agr Syst 61:109–121
34. Laborte AG, Schipper RA, Van Ittersum MK, Van Den Berg MM, Van Keulen H, Prins AG, Hossain M (2009) Farmers' welfare, food production and the environment: a model-based assessment of the effects of new technologies in the northern Philippines. NJAS Wageningen J Life Sci 6:345–373
35. Hengsdijk H, Guanghuo W, Van den Berg MM, Jiangdi W, Wolf J, Changhe L, Roetter RR, Van Keulen H (2007) Poverty and biodiversity trade-offs in rural development: a case study for Pujiang county, China. Agr Syst 94:851–861

36. Ponsioen TC, Hengsdijk H, Wolf J, Van Ittersum MK, Rötter RP, Son TT, Laborte AG (2006) TechnoGIN, a tool for exploring and evaluating resource use efficiemcy of cropping systems in East and Southeast Asia. Agr Syst 87:80–100
37. Abrecht DG, Robinson SD (1996) TACT: a tactical decision aid using a CERES based wheat simulation model. Ecol Model 86:241–244
38. Stone RC, Meinke H (2005) Operational seasonal forecasting of crop performance. Philos Trans Roy Soc B 360:2109–2124
39. Muchow RC, Bellamy JA (eds) (1991) Climatic risk in crop production: models and management for the semiarid tropics and subtropics. CAB International, Wallingford, UK, p 548
40. Stone RC, Hammer GL, Marcussen T (1996) Prediction of global rainfall probabilities using phases of the Southern Oscillation Index. Nature 384:252–255
41. Hammer GL, Nicholls N, Mitchell C (eds) (2000) Applications of seasonal climate forecasting in agricultural and natural ecosystems – the Australian experience. Kluwer, Dordrecht, The Netherlands, p 469
42. Carberry PS (2001) Are science rigour and industry relevance both achievable in participatory action research? Proceedings Australian Agronomy Conference, Hobart, January 2001. Available from http://www.regional.org.au/papers/agronomy/2001/plenery/5/Carberry,Peter.htm
43. Nelson RA, Hammer GL, Holzworth DP, McLean G, Pinington GK, Frederiks AN (1999) User's Guide for Whopper Cropper (CD-ROM) Version 2.1. QZ99013. Department of Primary Industries, Queensland, Brisbane, Australia
44. Hammer GL (2000) Applying seasonal climate forecasts in agricultural and natural ecosystems – a synthesis. In: Hammer GL, Nicholls N, Mitchell C (eds) Applications of seasonal climate forecasting in agricultural and natural ecosystems – the Australian experience. Kluwer, Dordrecht, The Netherlands, pp 453–462
45. Messina CD, Hansen JW, Hall AJ (1999) Land allocation conditioned on El Niño-Southern Oscillation phases in the Pampas of Argentina. Agr Syst 60:197–212
46. Hansen JW, Indeje M (2004) Linking dynamic seasonal climate forecasts with crop simulation for maize yield prediction in semi-arid Kenya. Agr Forest Meteorol 125:143–157
47. Wopereis MCS, Bouman BAM, Tuong TP, Ten Berge HFM, Kropff MJ (1996) ORYZA_W: Rice growth model for irrigated and rainfed environments. SARP Research Proceedings, AB-DLO, Wageningen, The Netherlands, p 159
48. Lansigan FP, Pandey S, Bouman BAM (1997) Combining crop modelling with economic risk-analysis for the evaluation of crop management strategies. Field Crops Res 51:133–145
49. MacRobert JF, Savage MJ (1998) The use of a crop simulation model for planning wheat irrigation in Zimbabwe. In: Tsuji GY, Hoogenboom G, Thornton PK (eds) Understanding options for agricultural production. Systems approaches for sustainable agricultural development. Kluwer, Dordrecht, The Netherlands, pp 205–220
50. Ko J, Piccinni G, Steglich E (2009) Using EPIC model to manage irrigated cotton and maize. Agr Water Manage 96:1323–1331
51. Richards QD, Bange MP, Johnston SB (2008) HydroLOGIC: an irrigation management system for Australian cotton. Agr Syst 98:40–49
52. Hearn AB (1994) OZCOT: a simulation model for cotton crop management. Agr Syst 44:257–299
53. Ten Berge HFM, Shi Q, Zheng Z, Rao KS, Riethoven JJM, Zhong X (1997) Numerical optimisation of nitrogen application to rice. II. Field evaluations. Field Crops Res 51:43–54
54. Ten Berge HFM, Thiyagarajan TM, Shi Q, Wopereis MCS, Drenth H, Jansen MJW (1997) Numerical optimisation of nitrogen application to rice. I. Description of MANAGE-N. Field Crops Res 51:29–42
55. Thiyagarajan TM, Stalin P, Dobermann A, Cassman KG, Ten Berge HFM (1997) Soil N supply and plant N uptake by irrigated rice in Tamil Nadu. Field Crops Res 51:55–64
56. ZhiMing Z, LiJiao Y, ZhaoQian W, Zheng Z, Yan L, Wang Z (1997) Evaluation of a model recommended for N fertilizer application in irrigated rice. Chin Rice Res Newslett 5:7–8
57. Wang E, Xu JH, Smith CJ (2008) Value of historical climate knowledge, SOI-based seasonal climate forecasting and stored soil moisture at sowing in crop nitrogen management in south eastern Australia. Agr Forest Meteorol 148:1743–1753
58. Aggarwal PK, Kalra N, Chander S, Pathak H (2006) InfoCrop: a dynamic simulation model for the assessment of crop yields, losses due to pests, and environmental impact of agroecosystems in tropical environments. I. Model description. Agr Syst 89:1–25
59. Yadav DS, Chander S (2009) Simulation of rice planthopper damage for developing pest management decision support tools. Crop Prot 29:67–76
60. Fischer A, Kergoat L, Dedieu G (1997) Coupling satellite data with vegetation functional models: review of different approaches and perspectives suggested by the assimilation strategy. Remote Sens Rev 15:283–303
61. Moulin S, Bondeau A, Delecolle R (1998) Combining agricultural crop models and satellite observations: from field to regional scales. Int J Remote Sens 19:1021–1036
62. Jongschaap REE, Quiroz RA (2000) Integrating remote sensing with process-based simulation models to assess primary production capacity for grazing lands in The Andes. Proceedings of the 5th seminar on GIS and developing countries: GISDECO 2000, Los Baños, Philippines, 2–3 November 2000
63. Clevers JPGW, Vonder OW, Jongschaap REE, Desprats DF, King C, Prévot L, Bruguier N (2002) Using SPOT data for calibrating a wheat growth model under Mediterranean conditions. Agronomie 22:687–694
64. Cabelguenne M (1996) Tactical irrigation management using real time EPIC-phase model and weather forecast: experiment on maize. In: ICD-CIID F (ed) Irrigation scheduling from theory to practice (Water Reports). FAO, Rome, Italy, pp 185–193
65. Doorenbos J, Kassam AH (1979) Yield response to water. Irrigation and Drainage Paper No. 33, Food and Agricultural Organisation, Rome, Italy

66. Jones CA, Kiniry JR (1986) CERES-Maize: a simulation model of maize growth and development. Texas A&M University Press, College Station, TX, USA
67. Williams JR, Jones CA, Dyke PT (1984) A modelling approach to determining the relationship between erosion and soil productivity. Trans Am Soc Eng 27:129–144
68. Cabelguenne M, Debaeke Ph, Puech J, Bose N (I997) Real time irrigation management using the EPIC-PHASE model and weather forecasts. Agr Water Manage 32:227–238
69. McGlinchey MG, Inman-Bamber N, Culverwell TL, Els M (1995) An irrigation scheduling method based on a crop model and an automatic weather station. Proceedings of the Annual congress of the South African Sugar Technologists' Association, vol 69, pp 69–73
70. Plauborg F, Heidmann T (1996) MARKVAND: an irrigation scheduling system for use under limited irrigation capacity in a temperate humid climate. In: ICD-CIID F (ed) Irrigation scheduling from theory to practice (Water Reports). FAO, Rome, pp 177–184
71. Hess TM (1990) Practical experiences of operating a farm irrigation scheduling service in England. Acta Hortic 278:871–878
72. Hess TM (1996) A microcomputer scheduling program for supplementary irrigation. Comput Electron Agr 15:233–243
73. Tollefson L (1996) Requirements for improved interactive communication between researchers, managers, extensionists and farmers. In: ICD-CIID F (ed) Irrigation scheduling from theory to practice (Water Reports). FAO, Rome, pp 217–226
74. Ines AVM, Honda K, Das Gupta A, Droogers P, Clemente RS (2006) Combining remote sensing-simulation modeling and genetic algorithm optimization to explore water management options in irrigated agriculture. Agr Water Manage 83:221–232
75. Bastiaanssen WGM, Menenti M, Feddes RA, Holtslag AAM (1998) A remote sensing surface energy balance algorithm for land (SEBAL). 1. Formulation. J Hydrol 212–213:198–212
76. Hansen JW, Ines AVM (2005) Stochastic disaggregation of monthly rainfall data for crop simulation studies. Agr Forest Meteorol 131:233–246
77. Groot JJR, Van Keulen H (1990) Prospects for improvement of nitrogen fertilizer recommendations for cereals: a simulation study. In: Van Beusichem ML (ed) Plant nutrition: physiology and applications. Developments in Plant and Soil Sciences, vol 41. Kluwer, Dordrecht, The Netherlands, pp 685–692
78. Li FY, Johnstone PR, Pearson A, Fletcher A, Jamieson PD, Brown HE, Zyskowskia RF (2009) AmaizeN: a decision support system for optimizing nitrogen management of maize. NJAS Wageningen J Life Sci 57:93–100
79. Zadoks JC (1981) EPIPRE: a disease and pest management system for winter wheat developed in The Netherlands. EPPO Bull 11:365–369
80. Rabbinge R, Rijsdijk FH (1983) EPIPRE: a disease and pest management system for winter wheat, taking account of micrometeorological factors. EPPO Bull 13:297–305
81. Smeets E, Vandenriessche H, Hendrickx G, De Wijngaert K, Geypens M (1992) Photosanitary balance of winter wheat in 1992 by the EPIPRE advice system. Parasitica 48:139–148
82. Djurle J (1988) Experience and results from the use of EPIPRE in Sweden. SROP Bulletin. In: Pest and disease models in forecasting crop loss appraisal and decision supported crop protection systems, vol 11, pp 94–95
83. Forrer HR (1988) Experience and current status of EPIPRE in Switzerland. SROP Bulletin. In: Pest and disease models in forecasting crop loss appraisal and decision supported crop protection systems, vol 11, pp 91–93
84. Macadam R, Britton I, Russell D, Potts W, Baillie B, Shaw A (1990) The use of soft systems methodology to improve the adoption by Australian cotton growers of the Siratac computer-based crop management system. Agr Syst 34:1–14
85. Hamilton WD, Woodruff DR, Jamieson AM (1991) Role of computer-based decision aids in farm decision-making and in agricultural extension. In: Muchow RD, Bellamy JA (eds) Climatic risk in crop production-models and management for the semi-arid tropics and subtropics. CAB International, Wallingford, UK, pp 411–423
86. Roetter RP, Hoanh CT, Laborte AG, Van Keulen H, Van Ittersum MK, Dreiser C, Van Diepen CA, De Ridder N, Van Laar HH (2005) Integration of Systems Network (SysNet) tools for regional land use scenario analysis in Asia. Environ Model Softw 20:291–307
87. Roetter RP, Laborte AG, Van Keulen H (2000) Using SysNet tools to quantify the trade-off between food production and environmental quality. International Rice Research Newsletter, December 2000:4–9
88. Van Ittersum MK, Roetter RP, Van Keulen H, De Ridder N, Hoanh CT, Laborte AG, Aggarwal PK, Ismail AB, Tawang A (2004) A systems network (SysNet) approach for interactively evaluating strategic land use options at sub-national scale in South and South-east Asia. Land Use Policy 21:101–113
89. Van Keulen H (2007) Quantitative analyses of natural resource management options at different scales. Agr Syst 94:768–783
90. Van Paassen A, Roetter RP, Van Keulen H, Hoanh CT (2007) Can computer models stimulate learning about sustainable land use? Experience with LUPAS in the humid (sub-) tropics of Asia. Agr Syst 94:874–887
91. Sterk B, Carberry P, Leeuwis C, Van Ittersum MK, Howden M, Meinke H, Van Keulen H, Rossingh WAH (2009) The interface between land use systems research and policy: multiple arrangements and leverages. Land Use Policy 26:434–442
92. David A (2001) Models implementation: a state of the art. Eur J Oper Res 134:459–480
93. Rykiel EJ Jr, Berkson J, Brown VA, Krewitt W, Peters I, Schwartz M, Shogren J, Van der Molen D, Blok R, Borsuk M, Bruins R, Cover K, Dale V, Dew J, Etnier C, Fanning L, Felix R, Nordin Hasan M, Hong H, King AW, Krauchi N, Lubinsky K, Olson J, Onigkeit J, Patterson G, Rajan KS, Reichert P, Sharma K, Smith V, Sonnenschein M, St-Louis R, Stuart D, Supalla R, Van Latesteijn H (2002) Science and decision making. In: Costanza R, Jörgensen SE (eds) Understanding and solving environmental problems in the 21st century. Toward a new, integrated hard problem science. Elsevier, Amsterdam, The Netherlands, pp 153–166

94. Walker DH (2002) Decision support, learning and rural resource management. Agr Syst 73:113–127
95. Carberry PS, Hochman Z, McCown RL, Dalgliesh NP, Foale MA, Poulton PL, Hargreaves JNG, Hargreaves DMG, Cawthray S, Hillcoat N, Robertson MJ (2002) The FARMSCAPE approach to decision support: farmers', advisers', researchers' monitoring, simulation, communication and performance evaluation. Agr Syst 74:141–177
96. Hargreaves DMG, Hochman Z, Dalgliesh N, Poulton P (2001) FARMSCAPE online – developing a method for interactive Internet support for farmers situated learning and planning. In: Proceedings of the Tenth Australian Agronomy Conference, Hobart, Australia. www.regional.org.au/au/asa/2001/5/a/hargreaves.htm)
97. McCown RL (2002) Changing systems for supporting farmers' decisions: problems, paradigms, and prospects. Agr Syst 74:179–220
98. Twomlow S (2001) Linking Logics II: taking simulation models to the farmers. British Society of Soil Science Newsletter, December 2001 (40):12–14
99. Carberry PS, Hochman Z, Hunt JR, Dalgliesh NP, McCown RL, Whish PM, Robertson MJ, Foale MA, Poulton MA, van Rees H (2009) Re-inventing model-based decision support with Australian dryland farmers. 3. Relevance of APSIM to commercial crops. Crop Pasture Sci 60: 1044–1056
100. Dalgliesh NP, Foale MA, McCown RL (2009) Re-inventing model-based decision support with Australian dryland farmers. 2. Pragmatic provision of soil information for paddock-specific simulation and farmer decision making. Crop Pasture Sci 60:1031–1043
101. McCown RL, Carberry PS, Hochman Z, Dalgliesh NP, Foale MA (2009) Re-inventing model-based decision support with Australian dryland farmers. 1. Changing intervention concepts during 17 years of action research. Crop Pasture Sci 60:1017–1030

Books and Reviews

European Journal of Agronomy (2003) Volume 18, Issues 3–4, special issue Modelling Cropping Systems: Science, Software and Applications. pp 187–393

Gary C, Heuvelink E (1998) Advances and bottlenecks in modelling crop growth: summary of a group discussion. Acta Hortic (ISHS) 456:101–104

Goudriaan J, Van Laar HH (1994) Modelling potential crop growth processes, textbook with exercises. Current Issues in Production Ecology, vol 2. Kluwer, Dordrecht, The Netherlands, p 238

Jongschaap REE (2006) Integrating crop growth simulation and remote sensing to improve resource use efficiency in farming systems. Ph.D. thesis, Wageningen University, Wageningen, The Netherlands

Leffelaar PA (ed) (1993) On system analysis and simulation of ecological processes, with examples in CSMP and FORTRAN. Current Issues in Production Ecology, vol 1. Kluwer, Dordrecht, The Netherlands, p 294

Matthews RB, Stephens W (2002) Crop-soil simulation models: applications in developing countries. CAB International, Wallingford, UK

Roetter RP, Van Keulen H, Kuiper M, Verhagen J, Van Laar HH (eds) (2007) Science for agriculture and rural development in low-income countries. Springer, Dordrecht, The Netherlands, p 222

Willocquet L, Savary S, Fernandez L, Elazegui FA, Castilla N, Zhu D, Tang Q, Huang S, Lin X, Singh HM, Srivastava KA (2002) Structure and validation of RICEPEST, a production situation-driven, crop growth model simulating rice yield response to multiple pest injuries for tropical Asia. Ecol Model 153:247–268

Wolf J, Van Ittersum MK (2009) Crop models: main developments, their use in CGMS and integrated modeling. Agro-Informatica 22:15–18

Socially Affected Traits, Inheritance and Genetic Improvement

Piter Bijma
Animal Breeding and Genomics Centre, Wageningen University, Wageningen, The Netherlands

Article Outline

Glossary

Animal breeding The process of selection and mating of parents to produce the next generation of a population, aiming to genetically improve populations of livestock and fish.

Associative effect Heritable effect of an individual on the phenotypic of another individual.

BLUP (best linear unbiased prediction) a method to estimate breeding values of individuals, making use of their pedigree and observed phenotypes.

Breeding value Expected phenotypic trait value of an individual given its genes

Genetic parameters Genetic component of the variances and covariances of traits in a population. Also encompasses derived parameters such as heritability.

Heritability Magnitude of the heritable variation in a population, expressed as a proportion of the observed variation among individuals.

Phenotype Observable trait value of an individual.

Response to selection Change in mean value of a phenotypic trait due to genetic selection.

Selection The choice of individuals to become parents of the next generation. The selection determines which genes are passed on to the next generation.

Social interactions The social process between individuals, which may affect the phenotypic trait values of those individuals.

Definition of the Subject

Livestock genetic improvement, also known as animal breeding, refers to the selection and mating of parents to produce the next generation of a population. The aim is to genetically improve populations of livestock and fish so that they better meet the future needs of markets and societies. Genetic improvement programs gradually change the genetic composition of populations and may create large changes over time. The social environment that individuals experience profoundly affects their productivity, health, and welfare, but has largely been overlooked in animal breeding. There is, however, increasing evidence that traits of individuals are affected not only by the genes carried by those individuals, but also by genes in the individuals present in their social environment. Thus there is a need to generalize the classical theory of inheritance and response to selection to make optimum use of the genetic variation present in the social environment. This involves both the basic mathematical description of the inheritance of socially affected traits, and methods for statistical genetic analysis of populations and designs of breeding schemes.

Introduction

Classical breeding has been very successful [1]. In many species, yields have increased dramatically, particularly in species with short generation intervals in which the focus has been on traits of moderate to high heritability recordable on the selection candidate, such as growth rate in broiler chickens [2, 3]. In many cases, however, there has been a trend for aspects of fitness to decline. Balanced genetic improvement in multiple traits is hampered by trade-offs between those traits, which are observed as unfavorable genetic correlations. Typical examples are trade-offs between yield and fertility in dairy cattle, between litter size and piglet survival in pigs, egg size and egg number in laying hens, and between growth rates and female fertility in broilers. Particularly the widespread trade-off between production and reproduction traits appears to be a systematic biological phenomenon [4].

Genetic improvement in the presence of trade-offs has compromised animal welfare, at least in some livestock species. This has partly been due to a lack of tools

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

to efficiently breed for health-related traits. In many cases, production traits have been most easy to record, have higher heritabilities than reproduction or health-related traits, and are available earlier in life. Breeding for longevity in dairy cattle, for example, has long been hampered by the lack of suitable statistical tools to account for censored records [5]. Moreover, in cases where yield related traits have greater heritability and better or earlier available phenotypic information, selection for the economically optimal index tends to yield negative responses in traits related to fitness. In addition, there has been a difficulty to market traits related to health and welfare of livestock. Profit margins in livestock production are often narrow, so that there is a strong emphasis on the economics of production. Genomic selection [6] is a very promising tool for a more balanced genetic improvement of productivity versus health and welfare, mainly because it provides estimated breeding values early in life for all recordable traits. However, though genomic selection largely removes trade-offs related to breeding scheme design, it does not remove the biological trade-offs between traits. In the absence of genetic engineering, traditional genetic parameters, such as the genetic correlation, remain the key parameters reflecting the prospects for genetic improvement.

There are different levels of modeling the causal mechanisms underlying the observed trade-offs. From a physiological perspective, for example, trade-offs can be understood from a resource allocation point of view. When resource intake becomes increasingly limiting, traits sharing the same resources will show an increasingly negative relationship [7, 8]. This perspective suggests that breeding for increased efficiency, which should be the overall goal of livestock genetic improvement [9], will lead to increasingly negative relationships between traits. From a genetic perspective, systematic trade-offs are the result of unfavorable pleiotropic effects of segregating genes. Sustained multitrait selection will increase such trade-offs because genes with favorable pleiotropic effects will become fixed by selection, while genes with unfavorable pleiotropic effects will remain segregating for much longer. There is no contradiction between the physiological and the genetic perspective; they merely represent different levels of causality. The evolutionary history of a population represents the ultimate level of causality because millions of years of evolution have shaped the biology of current livestock species. Hence, an evolutionary perspective may help to identify new opportunities for genetic improvement because it provides some understanding of the forces that have shaped the current livestock populations.

Denison et al. [10] consider opportunities for genetic improvement of agricultural populations from an evolutionary perspective. They argue that breeders cannot compete with the natural selection that has occurred in the evolutionary history of livestock populations because of the difference in time scales, being years for breeders and millions of years for natural selection. Consequently, it is unlikely that breeders can improve traits that have always been a target of natural selection. Typical examples are general disease resistance and basic efficiencies of metabolic processes. Hence, rather than trying to mimic natural selection, breeders should investigate other directions for genetic improvement.

A second reason why breeders should not try to mimic natural selection is that they have a fundamentally different objective. While natural selection acts on individual fitness, breeders are interested in the average performance of groups of animals or entire populations. Because natural selection acts on individual fitness, it has the intrinsic tendency to increase competition among population members. Thus natural selection may result in selfish individuals that are successful at the expense of others, rather than in maximization of output on the population, flock, or herd level. A clear example comes from the difference between wild wheat and rice versus modern cultivars. In natural populations, individual plants compete for sunlight. Natural varieties and traditional cultivars, therefore, have long stems making them sensitive to lodging (falling over). Modern wheat and rice cultivars, however, owe their high-yield potential largely to their short stature, relative to traditional cultivars. Short stature reduces lodging and increases growth of grain at the expense of stems, but artificial selection for these traits has resulted in decreased individual competitiveness. Forced to compete with traditional, low-yielding cultivars, modern high-yield rice cultivars completely disappear within as few as three generations [10]. This result clearly demonstrates that natural populations do not provide a useful blueprint for agriculture.

Moreover, it illustrates the relevance of competition among individuals in natural populations, which may also be relevant for livestock genetic improvement.

Denison et al. [10], therefore, conclude that breeders should investigate directions of human interest that are not already exhausted by natural selection. They identify two such directions. First, since domestication has greatly changed the environment to which populations are exposed, certain traits that are essential in nature may no longer be useful in domestic populations. Thus this change in environment releases usable genetic variation. Most modern agricultural species do, e.g., not need the capacity to escape from predators, or to deal with seasonal starvation. Hence, to achieve progress in traits of human interest, breeders should provide good environments and accept sacrificing traits that are essential in natural populations, rather than trying to breed robust individuals that can deal with very challenging environments. This direction has already been exhausted to a great deal in livestock. Improvement of genetics, husbandry, and feeding have gone together ever since domestication, and classical selection strategies implicitly utilize the genetic variance released by improvement of the environment. This approach, however, is gradually reaching limits. Concerns about animal welfare are on the increase in Western societies, and producers sometimes have difficulty providing the environment allowing animals to express their full genetic potential (personal communication Egbert Knol (IPG) and Frans van Sambeek (ISA)). Hence, breeders already utilize opportunities arising from improved environments, and it seems unlikely that current rates of genetic improvement can be accelerated by further improvement of the environment.

Second, Denison et al. [10] argue that breeders should target traits beneficial to the population but not to the individual expressing the trait. This is because natural selection acts on individual fitness and does, therefore, not exhaust the genetic variance in the social effect that individuals have on each other. Hence, they suggest breeding for decreased competition among population members. Thus the quantity of interest here is the heritable effect of an individual on the trait values of its group mates, known as the associative or indirect genetic [11, 12], rather than on its own trait value. Most livestock populations are kept in groups, ranging from cages of four laying hens, groups of moderate size in pigs and beef cattle in feed lots, to large flocks of broilers, sheep or beef cattle. In those systems, performance of individuals may depend on (social) characteristics of the other individuals present in the same group. Well-known examples are mortality due to cannibalistic behaviors in non-beak-trimmed laying hens [13], and tail biting and aggression at mixing in swine [14]. Results in pigs, moreover, indicate that growth rate is affected by heritable social effects, even in ad libitum feeding [15]. Also aquaculture species provide a typical example of social effects, where variation among individuals is often considerably increased due to competition for feed [16].

When trait values depend on social interactions among individuals, selection of the best individuals may cause the average performance of a population to decline, rather than increase. In non-beak-trimmed laying hens, for example, selection of the survivors has decreased survival in the next generation because the survivors had a negative heritable effect on survival of their cage mates [17]. Results in plant breeding, however, illustrate that selection for increased yield in the presence of social interactions can be successful. Properties of modern crops show that plant breeders have successfully improved yields by decreasing competition (see above). Reasons for this success may be that plant breeders are more aware of competition among individuals, and that breeding for reduced competition is much easier in plants. (Theory shows that selection between clonal plots exploits the full heritable variance in traits, including the component due to competition; see below and in [18]). In livestock breeding, in contrast, relatively little is known of the magnitude of associative effects. With the exception of maternal genetic effects, breeders have hardly attempted to utilize associative effects for genetic improvement (But see Muir's selection experiments; [17, 19–21]). Hence, there is a need for further research on the magnitude of associative effects in livestock populations.

At first glance, breeding for improved social effects may seem difficult to apply in practice because social effects will often be the result of certain behaviors that may be difficult to record. Breeding for improved behavior is rarely applied in modern breeding practice

because recording behaviors is usually very labor intensive. [22], however, showed that heritable social effects can be estimated in a similar way as maternal genetic effects [23] without the need to record the behaviors underlying the associative effects. At present, flexible tools are available to estimate social genetic effects without the need to observe the traits underlying them (See section on "Estimation of Genetic Parameters" below). Hence, nowadays breeders have the opportunity to address improvement of social interactions in their improvement programs.

Whether or not social interactions are of interest to breeders will depend on the trait considered and on the environment in which individuals are kept. Selection experiments on mortality in non-beak-trimmed layers kept in cages illustrate the relevance of social genetic effects in that situation [17, 20, 21]. For non-beak-trimmed laying hens, Ellen et al. [24] found that more than half of the total heritable variation in survival days originated from social effects. Bergsma et al. [15] found that social genetic effects contribute to heritable variation in growth rate and feed intake in pigs. In contrast, results of Arango et al. [25] in growing pigs indicate that social genetic effects may be difficult to estimate and not deviate significantly from zero. At present, knowledge of the importance of social genetic effects in populations of livestock and fish is still limited. In aquaculture, the large size differences among individual fish indicate that competition is important and that it affects uniformity [16], but the heritable effects underlying such competition are largely unknown. It would be interesting to see whether fish breeders can achieve something similar as plant breeders, who have effectively reduced competition among individuals in wheat [10]. Though social genetic effects may not always be important and may be difficult to utilize in some cases, the promising results observed in layer chickens should be sufficient incentive for further research in this area. Moreover, genetic improvement of productivity resulting from improved social interactions may cause less trade-offs in health and welfare traits because it may reduce energy waste rather than shifting resources from fitness traits toward production traits [17]. This chapter summarizes the quantitative genetic theory of socially affected traits, covering the basic models, expressions for response to selection, and estimation of genetic parameters.

Quantitative Genetics of Socially Affected Traits

Quantitative Genetic Model

Classical Model In classical quantitative genetics and animal breeding theory, individual phenotypic values, P, are modeled as the sum of a heritable component, A, known as breeding value, and a residual, E, known as environment (Table 1) [26, 27],

$$P = A + E \tag{1}$$

The breeding value is the sum of the so-called average effects of the genes of the individual on its phenotype [28, 29]. It represents the heritable component of an individual's phenotype, whereas the "environment" comprises all non-heritable effects, including the non-heritable genetic effects arising from dominance and epistasis.

The absolute magnitude of the heritable effects is measured by the variance of the breeding values, σ_A^2, known as the additive genetic variance. The relative magnitude of the heritable effects is measured by the heritability, the ratio of heritable variance over phenotypic variance [26, 27],

$$h^2 = \sigma_A^2/\sigma_P^2 \tag{2}$$

Heritability expresses the relative contribution of heritable effects to phenotypic variance among individuals, taking values from 0 through 1. In the classical model, heritability also has the natural interpretation as the regression coefficient of offspring phenotype on mean phenotype of the parents; it is the proportion of phenotypic superiority in the parents that is recovered in the offspring. In the context of selection experiments, this quantity is known as the realized heritability [26].

Social Model As summarized in the Introduction, individuals may affect each other's phenotypes. The "environmental" component of the phenotype, therefore, also includes effects of the social environment provided by other individuals that interact with the focal individual. In contrast to the physical environment, however, the social environment is biological in origin and may therefore contain a heritable component. Hence, the E-term in Eq. 1 may contain heritable components due to other individuals. Such heritable effects on trait values of others are known as indirect genetic effects in the evolutionary genetic literature [12, 30],

Socially Affected Traits, Inheritance and Genetic Improvement. Table 1 Notation key

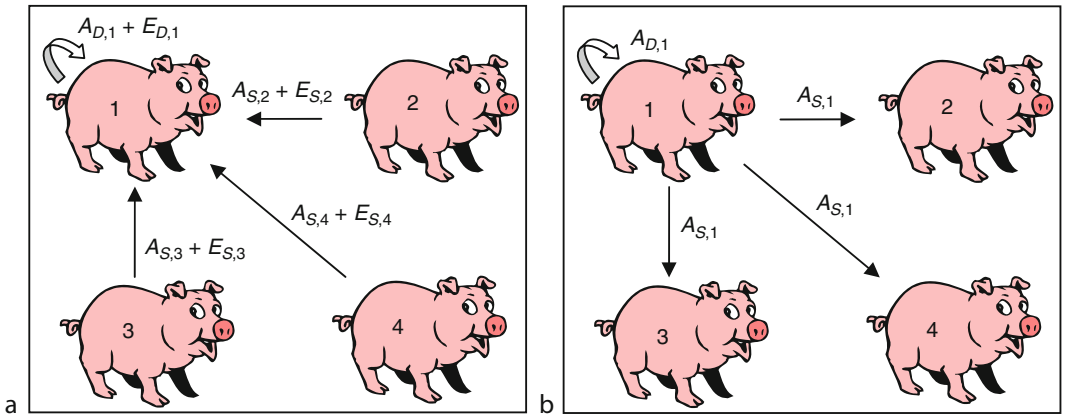
Symbol	Meaning
P, A, h^2	Phenotypic value, breeding value, heritability
i, j, n	Focal individual, group mate of focal individual, group size
$P_{D,i}$, $P_{S,i}$	Direct effect of i, (full) associative effect of i
$A_{D,i}$, $A_{S,i}$	Direct genetic effect of i, associative genetic effect of i
$E_{D,i}$, $E_{S,i}$	Direct non-genetic effect of i, associative non-genetic effect of i
$A_{T,i}$, T^2	Total breeding value of i, relative heritable variance
$A_{M,i}$, $E_{M,i}$	Breeding value for maternal effect of i, non-genetic maternal effect of i
R	Response to selection
ρ, ρ_T	Traditional accuracy of selection, total accuracy of selection
ρ_D, ρ_S	Direct and social accuracy of selection
g, r	Degree of between-group selection, relatedness between group mates
r_f, τ	relatedness between candidate and relatives, intraclass correlation between relatives
$\sigma^2_{A_D}$, $\sigma^2_{A_S}$	Direct genetic variance, associative genetic variance
$\sigma_{A_{DS}}$, r_g	Covariance and correlation between direct and associative genetic effects
$\sigma_{E_{DS}}$, r_E	Covariance and correlation between direct and non-genetic associative effects
σ^2_P, $\sigma_{P_{DS}}$	Phenotypic variance, full covariance between direct and associative effects
$\sigma^2_{A_T}$	Total heritable variance
σ^2_g, σ^2_e	Between-group non-genetic variance, residual variance
d	Degree of dilution of associative effects with group size
N_f, m	Number of families, effective number of records per family
σ^2_z, σ^2_f, σ^2_e	Full variance of records, between-family variance, residual variance
SE, $n_{opt,x}$	Standard error, optimum family size for parameter x

and as associative effects or social effects in the animal breeding literature [11, 17, 31]. Associative effects are of interest to animal breeders because they are heritable and thus can contribute to response to selection. Hence, for socially affected traits, the classical model in Eq. 1 needs to be modified because it treats associative effects as part of the non-heritable environment [11]. Also the definition of heritable variance may be modified because the classical additive genetic variance excludes the contribution of associative effects.

In most livestock and aquaculture populations, individuals are kept in groups, and interactions among individuals occur within group. In a population consisting of groups of n individuals each, the phenotype of an individual can be expressed as the sum of a direct effect rooted in the individual itself, and the associative effects of each of its $n-1$ group mates [11],

$$P_i = P_{D,i} + \sum_{i \neq j}^{n-1} P_{S,j}, \tag{3}$$

where $P_{D,i}$ denotes the direct effect due to the focal individual i, $P_{S,j}$ the associative effect of its group mate j, and the summation is over the $n-1$ group mates of the focal individual. Both the direct and associative effect may be decomposed into a heritable component, A, and a non-heritable component, E. The



Socially Affected Traits, Inheritance and Genetic Improvement. Figure 1

(**a**) The phenotype of individual 1 is the sum of its own direct effect plus the associative effects of its group mates, $P_1 = A_{D,1} + E_{D,1} + \sum_{j=2,3,4} \left(A_{S,j} + E_{S,j}\right)$. (**b**) The total breeding value of individual 1 is the sum of its heritable effects on all group members, including itself, $A_{T,1} = A_{D,1} + 3A_{S,1}$

phenotype, therefore, is the sum of the direct breeding value and direct environmental effect of the focal individual, and the summed associative breeding values and associative environmental effects of each of its $n - 1$ group mates [11, Fig. 1a],

$$P_i = A_{D,i} + E_{D,i} + \sum_{i \neq j}^{n-1} A_{S,j} + \sum_{i \neq j}^{n-1} E_{S,j} \qquad (4)$$

In this expression, $A_{D,i}$ is the direct breeding value (DBV) of individual *i*, and $A_{S,j}$ the associative breeding value (SBV) of its group mate *j*. The DBV corresponds to the classical (direct) breeding value, whereas the SBV is a generalization of a breeding value for maternal effect (See section on "A Special Case: Maternal Genetic Effects" below). An individual's DBV is the sum of the direct average effects of its genes, whereas its SBV is the sum of the associative effects of its genes. In total, the $A_{D,i} + \sum_{i \neq j}^{n-1} A_{S,j}$ is the best predictor of the phenotype of the focal individual from the average effects of its own genes and those in its group mates, obtained using the method of least squares [27–29, 32]. The $E_{D,i}$ and $E_{S,i}$ are the corresponding non-heritable effects ("environment").

At first glance, it seems that the summation in Eq. 4 implies additivity of associative effects, whereas, in reality, an individual's associative effect may depend on the individual it interacts with. For example, an individual may interact in a positive manner with a certain group mate, but have adverse effects on other group mates. Equation 4, however, considers the average associative effect of a genotype, which is additive by definition. This is an analogy of the average effect of an allele in, e.g., a single locus model [26, 29]. In the presence of dominance, the effect of an allele depends on the other allele at the locus. Nevertheless, one can still define the average effect of an allele, which is the effect relative for heritable variance and response to selection (at least in the short term). Hence, Eq. 4 does not assume additivity of the social interactions, but focuses on the heritable component of these interactions.

Breeding Value The objective of livestock genetic improvement is to generate response to selection, that is, to genetically change the mean trait values of the population. For this purpose, an individual's quality is measured by its heritable effect on the mean trait value of the population. In the classical model, an individual's heritable effect on the population mean simply equals the classical breeding value, *A*, as defined in Eq. 1. With associative effects, an individual's full heritable effect on the mean trait value of the population is the sum of its direct heritable effect on its own phenotype, plus its social heritable effect on the phenotypes of its group mates. Hence, an individual's total breeding value may be defined as [31; Fig. 1b]

$$A_{T,i} = A_{D,i} + (n-1)A_{S,i} \qquad (5)$$

In contrast to an individual's phenotype, its total breeding value is entirely a property of its own genes (Fig. 1a vs 1b). The total breeding value is a generalization of the classical breeding value and is the heritable property relevant for response to selection in socially affected traits. Taking the average phenotype of the population shows that response to selection equals the change in mean total breeding value,

$$R = \Delta \bar{A}_T \tag{6}$$

In contrast to the classical breeding value, however, the total breeding value contains a part that is not expressed in the individual's own phenotype, nor in the phenotypes of its offspring, but in the group mates of the individual and of its offspring.

Heritable Variance In the classical model, heritable variance equals the variance of the breeding values among individuals. By analogy, for socially affected traits, the total heritable variance may be defined as the variance in total breeding values among individuals [31],

$$\sigma^2_{A_T} = \sigma^2_{A_D} + 2(n-1)\sigma_{A_{DS}} + (n-1)^2\sigma^2_{A_S}, \tag{7}$$

where $\sigma^2_{A_D}$ is the variance in direct breeding values, $\sigma^2_{A_S}$ the variance in associative breeding values, and $\sigma_{A_{DS}}$ the covariance. The total heritable variance reflects the magnitude of the heritable differences among individuals that determine the potential of the population to respond to selection (See section "Response to Selection" below). The $(n-1)^2\sigma^2_{A_S}$ term represents the heritable variance present in the social environment. This term shows that even small associative effects can substantially increase the total heritable variance, particularly when groups are large. (But see the section "The Effect of Group Size on Heritable Variance" below.) This may explain the rapid response to group selection observed by Muir [19, 21]. The term $2(n-1)\sigma_{A_{DS}}$ shows that a negative genetic covariance between direct and associative breeding values reduces the total heritable variance. With negative $\sigma_{A_{DS}}$, individuals with positive breeding values for their own phenotype have on average negative associative effects on the phenotypes of their group mates, and vice versa. Thus a negative $\sigma_{A_{DS}}$ may be interpreted as "heritable competition." Heritable competition, therefore, reduces total heritable variance and thus the potential of the population to respond to selection. Heritable cooperation ($\sigma_{A_{DS}} > 0$), in contrast, increases total heritable variance.

The $\sigma^2_{A_T}$ expresses heritable variance in absolute units. The interpretation of the magnitude of the heritable variation is facilitated by expressing heritable variance relative to phenotypic variance, analogous to classical heritability (Eq. 2). By analogy, for socially affected traits, the ratio of total heritable variance over phenotypic variance equals [15],

$$T^2 = \frac{\sigma^2_{A_T}}{\sigma^2_P} \tag{8}$$

A comparison of h^2 and T^2 reveals the proportional contribution of associative to heritable variance. When group members are unrelated, phenotypic variance equals

$$\sigma^2_{P_{r=0}} = \sigma^2_{A_D} + \sigma^2_{E_D} + (n-1)\left(\sigma^2_{A_S} + \sigma^2_{E_S}\right) \tag{9}$$

With associative effects, phenotypic variance depends on relatedness between group members [15, 33]. As a consequence, T^2 depends on relatedness between group members when using the realized phenotypic variance in Eq. 8, which is inconvenient for a standardized parameter. Hence, to achieve standardization, it is preferable to scale total heritable variance by the phenotypic variance for populations with unrelated group members, using $T^2 = \sigma^2_{A_T}/\sigma^2_{P_{r=0}}$, rather than using the realized phenotypic variance in Eq. 8.

Comparison of Eqs. 7 and 9 shows that total heritable variance, $\sigma^2_{A_T}$, is not a component of phenotypic variance, illustrating that an individual's total breeding value is not a component of its trait value (Eq. 4 vs Eq. 5). Because an individual's total breeding value is dispersed over multiple individuals, it is hidden to direct observation. As a consequence, total heritable variance may exceed phenotypic variance, and T^2 may take values greater than one, whereas classical heritability has an upper limit of one. When group members are unrelated, the difference between the genetic component of phenotypic variance and total heritable variance equals (personal communication J. Bruce Walsh)

$$\sigma^2_{A_T} - \sigma^2_P = (n-1)\left[2\sigma_{A_{DS}} + (n-2)\sigma^2_{A_S}\right], \tag{10}$$

illustrating that heritable variance may substantially exceed phenotypic variance particularly when groups

are large. (But see the following section on "The Effect of Group Size".) Social interactions, therefore, result in hidden heritable variation. Empirical results indeed indicate that T^2 may considerably exceed h^2, but values greater than one have not been found so far (See Table 3 below). Note, however, that greater heritable variance than phenotypic variance does not necessarily imply that associative effects increase response to selection. Whether or not the available heritable variance translates into response to selection depends on selection criterion and on relatedness between group members [11, 18, 33] (See section on "Response to Selection" below).

The Effect of Group Size on Heritable Variance The careful reader will have noted that total heritable variance approaches infinity when groups become large (Eq. 7), which is unrealistic. Indeed, when $\sigma^2_{A_S}$ remains constant while group size increases, the $\sigma^2_{A_T}$ in Eq. 7 increases continuously. The magnitude of the associative genetic variance, $\sigma^2_{A_S}$, however, will probably also depend on group size because interactions between a specific pair of individuals are likely to be less intense in large groups [25]. The relationship between the magnitude of associative effects and group size is relevant because it affects the dynamics of response to selection, heritable variance, and group size. It determines, for example, whether or not selection is more efficient with larger groups.

Models accounting for the relationship between group size and associative effects have been provided in Refs. [25, 42, 43]. In [43], the relationship between associative effects and group size is modeled as

$$A_{S,i,n} = \left(\frac{\bar{n}-1}{n-1}\right)^d A_{S,i,\bar{n}}, \tag{11}$$

where $A_{S,i,n}$ is the associative effect of individual i when it would be expressed in a group of n members, $A_{S,i,\bar{n}}$ the associative effect of i when expressed in a group of the average size, $\bar{n}$, and d the degree of dilution. The degree of dilution measures the decrease of an individual's associative effect when group size increases.

With no dilution, $d = 0$, an individual's associative effect is the same for all group sizes, $A_{S,i,n} = A_{S,i,\bar{n}}$, so that its total associative effect summed over all group mates is proportional to the number of group mates, $(n-1)A_{S,i,n} = (n-1)A_{S,i,\bar{n}}$. This may occur, for example, in trees, where a large tree's associative effect may result from shading all individuals under its canopy, the effect on each individual being independent of the total number of individuals under the canopy (personal communication J. Bruce Walsh). With full dilution, $d = 1$, in contrast, an individual's total associative effect summed over all group mates is independent of group size, $(n-1)A_{S,i,n} = (\bar{n}-1)A_{S,i,\bar{n}}$, while its associative effect on each individual group mate is inversely proportional to the number of group mates, $A_{S,i,n} = [(\bar{n}-1)/(n-1)]\ A_{S,i,\bar{n}}$. This may occur, for example, with restricted feeding on group level, where an individual consuming 1 kg of feed has a total associative effect of -1 kg, and an average associative effect of $A_{S,i,n} = -1/(n-1)$ kg on each of its group mates. More generally, the magnitude of associative effects may be modeled as a function of the intensity of the interaction. In trees, for example, the associative effect of one tree on another may be modeled inversely proportional to the distance between both trees [17]. The degree of dilution of associative effects is an empirical issue, which may be trait and population specific, and needs to be estimated for the population of interest (See section on "Estimation of Genetic Parameters" below).

Dilution of associative effects alters the relationship between genetic variances and group size. The associative genetic variance for groups of $n$members equals,

$$\sigma^2_{A_S,n} = \left(\frac{\bar{n}-1}{n-1}\right)^{2d} \sigma^2_{A_S,\bar{n}}, \tag{12}$$

which decreases with group size for $d > 0$. The total heritable variance for groups of n members equals

$$\begin{aligned}\sigma^2_{A_T,n} = \sigma^2_{A_D} + 2(\bar{n}-1)^d(n-1)^{1-d}\sigma_{A_{DS},\bar{n}} \\ +(\bar{n}-1)^{2d}(n-1)^{2-2d}\sigma^2_{A_S,\bar{n}}\end{aligned} \tag{13}$$

(This expression may seem complex, but note that $\bar{n}$ is a constant for any particular population). Hence, for $\sigma_{A_{DS}} = 0$, total heritable variance increases with group size as long as dilution is incomplete ($d < 1$), and total heritable variance is independent of group size with full dilution ($d = 1$). Since total heritable variance depends on group size and on the degree of dilution, response to selection will also depend on those factors. To account for the effect of dilution on response to selection, the $\sigma^2_{A_S}$ and $\sigma^2_{A_T}$ should be substituted by Eqs. 12 and 13 in the expressions for response to selection provided below.

When genetic and non-genetic associative effects show the same dilution with group size, the phenotypic variance in populations of groups of $n$unrelated members equals

$$\sigma^2_{P,n} = \sigma^2_{P_D} + (\bar{n}-1)^{2d}(n-1)^{1-2d}\sigma^2_{P_S,\bar{n}} \qquad (14)$$

showing that phenotypic variance increases with group size for $d < 0.5$, is independent of group size for $d = 0.5$, and decreases with group size for $d > 0.5$. Hence, the relationship between phenotypic variance and group size provides an approximate impression of the degree of dilution.

Response to Selection

In a series of papers, Griffing [11, 33, 44–46] showed theoretically that associative effects on trait values alter response to genetic selection, not only in magnitude but potentially also in direction, and demonstrated that response depends strongly on relatedness among interacting individuals and on the selection criterion. Results of Griffing, however, do not fit easily in the common animal breeding framework for response to selection and have largely been overlooked. Griffing [44] summarized his results into a more common theoretical framework, but this work was published in a conference proceeding and was also largely overlooked. Muir [17] and Bijma et al. [31] rediscovered those results and expressed them in terms familiar to animal breeders. This section summarizes the resulting expressions for response to artificial selection, accounting for associative effects, genetic relatedness among interacting individuals, and selection acting on multiple levels, such as individual or group, or on an (optimum) index of both.

General Expression for Response to Selection In classical animal breeding theory, i.e., in the absence of associative effects, response to selection is commonly expressed as the product of the intensity of selection, $\imath$, the accuracy of selection, ρ, and the additive genetic standard deviation in the trait, σ_A [e.g., 47],

$$R = \imath\rho\sigma_A \qquad (15)$$

In this expression, R is the genetic change in mean trait value from one generation to the next. The selection intensity expresses the phenotypic superiority of the selected parents in standard deviation units, $i = S/\sigma$, where S is the superiority of the selected parents for the selection criterion and σ is the standard deviation of the selection criterion among the candidates for selection. The accuracy is the correlation between the value of the selection criterion (SC) and the additive genetic merit for the trait (the breeding value) in the candidates for selection,

$$\rho = \mathrm{corr}(A, \mathrm{SC}) \qquad (16)$$

Equation 15 partitions response to selection into three clearly distinct components; a scale-free measure of the strength of selection, $\imath$, a scale-free measure of how accurately the selection criterion resembles an individual's true breeding value for the trait of interest, ρ, and a measure of the magnitude of the heritable differences in the population, σ_A. The intensity of selection is largely determined by the reproductive potential of the species and the availability of reproductive technologies such as artificial insemination. The accuracy reflects the quality of the selection criterion, which depends on the type and amount of information collected. The genetic standard deviation is an intrinsic biological property of the population, reflecting its potential to respond to selection, and is outside human control. (Equation 15 can easily be extended to account for two sexes [26]).

The classical expression for response can be generalized to account for associative effects [48]. From the trait model (Eq. 4), it follows that response to selection equals the increase in mean direct breeding value plus the increase in mean associative breeding value weighted by the number of group mates, $R = \Delta[\bar{A}_D + (n-1)\bar{A}_S]$, which is the change in mean total breeding value, $\Delta\bar{A}_T$ (Eq. 5). The change in mean total breeding value equals the mean total breeding value of the selected parents expressed as a deviation from the overall mean and follows from regression of the total breeding value on the selection criterion, $R = b_{A_T,\mathrm{SC}}(\mathrm{SC} - \overline{\mathrm{SC}})$. From the definitions of regression and correlation coefficients, and using $\mathrm{SC} - \overline{\mathrm{SC}} = \imath\sigma_{\mathrm{SC}}$, it follows that

$$R = \imath\rho_T\sigma_{A_T}, \qquad (17)$$

where the total accuracy of selection, ρ_T, is the correlation between the selection criterion and the total breeding value in the candidates for selection, and σ_{A_T} is the total genetic standard deviation in the

trait (Eq. 7). Equation 17 applies to any selection strategy and inheritance model, as long as the total breeding value represents the average effects of an individual's genes on the mean trait value of the population. (See [49] for an application to maternal effects, and [49] for an application to both associative and maternal effects). Equation 17 corresponds to the first term of Price's Theorem [50] and represents the change in trait value due to change in allele frequency, keeping average effects of alleles constant for all elements of the inheritance model. Because intensity and accuracy are scale-free parameters depending on the breeding design, the total genetic standard deviation reflects the intrinsic potential of a population to respond to selection. Thus the total heritable variance defined in Eq. 7 bears a direct relationship to response to selection. In theory, σ_{A_T} may exceed σ_P (see above), suggesting that response in socially affected traits can be very large relative to phenotypic standard deviation. While this agrees with the very large response observed by Muir [19, 21] in cannibalistic laying hens, it is unlikely that this phenomenon is widespread in livestock. Nevertheless, the potential for genetic improvement of socially affected traits may be larger than suggested by classical theory (see Table 3 below).

Accuracies of Selection Traditional selection methods include mass selection, selection based on information recorded on relatives, mostly full sibs, half sibs, or progeny, index selection [51, 52], and selection on estimated breeding values (EBV) obtained using Best Linear Unbiased Prediction (BLUP, [53]). Those methods are aimed at the classical (direct) breeding value and do therefore not fully utilize the available heritable variation when trait values are affected by associative effects. Efficient improvement of socially affected traits, therefore, requires modification of classical selection methods. Research in the field of evolutionary biology [12, 18, 30, 54–57] and early work on associative effects [11, 33] indicates that genetic relatedness among interacting individuals and selection acting on the group rather than the individual level strongly affect response to selection. Relatedness between group members and selection between groups are, therefore, important factors in response to selection for socially affected traits.

The following summarizes the accuracies for direct, associative, and total breeding value for a number of selection strategies (Table 2). In all cases, the individuals that provide the information for selection are kept in groups of n members, but the selection criterion may differ. For any parameter, response to selection is the product of intensity ($\imath$), accuracy (ρ), and genetic standard deviation (σ_A) for the parameter of interest,

$$R_D = \imath \rho_D \sigma_{A_D} \tag{18a}$$

$$R_S = \imath \rho_S \sigma_{A_S} \tag{18b}$$

$$R = R_D + (n-1)R_S = \imath \rho_T \sigma_{A_T} \tag{18c}$$

Individual Selection (IS) With individual selection (also known as "mass selection"), the individuals with the best phenotypes are selected as parents of the next generation. The total accuracy of individual selection equals (Ellen et al. 2007),

$$\rho_{T,\mathrm{IS}} = \frac{r\,\sigma^2_{A_T} + (1-r)[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{\sigma_{A_T}\sigma_P} \tag{19}$$

In the numerator of Eq. 19, relatedness acts as a weighting factor; greater relatedness increases the $\sigma^2_{A_T}$ term, while decreasing the $\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}$ term (Fig. 2a).

When group members are unrelated, IS does not directly target the associative breeding value, as illustrated by the accuracy of associative effects, $\rho_{S,\mathrm{IS}}(r=0) = \sigma_{A_{DS}}/(\sigma_{A_S}\sigma_P)$ (Table 2). Hence, when group member are unrelated, response in associative effects is entirely dependent on the covariance between direct and associative effects (Fig. 2b). When this covariance is negative, IS yields a negative response in associative effects, meaning that competition among individuals increases. Moreover, with unrelated group members, the negative response in associative effects may exceed the positive response in direct effect, $-(n-1)R_S > R_D$, yielding a negative net response and a negative total accuracy. This occurs when $\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}} < 0$. The risk of negative response is not merely a theoretical possibility, but has been observed for egg number in cannibalistic laying hens by Muir [17].

When relatedness between group members increases, components in the numerator shift away from a potentially negative term, $\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}$,

Socially Affected Traits, Inheritance and Genetic Improvement. Table 2 Accuracies of selection

		Total	Direct	Associative
IS[a]	$r = 0$	$\frac{\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}}{\sigma_P \sigma_{A_T}}$	$\frac{\sigma_{A_D}}{\sigma_P}$	$\frac{\sigma_{A_{DS}}}{\sigma_P \sigma_{A_S}}$
	$r \neq 0$	$\frac{r\sigma^2_{A_T} + (1-r)[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{\sigma_{A_T}\sigma_P}$	$\frac{\sigma^2_{A_D} + r(n-1)\sigma_{A_{DS}}}{\sigma_{A_D}\sigma_P}$	$\frac{\sigma_{A_{DS}} + r(n-1)\sigma^2_{A_S}}{\sigma_{A_S}\sigma_P}$
GS[b]	$r = 0$	$\frac{\sigma_{A_T}}{n\sigma_{\bar{P}}}$	$\frac{\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}}{n\sigma_{A_D}\sigma_{\bar{P}}}$	$\frac{\sigma_{A_{DS}} + (n-1)\sigma^2_{A_S}}{n\sigma_{A_S}\sigma_{\bar{P}}}$
	$r \neq 0$	$\frac{[1+(n-1)r]\sigma_{A_T}}{n\sigma_{\bar{P}}}$	$\frac{[1+(n-1)r]\,[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{n\sigma_{A_D}\sigma_{\bar{P}}}$	$\frac{[1+(n-1)r]\,[\sigma_{A_{DS}} + (n-1)\sigma^2_{A_S}]}{n\sigma_{A_S}\sigma_{\bar{P}}}$
Indx[c]	$r = 0$	$\frac{g\sigma^2_{A_T} + (1-g)[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{\sigma_{A_T}\sigma_I}$	$\frac{g[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}] + (1-g)\sigma^2_{A_D}}{\sigma_{A_D}\sigma_I}$	$\frac{g[\sigma_{A_{DS}} + (n-1)\sigma^2_{A_S}] + (1-g)\sigma_{A_{DS}}}{\sigma_{A_S}\sigma_I}$
	$r \neq 0$	$\left\{[g + r + (n-2)gr]\,\sigma^2_{A_T} + (1-g)(1-r)[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]\right\}/(\sigma_{A_T}\sigma_I)$	$\left\{[g + r + (n-2)gr][\,\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}] + (1-g)(1-r)\sigma^2_{A_D}\right\}/(\sigma_{A_D}\sigma_I)$	$\left\{[g + r + (n-2)gr][\,\sigma_{A_{DS}} + (n-1)\sigma^2_{A_S}] + (1-g)(1-r)\sigma_{A_{DS}}\right\}/(\sigma_{A_D}\sigma_I)$
Rel's[d]	$r = 0$[e]	$\frac{r_f[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{\sigma_{\bar{P}_f}\sigma_{A_T}}$	$\frac{r_f\sigma_{A_D}}{\sigma_{\bar{P}_f}}$	$\frac{r_f\sigma_{A_{DS}}}{\sigma_{\bar{P}_f}\sigma_{A_S}}$
	$r = r_w$[f]	$\frac{r\sigma_{A_T}}{\sigma_{\bar{P}_f}} = \frac{r_f\eta}{\sqrt{\tau + (1-\tau)/mn}}$	$\frac{r_f[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{\sigma_{A_D}\sigma_{\bar{P}_f}}$	$\frac{r_f[\sigma_{A_{DS}} + (n-1)\sigma^2_{A_S}]}{\sigma_{A_S}\sigma_{\bar{P}_f}}$

[a]Individual selection.

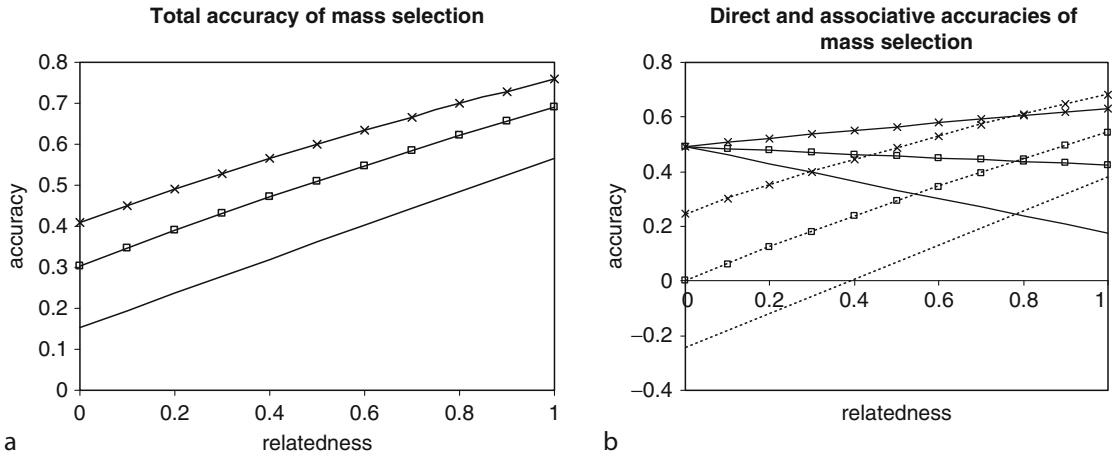
[b]Group selection; $\sigma_{\bar{P}}$ is the standard deviation of the group means (Eq. 20b).

[c]Index selection, $I_i = P_i + g\sum_{n-1} P_j$, g represents the degree of group selection, σ_I is the standard deviation of the index.

[d]Selection based on phenotypes of relatives; r_f is the additive genetic relatedness between the candidate and the relatives providing information, which equals 0.25 for half sibs, and 0.5 for full sibs and progeny; $\sigma_{\bar{P}_f}$ is the standard deviation of the family mean.

[e]The relatives of the selection candidate are mixed at random over groups, so that group members are on average unrelated.

[f]The relatives of selection candidates are kept together in groups, so that relatedness within groups equals within-family relatedness (r_w), which equals 0.25 for half sib families and 0.5 for full sib families. The m is the number of groups of relatives per candidate, so that mn is the total number of relatives per candidates. See Eq. 35 for η and τ.



Socially Affected Traits, Inheritance and Genetic Improvement. Figure 2
Accuracies of mass selection. (**a**) Total accuracy. *No symbols*: $r_{A_{DS}} = -0.5$; *boxes*: $r_{A_{DS}} = 0$; *crosses*: $r_{A_{DS}} = +0.5$. (**b**) Direct and associative accuracies. *Solid lines*: direct accuracy; *dotted lines*: associative accuracy. Symbols as in panel **a**. Other genetic parameters are $n = 8$, $\sigma^2_{A_D} = 1.5$, $\sigma^2_{A_S} = 0.05$, $h^2_D = h^2_S = 0.3$ and $r_{E_{DS}} = r_{A_{DS}}$. These parameters yield a phenotypic variance of $\sigma^2_P = 6.17$ when relatedness equals zero, and total heritable variances of $\sigma^2_{A_T} = 2.03$ for $r_{A_{DS}} = -0.5$, $\sigma^2_{A_T} = 3.95$ for $r_{A_{DS}} = 0$, and $\sigma^2_{A_T} = 5.87$ for $r_{A_{DS}} = +0.5$, respectively

toward a positive term, $\sigma^2_{A_T}$, which increases the accuracy (Fig. 2a). In the extreme case with fully related group members (clones), $\rho_{T,\mathrm{IS}}(r = 1) = \sigma_{A_T}/\sigma_P$, which is an analogy of the classical accuracy of individual selection, $\rho_{\mathrm{mass}} = h = \sigma_A/\sigma_P$ [26]. This illustrates that a population consisting of clone groups is genetically equivalent to a population consisting of noninteracting individuals with heritability $h^2 = \sigma^2_{A_T}/\sigma^2_P$. In other words, the "group becomes an individual" when group members are fully related. In summary, with individual selection, relatedness between group members can prevent negative accuracies.

Group Selection (GS) With group selection, selection is based solely on mean trait values of the groups, so that parents are selected from the best groups [33, 58, 59]. The accuracy of group selection equals [48]

$$\rho_{T,\mathrm{GS}} = \frac{[1 + (n-1)r]\sigma_{A_T}}{n\sigma_{\bar{P}}}, \tag{20a}$$

where $\sigma_{\bar{P}}$ is the standard deviation of the group means,

$$\sigma^2_{\bar{P}} = \frac{1}{n}\left[\sigma^2_P + (n-1)\mathrm{Cov}(P_i, P_j)\right] \tag{20b}$$

in which $\mathrm{Cov}(P_i, P_j)$ is the covariance between the phenotypes of two group members,

$$\begin{aligned}\mathrm{Cov}(P_i, P_j) = {} & 2\sigma_{P_{DS}} + (n-2)\sigma^2_{P_S} \\ & + r\left[\sigma^2_{A_D} + 2(n-2)\sigma_{A_{DS}}\right. \\ & \left. + (n^2 - 3n + 3)\sigma^2_{A_S}\right].\end{aligned} \tag{20c}$$

Because both the numerator and denominator of Eq. 20a are nonnegative, accuracy of group selection is nonnegative, irrespective of relatedness. Accuracy of GS has lower limit of zero, which occurs when all groups are composed in exactly the same manner; for example, when 10 clones are allocated to 10 groups, each group containing precisely one individual of each clone. In that case, $r = -1/(n-1)$ and accuracy is zero [60]. In this case, there is no between-group genetic variance, so that group selection cannot create response. In all other cases, GS directly targets the total breeding value, as illustrated by the σ_{A_T} in the numerator of Eq. 20a. This is because an individual's total breeding value surfaces in the group mean; the direct breeding value via its own phenotype and the associative breeding value via the phenotypes of each of its $(n-1)$ group mates. Group selection, therefore, also directly targets

both direct and associative breeding values, as illustrated by the $\sigma^2_{A_D}$ and $\sigma^2_{A_S}$ in the numerator of the direct and associative accuracy, respectively (Table 2).

When group members are unrelated, however, the n in the denominator causes accuracy to be low particularly when groups are large. This is because the between-group genetic variance is proportional to $1/n$ when group members are unrelated. Hence, with unrelated group members, the genetic variance that can be utilized by group selection decreases with group size, and approaches zero for large groups. Relatedness between group members considerably increases the accuracy of group selection because it increases the between-group genetic variance. In the extreme case of fully related group members (clones), $\rho_{T,\mathrm{GS}}\,(r=1)=\sigma_{A_T}/\sigma_{\bar{P}}$, which may be interpreted as the square root of the group heritability, $\sigma^2_{A_T}/\sigma^2_{\bar{P}}$. Note that GS outperforms IS when group members are fully related because $\sigma_{A_T}/\sigma_{\bar{P}} > \sigma_{A_T}/\sigma_P$. This occurs because GS does not waste effort on within-group selection, which is useless when group members are genetically identical. In summary, group selection prevents negative accuracy, but is efficient only when group members are sufficiently related.

Multilevel Selection Rather than selecting solely on individual or mean group phenotype, one may select on a linear combination of phenotypes of group mates and individual phenotype. In terminology used in evolutionary biology, this is an application of multilevel selection, where the levels of selection are the individual and the group. In natural populations, multilevel selection occurs when individual fitness depends on both properties of the individual itself and on properties of its groups. Multilevel selection is widespread in nature [61], but can also be applied as a breeding strategy in livestock and aquaculture [17, 31, 44].

Multilevel selection may be described by the following index [17, 31, 44]

$$I_i = P_i + g\sum_{n-1} P_j \tag{21}$$

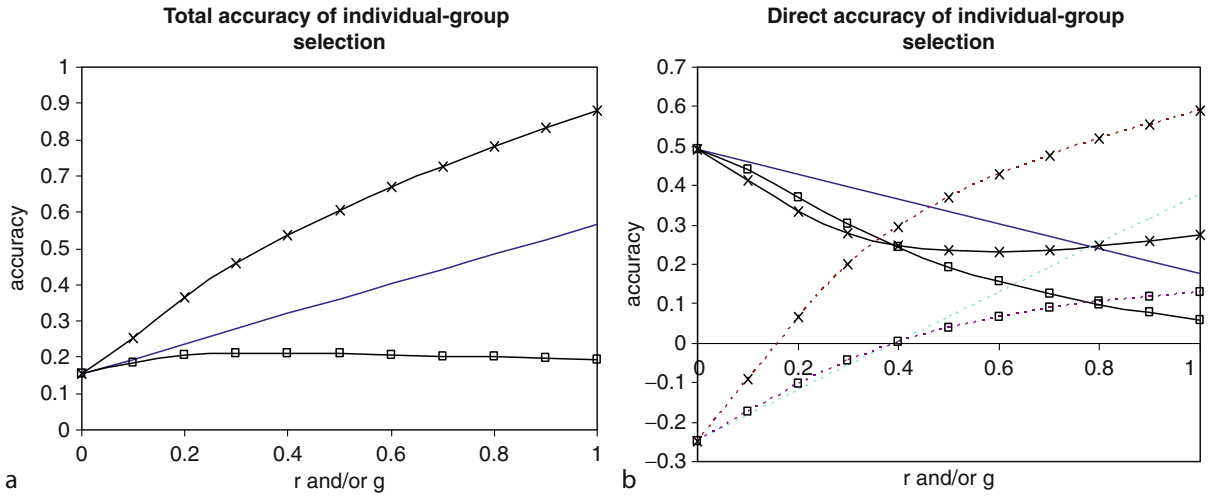
where g represents the weight on the summed phenotypes of the $(n-1)$ group mates. A $g=0$ corresponds to individual selection, whereas a $g=1$ corresponds to selection on $I_{i,g=1}=\sum_n P_j$, where the summation is over all $n$group members, including the individual of interest. Hence, a $g=1$ corresponds to between-group selection. Thus the $g$may be interpreted as a measure of the degree of group versus individual selection. A $g=-1/(n-1)$ corresponds to within-group selection, where individuals are selected based on the deviation of their phenotype from the mean phenotype of their group. (Interestingly, the value of $-1/(n-1)$ is also the lower bound for relatedness between group members, indicating a symmetry between $g$and r; [60]).

The accuracy of multilevel selection equals [62]

$$\rho_{T,I} = \frac{[g+r+(n-2)gr]\,\sigma^2_{A_T} + (1-g)(1-r)[\sigma^2_{A_D}+(n-1)\sigma_{A_{DS}}]}{\sigma_{A_T}\sigma_I} \tag{22}$$

Note that the numerator of this expression is fully symmetric in the degree of group selection, g, and relatedness, r, indicating that group selection and relatedness have precisely the same effect on the covariance between the index value and the total breeding value of selection candidates. The first term in the numerator, $[g+r+(n-2)gr]\,\sigma^2_{A_T}$, shows that group selection and relatedness cause selection to target the total breeding value. The second term, $(1-g)(1-r)[\sigma^2_{A_D}+(n-1)\sigma_{A_{DS}}]$, shows that the complement of group selection and relatedness acts on a term that is potentially negative. The $[\sigma^2_{A_D}+(n-1)\sigma_{A_{DS}}]$ is the numerator of the accuracy of individual selection with unrelated group members, which may indeed give negative response (See section on "Individual Selection" above). Hence, group selection and relatedness shift selection away from a potentially negative term toward the total genetic variance, thereby preventing negative response due to increased competition. Figure 3a illustrates this effect, showing that the combination of multilevel selection and relatedness may strongly increase accuracy. When group members are unrelated, the effect of multilevel selection on accuracy may be only moderate, and an intermediate value of $g$may be optimal. Figure 3b shows that multilevel selection and relatedness increase the accuracy of the associative effect, which may be at the expense of the direct effect when the direct-associative genetic correlation is negative.

Optimum Index Selection If the genetic parameters of direct and associative effects are known, then the degree of between-group selection, g, can be optimized using



Socially Affected Traits, Inheritance and Genetic Improvement. Figure 3

Accuracy of multilevel selection, $I_i = P_i + g\sum_{n-1} P_j$. (**a**) Total accuracy. *No symbols*: $g = 0$, $r \in [0, 1]$; *boxes*: $r = 0$, $g \in [0, 1]$; crosses: $r = g \in [0, 1]$. (**b**) Direct and associative accuracies. *Solid lines*: direct accuracy; *dotted lines*: associative accuracy. Symbols as in panel **a**. Other genetic parameters are $n = 8$, $\sigma^2_{A_D} = 1.5$, $\sigma^2_{A_S} = 0.05$, $r_{A_{DS}} = -0.5$, $h^2_D = h^2_S = 0.3$ and $r_{E_{DS}} = r_{A_{DS}}$. These parameters yield a phenotypic variance of $\sigma^2_P = 6.17$ when relatedness equals zero, and a total heritable variance of $\sigma^2_{A_T} = 2.03$

selection index theory [17, 44, 48, 51]. For this purpose, it is convenient to rescale the index to

$$I_i = b_1 P_i + b_2 \sum_{n-1} P_j \tag{23}$$

This index is equivalent to $I_i = P_i + (b_2/b_1)\sum_{n-1} P_j$, so that the optimum degree of group selection is

$$g_{\text{opt}} = \frac{b_{2,\text{opt}}}{b_{1,\text{opt}}} \tag{24}$$

From selection index theory, optimum index weights are [52]

$$\mathbf{b}_{\text{opt}} = \mathbf{P}^{-1}\mathbf{G}\mathbf{v}, \tag{25}$$

where $\mathbf{b}_{\text{opt}}$ is a column-vector of optimum index weights, $\mathbf{b}'_{\text{opt}} = [\, b_{1,\text{opt}} \quad b_{2,\text{opt}} \,]$, $\mathbf{P}$ is the symmetric matrix of covariances between the information sources in the index,

$$\mathbf{P} = \begin{bmatrix} \text{Var}(P_i) & \text{Cov}\left(P_i, \sum_{n-1} P_j\right) \\ \text{Cov}\left(P_i, \sum_{n-1} P_j\right) & \text{Var}\left(\sum_{n-1} P_j\right) \end{bmatrix},$$

$\mathbf{G}$ is the matrix of covariances between the information sources in the index (rows of $\mathbf{G}$) and the true direct and associative breeding values of the individual of interest (columns of $\mathbf{G}$),

$$\mathbf{G} = \begin{bmatrix} \text{Cov}(P_i, A_{D,i}) & \text{Cov}(P_i, A_{S,i}) \\ \text{Cov}\left(\sum_{n-1} P_j, A_{D,i}\right) & \text{Cov}\left(\sum_{n-1} P_j, A_{S,i}\right) \end{bmatrix},$$

and $\mathbf{v}$ is a vector of weights on direct and associative breeding values (also known as "economic values"). Because response equals $R = \Delta[A_D + (n-1)A_S]$, the weights required to optimize response to selection are given by $\mathbf{v}' = [1\, n-1]$.

Elements of $\mathbf{P}$ are

$$P_{11} = \sigma^2_P \tag{26a}$$

$$P_{12} = P_{21} = (n-1)\text{Cov}(P_i, P_j) \tag{26b}$$

$$P_{22} = (n-1)\text{Var}(P_i) + (n-1)(n-2)\,\text{Cov}(P_i, P_j), \tag{26c}$$

where $\text{Cov}(P_i, P_j)$ is the covariance between the phenotypes of two group members, which equals

$$\begin{aligned}\text{Cov}(P_i, P_j) = {} & 2\sigma_{P_{DS}} + (n-2)\sigma^2_{P_S} \\ & + r\left[\sigma^2_{A_D} + 2(n-2)\sigma_{A_{DS}}\right. \\ & \left. + (n^2 - 3n + 3)\sigma^2_{A_S}\right].\end{aligned} \tag{27}$$

The first two terms of this expression can be obtained from mixed model analysis using, $2\sigma_{P_{DS}} + (n-2)\sigma^2_{P_S} = 2\sigma_{A_{DS}} + (n-2)\sigma^2_{A_S} + \sigma^2_g$, where σ^2_g is the between-group non-genetic variance (See section "Estimation of Genetic Parameters" below). Elements of **G** are

$$G_{11} = \sigma^2_{A_D} + (n-1)r\sigma_{A_{DS}} \tag{28a}$$

$$G_{12} = \sigma_{A_{DS}} + (n-1)r\sigma^2_{A_S} \tag{28b}$$

$$G_{21} = (n-1)\left[r\sigma^2_{A_D} + \sigma_{A_{DS}} + (n-2)r\sigma_{A_{DS}}\right] \tag{28c}$$

$$G_{22} = (n-1)\left[r\sigma_{A_{DS}} + \sigma^2_{A_S} + (n-2)r\sigma^2_{A_S}\right] \tag{28d}$$

Using those **P** and **G**, the optimum weight on group versus individual, g_{opt}, follows from Eqs. 25 and 24. Note that g_{opt} may fall outside the 0–1 range. For example, when associative effects are large, g_{opt} may exceed one, indicating that the individual phenotype is weighted negatively relative to the overall group mean.

The accuracy of the optimum index can be obtained either by substituting g_{opt} into Eq. 22, or from

$$\rho_{T,I_{\text{opt}}} = \sigma_I/\sigma_{A_T}, \tag{29}$$

where σ_I is the standard deviation of the index,

$$\sigma_I = \sqrt{\mathbf{b}'\mathbf{P}\mathbf{b}} \tag{30}$$

For any index, i.e., for any combination of weights on the phenotype of the individual versus the summed phenotypes of its group mates, accuracy is given by

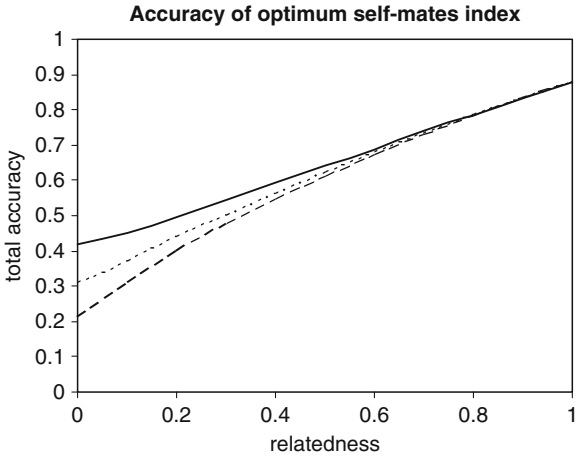
$$\rho_T(\mathbf{b}) = \frac{\mathbf{b}'\mathbf{G}\mathbf{v}}{\sigma_I\sigma_{A_T}} \tag{31}$$

This expression is equivalent to Eq. 22, where $g = b_2/b_1$. Total response to selection follows from Eq. 17, and responses in direct and associative effects are given by

$$\begin{bmatrix} \Delta\bar{A}_D \\ \Delta\bar{A}_S \end{bmatrix} = \mathbf{b}'\mathbf{G}\frac{\imath}{\sigma_I}, \tag{32}$$

where $\imath$ is the intensity of selection [26].

Figure 4 illustrates the accuracy of index selection, showing that accuracy is always positive and increases considerably with relatedness among group members.



Socially Affected Traits, Inheritance and Genetic Improvement. Figure 4
Accuracy of an optimum index of own performance and group mates, $I = b_1P_i + b_2\sum_{n-1}P_j$, as a function of relatedness among group members. For three genetic correlations between direct and associative effects; *dashed line* $r_{A_{DS}} = -0.5$, *dotted line* $r_{A_{DS}} = 0$, *solid line* $r_{A_{DS}} = +0.5$. Other genetic parameters are $n = 8$, $\sigma^2_{A_D} = 1.5$, $\sigma^2_{A_S} = 0.05$, $h^2_D = h^2_S = 0.3$ and $r_{E_{DS}} = r_{A_{DS}}$. These parameters yield a phenotypic variance of $\sigma^2_P = 6.17$ when relatedness equals zero, and total heritable variances of $\sigma^2_{A_T} = 2.03$ for $r_{A_{DS}} = -0.5$, $\sigma^2_{A_T} = 3.95$ for $r_{A_{DS}} = 0$, and $\sigma^2_{A_T} = 5.87$ for $r_{A_{DS}} = +0.5$, respectively

Hence, also when index weights are optimized, there is still a clear benefit of using groups composed of related group members. This result extends to selection on estimated breeding values using best linear unbiased prediction (see below).

Selection Based in Information of Relatives The above-mentioned selection methods rely on phenotypic information collected on the selection candidates themselves, which requires that the selection candidates are kept in groups (When selection candidates are housed individually, their phenotypes provide no information on their associative effect). Keeping selection candidates in groups, however, is often undesirable or difficult to apply in practice, particularly in laying hens and broilers. This is because group housing may interfere with recording data on an individual basis for important traits such as egg number or individual feed intake. Moreover, for behavioral traits

such as cannibalism, group housing may increase loss of selection candidates which is costly and reduces the intensity of selection. Also for veterinary reasons, it is often required that selection candidates are kept in clean conditions, such as Specific Pathogen Free environments, which are very different from the average production environment. Hence, response to selection based on information collected on the candidates themselves may be sensitive to genotype by environment interaction. Finally, recording of certain traits, such as carcass quality, may require sacrificing the individual, in which case selection cannot be based on own performance information.

For those reasons, selection decisions are often based on phenotypic information collected on relatives of the selection candidates, particularly sibs or progeny. Ideally, those relatives are kept in environments resembling commercial production environments, such as group housing and increased levels of disease. The following provides expressions for response to selection in socially affected traits when selection decisions are based on phenotypes of relatives kept in groups [48].

Consider selection on the average phenotype of relatives of the selection candidate, $\bar{P}_{\text{rels}}$. The phenotypic value of a relative j consists of the direct effect of j, and the summed associative effects of its group mates, $P_j = P_{D_j} + \sum_{n-1} P_{S_k}$, k denoting a group mate. When the group mates of the relative are unrelated to the candidate ($r_{ik} = 0$, $i$denoting the candidate), then the phenotype of the relative provides information only on the direct effect of the candidate, so that response in associative effect is entirely dependent on the genetic correlation between direct and associative effects. This is illustrated by the covariance between the total breeding value of the selection candidate and the phenotype of its relative, of which the component $\text{Cov}(A_{T,i}, \sum_{n-1} P_{S_k}) = 0$, so that $\text{Cov}(A_{T,i}, P_j) = r_{ij}[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]$ (Beware of a typo in this expression in [48]). In this case, accuracy is simply a scaled version of that with mass selection and unrelated group members,

$$\rho_{T,\text{rels}} = \frac{r_{\text{rels}}[\sigma^2_{A_D} + (n-1)\sigma_{A_{DS}}]}{\sigma_{A_T}\sigma_{\bar{P}_{\text{rels}}}}, \tag{33}$$

where r_{rels} is relatedness between the candidate and its relatives providing the information for selection. Hence, just as with mass selection, when using information recorded on sibs kept in groups with unrelated members, there is a risk of negative accuracy and thus negative response.

To capture the entire total breeding value of the selection candidates, relatedness between a candidate and the group mates of its relatives needs to be equal to relatedness between the candidate and its relatives, $r_{ik} = r_{ij}$, i denoting the candidate, j its relative, and k a group mate of j. In that case, $\text{Cov}(A_{T,i}, P_j) = \text{Cov}(A_{T,i}, A_{D,j} + \sum_{n-1} A_{S,k}) = r_{\text{rels}}\sigma^2_{A_T}$, showing that the phenotype of the relative captures the total breeding value of the candidate. This can be achieved by keeping relatives in family groups. For example, when selection is based on sib information, groups may be composed of full sibs of the candidate, so that $r_{ik} = r_{ij} = 0.5$. The following, therefore, describes the accuracy of selection based on relatives when they are kept in family groups.

The accuracy of selection based on the mean phenotypic value of relatives kept in family groups is an analogy of the classical expression for traits not affected by social interactions [48]. In the absence of social interactions, the accuracy of selection based on relatives is commonly expressed in terms of relatedness between the candidate and its relatives, r_{rels}, the square root of heritability, h, and the intraclass correlation t between the relatives (e.g., [63]),

$$\rho = \frac{r_{\text{rels}}h}{\sqrt{t + (1-t)/N}}, \tag{34}$$

where $t = r_w h^2$, being the product of mutual relatedness between the relatives, i.e., the within-family relatedness r_w, and heritability, and N is the number of records of relatives for each selection candidate. Note that there may be a difference between relatedness between the candidate and its relatives, r_{rels}, and mutual relatedness r_w within the group of relatives. For example, for half-sib progeny of the candidate, $r_{\text{rels}} = \frac{1}{2}$, whereas $r_w = \frac{1}{4}$. For full sibs of the candidate, $r_{\text{rels}} = r_w = \frac{1}{2}$, and for half sibs of the candidate, $r_{\text{rels}} = r_w = \frac{1}{4}$. When relatives are kept in family groups, with m groups per candidate each containing n members, yielding a total of mn records of relatives per candidate, the accuracy for socially affected traits equals,

$$\rho_{\text{rel}} = \frac{r_{\text{rels}}\eta}{\sqrt{\tau + (1-\tau)/mn}}, \tag{35a}$$

which is an analogy of Eq. 34 [48]. The Greek symbols in Eq. 35 are analogies of the "heritability" and the intraclass correlation;

$$\eta = \frac{\sigma_{\mathrm{TBV}}}{\sigma_{\mathrm{TPV}}} \tag{35b}$$

is an analogy of the square root of heritability, $h = \sigma_A/\sigma_P$, and

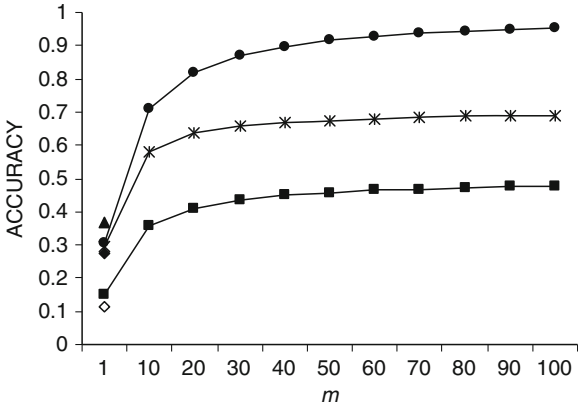
$$\tau = r_w \eta^2 \tag{35c}$$

is an analogy of the intraclass correlation between relatives $t = r_w h^2$. The η and τ account for interactions among individuals, and, therefore, depend on the total breeding value (TBV) and on the total phenotypic value (TPV) contributed by an individual. The TPV is the phenotypic analogy of the TBV (Eq. 5). It is not the observed phenotype of the individual, but represents an individual's effect on all phenotypes in the population, which is the sum of its direct phenotypic effect and $n-1$ times its associative phenotypic effect, $P_{T,i} = P_{D,i} + (n-1)P_{S,i}$, so that,

$$\sigma^2_{P_T} = \sigma^2_{P_D} + 2(n-1)\sigma_{P_{DS}} + (n-1)^2\sigma^2_{P_S} \tag{35d}$$

Thus, the TPV measures the total effect of an individual on performance of its group, the total breeding value is the heritable component of the TPV, and $\eta^2 = \sigma^2_{A_T}/\sigma^2_{P_T}$ is the proportion of the variance of the TPV which is heritable, analogous to the classical heritability. The intraclass correlation τ equals the correlation between TPVs of relatives, analogous to the classical intraclass correlation t, which equals the correlation between phenotypes of relatives for traits not affected by interactions [26]. In the absence of interactions, η reduces to h, τ reduces to t, and σ_{TBV} reduces to σ_A, so that Eq. 35a reduces to Eq. 34.

Hence, Eq. 35a shows that response to selection based on relatives kept in family groups can be obtained from the classical expression for response to selection based on relatives (Equation 34), when replacing heritability by $\sigma^2_{A_T}/\sigma^2_{P_T}$ and the intraclass correlation between relatives by $r_w\sigma^2_{A_T}/\sigma^2_{P_T}$. The key issue is that the sibs need to be kept in family groups. For example, when 16 half sibs are available and groups consist of 4 individuals, 4 groups of 4 individuals each should be used; the 16 half sibs should not be distributed over 16 groups each also containing 3 unrelated individuals.



Socially Affected Traits, Inheritance and Genetic Improvement. Figure 5

Accuracy of selection methods. The accuracy is shown for individual selection when the animals in a group are either full sibs (♦) or unrelated (◇), for group selection with groups of full sibs (▲), and for selection based on relatives kept in family groups, as a function of the number of groups per candidate (m), where relatives can be either half sibs (■), full sibs (✳) or half-sib progeny (●). (For $n = 4$; $\sigma^2_{P_D} = 1$; $\sigma^2_{P_S} = 0.33$; $h^2_D = 0.10$; $h^2_S = 0.10$; $r_A = r_E = 0$; Taken from Ellen et al. 2007)

Figure 5 illustrates the accuracy of selection based on relatives as a function of the number of groups of relatives per candidate. With many groups of relatives per candidate, $m \to \infty$, accuracy approaches

$$\max(\rho_{T,\mathrm{rels}}) = \frac{r_{\mathrm{rels}}}{\sqrt{r_w}}, \tag{36}$$

which is 0.5 for half sib information, $\sqrt{0.5}$ for full sib information, and 1 for information on half-sib progeny of the candidate [26]. Those values are the same as for classical selection based on relatives. Hence, with selection based on phenotypes of relatives, limiting accuracies for socially affected traits are the same as those for classical traits. For example, it is possible to obtain an accuracy approaching unity by using information on a large number of half-sib progeny kept in groups consisting of half sibs.

Selection on Estimated Breeding Values (EBVs) In animal breeding practice, breeding values are usually estimated using the so-called BLUP-procedure (Best Linear Unbiased Prediction; [53]. In the traditional

BLUP approach, phenotypes of individuals are analyzed using a mixed "animal model" containing fixed correction factors, such as herd, sex, or age of the individual, and a random genetic effect of the animal [27, 53, 64],

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{e}, \tag{37}$$

where $\mathbf{y}$ is a vector of phenotypes, $\mathbf{b}$ a vector of fixed correction factors with incidence matrix $\mathbf{X}$, $\mathbf{a}$ a vector of random additive genetic effects ("breeding values") of individuals with incidence matrix $\mathbf{Z}$, and $\mathbf{e}$ a vector of residuals (Note that elements of $\mathbf{y}$ in Eq. 37 correspond to P_i in Eq. 4.). In animal breeding, interest is in the vector of estimated breeding values, $\hat{\mathbf{a}}$. Estimates for fixed effects and breeding values are obtained from the so-called Mixed Model Equations (MME). When residuals are independent and identically distributed, the MME are [27, 53, 64]

$$\begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \alpha\mathbf{A}^{-1} \end{bmatrix} \begin{bmatrix} \mathbf{b} \\ \mathbf{a} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \end{bmatrix}, \tag{38a}$$

so that the estimates follow from

$$\begin{bmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} \\ \mathbf{Z}'\mathbf{X} & \mathbf{Z}'\mathbf{Z} + \alpha\mathbf{A}^{-1} \end{bmatrix}^{-1} \begin{bmatrix} \mathbf{X}'\mathbf{y} \\ \mathbf{Z}'\mathbf{y} \end{bmatrix}, \tag{38b}$$

where $\mathbf{A}$ is a matrix of relatedness coefficients between all individuals in $\mathbf{a}$, and $\alpha = \sigma_E^2/\sigma_A^2$. The use of BLUP requires knowledge of heritability of the trait to calculate the $\alpha = (1 - h^2)/h^2$. Further details and extensions are in [27, 64].

In contrast to selection index theory, BLUP is extremely flexible. It utilizes all available information, it allows correction for systematic non-genetic effects such as herd, sex or age, it allows for any degree of relatedness among individuals, any number of relatives, it provides also estimated breeding values for individuals without records, and it accounts for selection (under certain conditions).

Deterministic prediction of the accuracy of selection on BLUP-EBV is possible with selection index theory [65, 66], but is tedious, particularly when the model contains additional random effects such as maternal or associative effects. For this reason, consequences of selection on BLUP-EBV are often investigated using stochastic simulations. The following discusses the consequences of selection on BLUP-EBV when trait values are affected by associative effects, first when the mixed model ignores the associative effects, and afterward when accounting for associative effects.

BLUP Ignoring Associative Effects When only direct breeding values are modeled (i.e., when using Eq. 37), the consequences of selection on BLUP-EBV will depend critically on relatedness among group members [67]. When group members are unrelated, the EBV captures only the direct genetic effect of individuals, so that response in associative effects depends entirely on the genetic correlation between direct and associative effects. The accuracy derived from the MME, therefore, refers to the direct breeding value, not to the total breeding value. The actually achieved accuracy for the total breeding value is given by

$$\rho_{T,\mathrm{BLUP}}(r=0) \approx \hat{\rho}_{\mathrm{MME}} \left[\frac{\sigma_{A_D}^2 + (n-1)\sigma_{A_{DS}}}{\sigma_{A_D}\sigma_{A_T}} \right] \tag{39}$$

The $\hat{\rho}_{\mathrm{MME}}$ is the ordinary accuracy of BLUP-EBV derived from the diagonal elements of the inverse of the coefficient matrix of the MME [64], and the term in square brackets is the correlation between the direct and total breeding value (Note that this result reduces to $\rho_{T,\mathrm{BLUP}}(r=0) \approx \hat{\rho}_{\mathrm{MME}}$ when associative effects are absent). This result shows that BLUP selection with unrelated group member yields a negative accuracy when $\sigma_{A_D}^2 + (n-1)\sigma_{A_{DS}} < 0$, which is the same condition as for mass selection with unrelated group members (Table 2). When $\sigma_{A_D}^2 + (n-1)\sigma_{A_{DS}} < 0$, the correlated response in associative effects is negative and greater in absolute magnitude than the direct response, yielding a negative net response. Results of Muir [17] in cannibalistic quail illustrate that this is not merely a theoretical possibility, but may indeed occur in practice. (See section "Results of Social Selection Experiments" below.)

When groups are composed of families, the EBV of the traditional BLUP model implicitly captures the total breeding value of individuals, even though the associative component of trait values is not explicitly included in the model (Eq. 37; [67]). The reason is that the covariance between family members equals $r\sigma_{A_T}^2$, where $r = 0.5$ for full sibs and 0.25 for half sibs, when

groups consist of family members (When groups are composed at random with respect to family, the covariance between sibs equals $r\sigma^2_{A_D}$). Consequently, the accuracy of BLUP-EBV obtained from data containing groups of family members is approximately equal to the ordinary accuracy derived from the MME,

$$\rho_{T,\mathrm{BLUP,fam}} \approx \hat{\rho}_{\mathrm{MME}} \tag{40}$$

Moreover, the estimated additive genetic variance from an ordinary mixed model as in Eq. 37 will yield an estimate of $\sigma^2_{A_T}$ rather than $\sigma^2_{A_D}$. This occurs because the covariance between relatives equals $r\sigma^2_{A_T}$ rather than $r\sigma^2_{A_D}$ when groups consist of family members. Hence, the accuracy of BLUP-EBVs will always be positive when it is obtained from data consisting of family groups. This theoretical prediction agrees with results of Muir et al. [67] in quail, who found a negative response with BLUP selection and unrelated group members, but a positive response to BLUP selection when group members were related (referred to as Kin-BLUP in [67]).

BLUP Including Associative Effects Muir and coworkers [17, 22] extended the traditional mixed animal model to include Griffing's associative effects. In this model, the phenotype of each individual contains two separate components: a direct breeding value of the individual itself and the summed associative breeding values of its group mates,

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S + \mathbf{e}, \tag{41}$$

where $\mathbf{y}$ is the usual vector of observations, $\mathbf{b}$ a vector of fixed effects with incidence matrix $\mathbf{X}$, $\mathbf{a}_D$ a vector of direct additive genetic effects with incidence matrix $\mathbf{Z}_D$ linking phenotypes of individuals to their own direct genetic effect, and $\mathbf{e}$ a vector of residuals. (Note that elements of $\mathbf{y}$ in Eq. 41 correspond to P_i in Eq. 4.) The $\mathbf{Z}_S\mathbf{a}_S$ represents the associative additive genetic effects, where $\mathbf{a}_S$ is a vector of associative effect, with incidence matrix $\mathbf{Z}_S$ linking phenotypes of individuals to the associative effects of their group mates. For example, for a population of 8 individuals kept in two groups of 4 individuals, individuals 1 through 4 in the first group and 5 through 8 in the second group, where individuals 2, 5, and 8 are female and the rest is male, a mixed model with a separate mean for both sexes and direct and associative effects for all records is

$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \\ y_7 \\ y_8 \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ 1 & 0 \\ 1 & 0 \\ 0 & 1 \\ 1 & 0 \\ 1 & 0 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} \mu_m \\ \mu_f \end{bmatrix} + \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} a_{D,1} \\ a_{D,2} \\ a_{D,3} \\ a_{D,4} \\ a_{D,5} \\ a_{D,6} \\ a_{D,7} \\ a_{D,8} \end{bmatrix}$$
$$+ \begin{bmatrix} 0 & 1 & 1 & 1 & 0 & 0 & 0 & 0 \\ 1 & 0 & 1 & 1 & 0 & 0 & 0 & 0 \\ 1 & 1 & 0 & 1 & 0 & 0 & 0 & 0 \\ 1 & 1 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 \\ 0 & 0 & 0 & 0 & 1 & 0 & 1 & 1 \\ 0 & 0 & 0 & 0 & 1 & 1 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 & 1 & 1 & 0 \end{bmatrix} \begin{bmatrix} a_{S,1} \\ a_{S,2} \\ a_{S,3} \\ a_{S,4} \\ a_{S,5} \\ a_{S,6} \\ a_{S,7} \\ a_{S,8} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \\ e_4 \\ e_5 \\ e_6 \\ e_7 \\ e_8 \end{bmatrix}$$

The covariance structure of the random genetic terms is: $\mathrm{Var}\begin{bmatrix} \mathbf{a}_D \\ \mathbf{a}_S \end{bmatrix} = \mathbf{C} \otimes \mathbf{A}$, where $\mathbf{C} = \begin{bmatrix} \sigma^2_{A_D} & \sigma_{A_{DS}} \\ \sigma_{A_{DS}} & \sigma^2_{A_S} \end{bmatrix}$, $\mathbf{A}$ is a matrix of relatedness coefficients between individuals, and $\otimes$ denotes the Kronecker product of matrices. Thus, Eq. 41 is involves

three genetic variance components, $\sigma^2_{A_D}$, $\sigma_{A_{DS}}$ and $\sigma^2_{A_S}$. (See section "Estimation of Genetic Parameters" below for more details, such as the residual variance structure.)

The estimated breeding values for the direct and associative effects follow from the solution of the mixed model equations,

$$\begin{bmatrix} \hat{\mathbf{b}} \\ \hat{\mathbf{a}}_D \\ \hat{\mathbf{a}}_S \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z}_D & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z}_S \\ \mathbf{Z}_D'\mathbf{R}^{-1}\mathbf{X} & \mathbf{Z}_D'\mathbf{R}^{-1}\mathbf{Z}_D + g^{11}\mathbf{A}^{-1} & \mathbf{Z}_D'\mathbf{R}^{-1}\mathbf{Z}_S + g^{12}\mathbf{A}^{-1} \\ \mathbf{Z}_S'\mathbf{R}^{-1}\mathbf{X} & \mathbf{Z}_S'\mathbf{R}^{-1}\mathbf{Z}_D + g^{21}\mathbf{A}^{-1} & \mathbf{Z}_S'\mathbf{R}^{-1}\mathbf{Z}_S + g^{22}\mathbf{A}^{-1} \end{bmatrix}^{-1} \times \begin{bmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}_D'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}_S'\mathbf{R}^{-1}\mathbf{y} \end{bmatrix} \tag{42}$$

where $\mathbf{R}$ is the covariance matrix of the residuals, $\mathbf{R} = \mathrm{Var}(\mathbf{e})$, and the g^{ij} denote elements of the inverse of the genetic covariance matrix, $\begin{bmatrix} g^{11} & g^{12} \\ g^{21} & g^{22} \end{bmatrix} = \begin{bmatrix} \sigma^2_{A_D} & \sigma_{A_{DS}} \\ \sigma_{A_{DS}} & \sigma^2_{A_S} \end{bmatrix}^{-1}$. The elements of $\mathbf{R}$ are given in the section on "Estimation of Genetic Parameters" below.

When fitting Eq. 41, fixed effects and potential additional random effects must be chosen carefully because not only an individual's direct effect but also its associative effect may be affected by fixed and random factors. Hence, in addition to the ordinary fixed effects, which refer to effects on the individual producing the record, one may need to fit fixed effects for its group mates. For example, when groups consist of mixed breeds, one may fit a fixed associative effect for the breed of each group mate. When associative breed effects are omitted, they may inflate the estimated associative effects and their variance. Similarly, when groups consist of mixed sexes, one may fit a fixed associative effect for the sex of each group mate. Moreover, when common-litter effects play a role, one may fit a random common-litter associative effect [41]. An example is given in the section on "Estimation of Genetic Parameters" below.

Deterministic prediction of the accuracy of selection on BLUB-EBV obtained from Model 41 is very tedious. In principle, selection index theory can be used to derive that accuracy, using the pseudo-BLUP selection index methodology suggested by [66]. However, for a population consisting of full and half sib families, a pseudo-BLUP selection index for a single trait contains 24 distinct sources of information. Hence, the variance matrix of the index contains 24^2 elements. Thus the use of stochastic simulation is more practical.

Some insight in the factors determining accuracy of BLUP selection can be obtained from a simplified index. The selection index of Muir et al. [67] shows that relatedness between group members may have greater impact on accuracy than extension of the mixed model to account for associative effects. They compared Kin-BLUP, in which the mixed model contained direct effects only (Eq. 37) and group members were related, to C-BLUP, in which the mixed model contained both direct and associative effects (Eq. 41), but group members were unrelated. The accuracy of Kin_BLUP exceeded that of C_BLUP, and the correlation of the true associative BV with the EBV of Kin_BLUP was greater than that with the associative EBV of C_BLUP. Hence, though Kin_BLUP does not explicitly account for associative effects, it can have greater accuracy for associative effects than C_BLUP. This result agrees with the empirical observations of Muir et al. [67] who observed greater response with Kin-BLUP than with C-BLUP in cannibalistic quail. Kin_BLUP picks up associative effects because they are hidden in the own performance and FS-performance when group members are related.

In conclusion, both empirical and theoretical results indicate that relatedness within groups is the key factor for response to selection; the use of C_BLUP does not make relatedness superfluous. C_BLUP, however, will always outperform Kin_BLUP when applied to the same breeding structure because it explicitly models the associative effects. When both methods are applied to data with full-sib groups, for example, C_BLUP will outperform Kin_BLUP [67].

A Special Case: Maternal Genetic Effects Maternal genetic effects are a common case of social interactions among individuals, where the mother affects the trait values of her offspring. Maternally affected traits usually depend on both the genes for maternal effect in the mother and genes for direct effect in the offspring [23, 27, 68, 69]. Preweaning growth rate and survival of piglets, for example, will depend on maternal milk yield and behavior of the sow, but also on the direct effect of the piglets for growth rate. Other

examples are calving ease in dairy cattle [70], and preweaning survival in sheep [71].

Maternal effects should not be confused with the genes for direct effect that the offspring inherits from its mother, but refers to the effect of the mother on her offspring that arises through the environment, for example, due to maternal care. Hence, direct and maternal effects are genetically distinct traits. For growth rate in piglets, for example, genes for direct effect will relate to juvenile growth rate, whereas genes for maternal effect may relate to milk yield or behavior of sows.

Maternal effects may be modeled as [68, 69]

$$P_i = A_{D,i} + E_{D,i} + A_{M,j} + E_{M,j}, \tag{43}$$

where i is the offspring, j the mother, P_i is the phenotype of the offspring, A_D and A_M the direct and maternal breeding values, and E_D and E_M the non-heritable direct and maternal effects, respectively. From Eq. 43, response to selection equals the sum of the changes in mean direct and mean maternal breeding value [68, 69],

$$R = \Delta\bar{A}_D + \bar{\Delta}A_M, \tag{44}$$

Based on Eq. 44, Eaglen and Bijma [49] defined a total breeding value for maternally affected traits, being the sum of an individual's breeding values for direct and maternal effect,

$$A_T = A_{D,i} + A_{M,i}, \tag{45}$$

so that response to selection equals $R = \Delta\bar{A}_T$. In contrast to the phenotypic value, the total breeding value includes the individual's own breeding value for maternal effect, rather than that of its mother. This is because an individual transmits its own genes for maternal effects to its offspring, not those of its mother. The total heritable variance that can be used to generate response to selection in maternally affected traits equals the variance in total breeding values among individuals,

$$\sigma^2_{A_T} = \sigma^2_{A_D} + 2\sigma_{A_{DM}} + \sigma^2_{A_M} \tag{46}$$

From Eq. 17, response to selection equals [49]

$$R = \imath\rho_M\sigma_{A_T}, \tag{47}$$

where ρ_M is the accuracy of selection for maternally affected traits, which is the correlation between the selection criterion and the total breeding value of an individual.

A number of studies have referred to a "total heritability" [72, 73], citing Willham [69],

$$h^2_r = \frac{\sigma^2_{A_D} + 1\frac{1}{2}\sigma_{A_{DM}} + \frac{1}{2}\sigma^2_{A_M}}{\sigma^2_P} \tag{48}$$

Willham [23, 69] referred to h^2_r as the "fraction of the selection differential realized if selection were on P_x" (P_x being the offspring phenotype). Hence, h^2_r represents the realized heritability for mass selection, $h^2_r = R/S$, the ratio of response over the selection differential, rather than the ratio of heritable variance over phenotypic variance [68]. Thus response to mass selection can be predicted as $R = h^2_r S$. Equation 47 yields the same result for mass selection, but applies to any selection strategy.

Maternal genetic effects can be estimated using mixed models that include both a direct and maternal genetic effect [64]. Issues related to the estimation of maternal genetic effects are discussed in [23, 64, 74–78].

Results of Social Selection Experiments Only a few social selection experiments have been applied in livestock. Muir [19] selected laying hens, housed in half-sib groups, based on total egg production per group. Hence, this experiment combined group selection with relatedness. In six generations, mortality decreased from 67% to 8%. Most of the response occurred within the first few generations of selection. Eggs per hen housed increased from 91 to 237 eggs, mainly as a result of increased survival. In the seventh generation, the group selected and control lines were compared to the commercial line from which the group selected line was derived [20, 79, 80]. In single-bird cages, egg production was significantly greater for the commercial line. In 12-bird cages, however, the reverse was seen. The most remarkable difference was for mortality at 58 weeks of age, which was 89% for the commercial line, 20% for the group selected line and 54% for the control. These results confirm the theoretical expectation that group selection with related group members yields large response to selection when associative effects are important.

The changes in mortality were accompanied by changes in behavior, stress physiology, and immunology [81, 82].Under conditions of social stress, birds from the low mortality population were more hesitant to attack other birds, showed less feather pecking, had a lower H/L ratio and had lower dopamine and corticosterone blood concentrations, indicating lower stress levels [83, 84]. Cheng and Muir [82] suggested that these changes in birds from the low mortality population may reflect a greater ability to cope with novel environments and to have a greater resistance to stressors than birds from the high mortality population.

Muir and coworkers [17, 22, 67] presented results of 25 cycles of selection for body weight in quail housed in groups of 16 birds. Three selection programs were compared. First, selection on conventional animal model BLUP EBV considering direct effects only (AM_BLUP, Eq. 37). Second, selection on estimated total breeding values derived from a mixed model, including both direct and associative effects (C_BLUP, Eq. 41). Group members were unrelated in those selection programs. The third selection program used groups consisting of half-sibs families, and selection on conventional animal model BLUP EBV considering direct effects only (Kin_BLUP). Results showed that the AM_BLUP program decreased body weight and increased mortality, whereas the Kin_BLUP program increased body weight and decreased mortality. The C_BLUP program was intermediate. Those results agree with expectations based on the theory presented above.

At Wageningen University, Ellen and coworkers initiated a selection experiment in laying hens, combining individual selection for egg number and group selection for low mortality in non-beak-trimmed kin-groups, based on the method of [48]. Selection candidates were housed individually, allowing recording of individual egg number. Non-beak-trimmed full sibs of these selection candidates were housed in family groups in which mortality was recorded. Selection was for a combination of egg number and mortality. Rodenburg et al. [85, 86] and Bolhuis et al. [87] investigated the behavioral and physiological consequences of this selection program in the second generation. Animals of the selected line were less fearful and less sensitive to stressors in a range of behavioral tests. Consistent results were found both in young chicks [87] and in adult birds [88] in two environments (cages and floor pens).

A number of group selection experiments have been performed in laboratory species, particularly in flour beetles (*Tribolium castaneum*). Wade [58, 59] was the first to demonstrate the power of group selection experimentally. Goodnight [89] reviewed 12 group selection experiments. Without exception, all group selection experiments showed a significant response to selection both in animals, plants, and microbial communities. Similar results have been found in bacteria [90]. Again, those results demonstrate that selection between groups and use of related group members increases response to selection in traits affected by social interactions, which agrees with theoretical expectations (see above).

Estimation of Genetic Parameters

Statistical Methodology

Some of the selection methods described above can be applied without knowledge of the genetic parameters, such as mass and group selection. Without knowledge of the genetic parameters, however, it is unclear whether associative effects are present and whether those selection strategies are needed and/or efficient. Moreover, most applied breeding programs make use of Best Linear Unbiased Prediction [53] for estimating breeding values, e.g., to correct for systematic environmental effects, which requires knowledge of the genetic parameters. Knowledge of the genetic parameters is also needed to quantify the expected response to selection, which may be required to make decisions on investment in breeding programs. Moreover, there is a wider biological interest in the heritable components affecting individual trait values and responses to selection, both in agriculture and in other fields of biology such as evolutionary genetics [30, 62]. Thus there is a need to estimate the genetic parameters for socially affected traits, which are the additive genetic variances of direct genetic effects, $\sigma^2_{A_D}$, the additive genetic variances of associative effects, $\sigma^2_{A_S}$, and the additive genetic covariance of direct and associative effects, $\sigma_{A_{DS}}$. This section addresses the estimation of those parameters, starting with a review of existing results.

Empirical Estimates of the Associative Variance Table 3 summarizes estimates of direct and associative heritabilities. Brichette et al. [37] were the first to publish estimates of the associative genetic variance. They analyzed growth in mussel cultures and found strongly significant direct and associative effects. Their model is somewhat different from the mixed models discussed in this chapter, and estimates may not be directly comparable. In each group, they tested a single focal family against a mixed reference set containing members of all families. In this design, the effect of the focal family on the mean phenotype of the reference group represents the associative effect of that family. Hence, in this setup, the associative effect is defined for a set of family members, rather than for a single individual, and the factors $(n-1)$ and $(n-1)^2$ in the total genetic variance do not apply (Eq. 7). Brichette et al. [37] obtained direct heritabilities of 0.104 and 0.232 for length measures, and 0.097 and 0.234 for area measures. Corresponding estimates for associative heritability were 0.010 and 0.087 for length and 0.220 and 0.082 for area. Direct-associative genetic correlations were negative, averaging −0.2. These results suggest that $\sigma^2_{A_t}/\sigma^2_P \approx 0.24$, and that associative effect contributed ∼1/3 of heritable variance in growth of mussel cultures.

Later studies focused primarily on laying hens and pigs. Particularly in pigs and beef cattle, it has proven

Socially Affected Traits, Inheritance and Genetic Improvement. Table 3 Genetic parameter estimates from agriculture and aquaculture populations

	$\hat{h}^2_D$	$\hat{h}^2_S$	$\hat{T}^2$	$\hat{r}_{A_{DS}}$
Beef cattle (*Bos taurus*)[a]				
Feed lot growth rate	0.06	0.003	2.01	0.69
Cod (*Gadus morhua*)[b]				
Condition factor	0.05	NR	0.21	NR
Dorsal fin erosion	0.83	NR	1.32	NR
Caudal fin erosion	0.08	NR	0.47	NR
Growth rate	0.11	NR	0.12	NR
Body weight	0.37	NR	0.57	NR
Laying hens (*Gallus gallus*)				
Survival time, Line W1[c]	0.07	0.010	0.19	0.18 (n.s.)
Survival time, Line WB[c]	0.10	0.014	0.15	−0.31
Survival time, Line WF[c]	0.02 (n.s.)	0.004 (n.s.)	0.06 (n.s.)	0.11 (n.s.)
Survival time, Cross W1×WB[d]	0.03	0.033	0.26	−0.37
Survival time, Cross WB×W1[d]	0.05	0.036	0.17	−0.83
Mussel cultures (*Mytilus galloprovincialis*)[e]				
Length	0.17	0.049[f]	0.21	−0.09 (n.s.)
Area	0.17	0.151[f]	0.27	−0.30 (n.s.)
Pigs (*Sus scrofa*)				
Growth rate fattening[g]	0.21	0.007	0.71	0.20 (n.s.)
Growth rate fattening[h]	0.13	0.002	0.23	0.11 (n.s.)
Growth rate fattening[i]	0.20	0.001	0.61	0.24
Body weight[j]	0.39	0.001 (n.s.)	0.47	0.07 (n.s.)

Socially Affected Traits, Inheritance and Genetic Improvement. Table 3 (Continued)

	$\hat{h}_D^2$	$\hat{h}_S^2$	$\hat{T}^2$	$\hat{r}_{A_{DS}}$
FI fattening[g]	0.17	0.006	0.70	0.38 (n.s.)
Back fat[g]	0.35	0.001 (n.s.)	0.41	−0.02 (n.s.)
Back fat[j]	0.45	0.001 (n.s.)	0.47	0.07 (n.s.)
Muscle depth[g]	0.21	<0.001 (n.s.)	0.32	0.33 (n.s.)
Muscle area[j]	0.29	0.001 (n.s.)	0.31	−0.63 (n.s.)
Growth suckling piglets[k]	0.07	0.001 (n.s.)	0.15	−0.27 (n.s.)
Quail (*Coturnix coturnix japonica*)				
Body weight[l]	0.17	0.014	2.56	−0.56

$\hat{h}_D^2 = \hat{\sigma}_{A_D}^2/\hat{\sigma}_P^2$, $\hat{h}_S^2 = \hat{\sigma}_{A_S}^2/\hat{\sigma}_P^2$, $\hat{T}^2 = \hat{\sigma}_{A_T}^2/\hat{\sigma}_P^2$, $\hat{r}_{A_{DS}} = \hat{\sigma}_{A_{DS}}/(\sigma_{A_D}\sigma_{A_D})$, see Eqs. 4–9 for further information.
[a][34], first 28 days of growth period, no evidence for associative effects found in other periods.
[b][35], significance levels not reported.
[c][24], individuals were not beak-trimmed.
[d][36], individuals were not beak-trimmed.
[e][37].
[f]The h_S^2 is defined somewhat different here, see text.
[g][15].
[h][38], for d= 1.
[i][39], model 2.
[j]H [40], model 2, significance levels tested against model 6.
[k][41], model 4.
[l] [17], non-genetic associative effects not accounted for, thus $\hat{\sigma}_{A_S}^2$ and $\hat{T}^2$may be inflated; significance levels not reported. NR: not reported. n.s. not significantly different from zero.

difficult to reliably estimate the associative genetic variance. Often the estimates suggested a considerable contribution of associative effects to total heritable variance ($T^2 >> h_D^2$), but the estimated associative genetic variance was nevertheless not significantly different from zero (Table 3). This occurred particularly when group sizes were relatively large, so that the number of groups was fairly small [25]. These observations agree with theoretical predictions, which indicate that the number of groups is the major determinant of statistical power, and that designs with small groups are often superior ([32]; see section on "Power and Optimum Designs" below). Hence, in large groups, even small values of the associative heritability can make a major contribution to total heritable variation (Eq. 7), but this contribution is difficult to estimate accurately, as reflected by the large standard errors of $\hat{\sigma}_{A_T}^2$. Estimates in laying hens, which come from data containing groups of four individuals, have been more accurate [24, 36].

Several studies have shown that the estimated associative genetic variance is very sensitive to the choice of the statistical model. Models not accounting for non-genetic associative effects, i.e., models that fit neither environmental associative effects nor pen effects or correlated residuals, generally yielded strongly inflated estimates [34, 40, 91, 92]. Hence, the choice of the statistical model requires careful consideration. To properly estimate the genetic variance components, additional fixed and random associative effects may be required to account for other sources of variation, such as litter environment or group size (see below).

QTL: There appears to be only a single QTL-study involving associative effects. Biscarini et al. [93] performed an association study to map direct and associative QTL for feather damage across nine different genetic lines of laying hens. A total population of 662 hens were genotyped for 1022 SNPs. Eleven significant SNPs were detected for direct effect and 81 for associative effect. From the significant associative SNPs, six were located at the sex chromosome. The SNPs identified suggest a relationship between behavior and immunology, and a role of the serotonergic system in feather pecking.

Mixed Models Muir and coworkers [17, 22] extended the traditional mixed animal model to include Griffing's associative effects (See Eq. 41 above). The following describes an extension of their model, taking the basic model as a starting point. In the basic model (Eq. 3), observed trait values are the sum of direct and associative effects, $P_i = P_{D,i} + \sum_{j=1}^{n-1} P_{S,j}$. The full direct and associative effects are not solely genetic, but may depend also on fixed effects, random genetic effects, and other random effects such as permanent or litter effects,

$$P_{D,i} = \text{fixed}_D + A_{D,i} + E_{D_P,i} + E_{D_C,i} + E_{D,i} \quad (49a)$$

$$P_{S,i} = \text{fixed}_S + A_{S,i} + E_{S_P,i} + E_{S_C,i} + E_{S,i}, \quad (49b)$$

where subscripts P and C indicate permanent and common-litter effects, respectively. (Note that Eqs. 49a and b are not exhaustive; they may be extended with, e.g., maternal genetic effects.) Just as in classical mixed models, omission of associative permanent effects or associative common-litter effects may lead to overestimation of $\sigma^2_{A_S}$. In pigs, for example, two full sibs may develop similar social skills because they experienced the same litter environment. In the fattening period, this similarity in social skills will create a covariance between the pen mates of both full sibs. When not accounted for, this covariance will largely be attributed to the associative genetic variance because the mixed model implies that $\text{Cov}(\bar{P}_{\text{pen mates}(FS1)}, \bar{P}_{\text{pen mates}(FS2)}) = \frac{1}{2}\sigma^2_{A_S}$ when associative litter effects are not included. A similar reasoning applies to permanent effects. For example, when an individual has been member of two distinct groups, the covariance between the phenotypes of two group mates, one taken from each group, equals $\sigma^2_{A_S} + \sigma^2_{E_S}$. When permanent associative effects are omitted from the mixed model, this covariance will be fully attributed to associative genetic effects, leading to overestimation of $\sigma^2_{A_S}$.

In general, therefore, the mixed model for socially affected traits is given by

$$\begin{aligned}\mathbf{y} = &\{\mathbf{X}_D\mathbf{b}_D + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_{D_P}\mathbf{e}_{D_P} + \mathbf{Z}_{D_C}\mathbf{e}_{D_C} + \cdots + \mathbf{e}_D\} \\ &+ \{\mathbf{X}_S\mathbf{b}_S + \mathbf{Z}_S\mathbf{a}_S + \mathbf{Z}_{S_P}\mathbf{e}_{S_P} + \mathbf{Z}_{S_C}\mathbf{e}_{S_C} + \cdots + \mathbf{e}_S\}\end{aligned} \quad (50)$$

where subscripts D and S indicate direct and associative effects, P and C indicate permanent and common-litter effects, $\mathbf{X}$ and $\mathbf{Z}$ denote incidence matrices, and $\mathbf{e}$ the residual. Whether or not a specific model component needs to be included will depend on the data structure. Direct and associative fixed effects may often be fully confounded, so that fitting a single effect is sufficient. For example, group members will usually be in the same herd at the same time. Hence, the direct and associative herd-year effects will be fully confounded, so that fitting a single herd-year effect suffices. In some cases, therefore, the simplest model, $\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S + \mathbf{e}$, may be appropriate. (But see the section on "Non-Genetic Associative Effects" below.)

In other cases, however, additional fixed or random associative effects may be required. Consider, for example, a pen of four beef cows, the first individual being a Hereford, and individuals two through four being Charolais. In this situation, fitting both a direct and an associative fixed breed-effect may be appropriate,

$$\begin{aligned}\mathbf{y} &= \mathbf{X}_D\mathbf{b}_D + \mathbf{X}_S\mathbf{b}_S + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S + \mathbf{e} \\ \begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \end{bmatrix} &= \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ 0 & 1 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} H_D \\ C_D \end{bmatrix} + \begin{bmatrix} 0 & 3 \\ 1 & 2 \\ 1 & 2 \\ 1 & 2 \end{bmatrix} \begin{bmatrix} H_S \\ C_S \end{bmatrix} \\ &\quad + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S + \mathbf{e}\end{aligned}$$

where H_D and C_D are the fixed direct breed effect of Hereford and Charolais, respectively, and H_S and C_S are the fixed associative breed effect of Hereford and Charolais, respectively. The record of individual 1, for example, contains three fixed Charolais associative effects, because its three pen mates are all of the Charolais breed. When associative breed effects are omitted, they may partly end up in the estimated associative genetic variance. Moreover, when groups consist of mixed sexes, fitting a fixed associative effect for sex of the group mates may be appropriate.

As a second example, consider social interactions in fattening pigs. Because litter-mates may develop similar social skills, fitting a common-litter associative effect is of interest in this case [15]. Consider a pen of 8 individuals originating from three distinct litters; individuals 1 through 3 are born in litter 1, individual

4 is born in litter 2, and individuals 5 through 8 are born in the third litter. The mixed model for this pen is

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S + \mathbf{Z}_{C_D}\mathbf{e}_{C_D} + \mathbf{Z}_{C_S}\mathbf{e}_{C_S} + \mathbf{e} \tag{51}$$

$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \\ y_7 \\ y_8 \end{bmatrix} = \mathbf{Xb} + \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} a_{D,1} \\ a_{D,2} \\ a_{D,3} \\ a_{D,4} \\ a_{D,5} \\ a_{D,6} \\ a_{D,7} \\ a_{D,8} \end{bmatrix}$$

$$+ \begin{bmatrix} 0 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 0 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 0 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 0 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 0 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 0 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 0 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 0 \end{bmatrix} \begin{bmatrix} a_{S,1} \\ a_{S,2} \\ a_{S,3} \\ a_{S,4} \\ a_{S,5} \\ a_{S,6} \\ a_{S,7} \\ a_{S,8} \end{bmatrix}$$

$$+ \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \\ 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} e_{C_D,1} \\ e_{C_D,2} \\ e_{C_D,3} \end{bmatrix}$$

$$+ \begin{bmatrix} 2 & 1 & 4 \\ 2 & 1 & 4 \\ 2 & 1 & 4 \\ 3 & 0 & 4 \\ 3 & 1 & 3 \\ 3 & 1 & 3 \\ 3 & 1 & 3 \\ 3 & 1 & 3 \end{bmatrix} \begin{bmatrix} e_{C_S,1} \\ e_{C_S,2} \\ e_{C_S,3} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ e_3 \\ e_4 \\ e_5 \\ e_6 \\ e_7 \\ e_8 \end{bmatrix}$$

The $\mathbf{Z}_S$ links the record of each individual to the associative genetic effect of its group mates, whereas $\mathbf{Z}_{C_S}$ links the record of each individual to the litter of its group mates. Hence, with groups of 8 individuals, each row of both $\mathbf{Z}_S$ and $\mathbf{Z}_{C_S}$ has a sum of 7, because each individual has 7 group mates. For this model, the variance structures for genetic effects is $\text{Var}\begin{bmatrix} \mathbf{a}_D \\ \mathbf{a}_S \end{bmatrix} = \mathbf{G} \otimes \mathbf{A}$, where $\mathbf{G} = \begin{bmatrix} \sigma^2_{A_D} & \sigma_{A_{DS}} \\ \sigma_{A_{DS}} & \sigma^2_{A_S} \end{bmatrix}$, $\mathbf{A}$ is the usual numerator relationship matrix [27], and $\otimes$ denotes the Kronecker product of matrices. The variance structure of the common-litter effects is $\text{Var}\begin{bmatrix} \mathbf{e}_{C_D} \\ \mathbf{e}_{C_S} \end{bmatrix} = \mathbf{C} \otimes \mathbf{I}$, where $\mathbf{C} = \begin{bmatrix} \sigma^2_{E_{C_D}} & \sigma(E_{C_D}, E_{C_S}) \\ \sigma(E_{C_D}, E_{C_S}) & \sigma^2_{E_{C_S}} \end{bmatrix}$, and $\mathbf{I}$ is an identity matrix of dimension equal to the number of litters. The $\sigma^2_{E_{C_D}}$ is the variance of the (usual) direct litter effects, $\sigma^2_{E_{C_S}}$ the variance of the associative litter effects, and $\sigma(E_{C_D}, E_{C_S})$ the covariance between the direct and associative effect of a litter. Hence, Eq. 51 involves the estimation of six variance components and the residual variance. (See below for the variance structure of the residual). Canario et al. [38] showed that an associative common-litter effect may be required to prevent overestimation of the associative genetic variance in fattening pigs.

Non-Genetic Associative Effects In both above examples, the direct residual effect of the focal individual and the associative residual effects of its group mates have been summarized into a single residual, $e_i = E_{D,i} + \sum_{j=1}^{n-1} E_{S,j}$. This is because the non-genetic parameters $\sigma^2_{E_D}$, $\sigma_{E_{DS}}$ and $\sigma^2_{E_S}$ are not uniquely identifiable when group size is constant [39, 40, 92]. Hence, a model including both E_S and a (direct) residual is overspecified. Nevertheless, non-genetic associative effects have consequences for the variance structure of the residuals. First, the residual variance becomes dependent on group size [39, 92],

$$\sigma^2_e = \sigma^2_{E_D} + (n-1)\,\sigma^2_{E_S}, \tag{52}$$

Hence, when group size varies in the data, it may be appropriate to fit a separate residual variance for each group size. Second, the residuals of group mates become correlated [39, 92],

$$\text{Cov}(e_i, e_j) = 2\sigma_{E_{DS}} + (n-2)\sigma^2_{E_S}, \tag{53}$$

where i and j are group mates. Equations 52 and 53 illustrate that the non-genetic variance components are

non-identifiable because there are only two observable (co)variances in the data, but three unknowns. In other words, there is an infinite number of combinations of $\sigma^2_{E_D}$, $\sigma_{E_{DS}}$ and $\sigma^2_{E_S}$ that yield the same values of σ^2_e and $\mathrm{Cov}(e_i, e_j)$ [15, 39, 92]. (This becomes different when group sizes vary; see below).

Equation 53 shows that the residual covariance may take negative values, particularly when groups are small. In that case, it may be required to allow for correlated residuals within group in the mixed model, using [92] $\mathrm{Var}(\mathbf{e}) = \mathbf{R}\sigma^2_e$, with

$$R_{ii} = 1, \tag{54}$$

$R_{ij} = \rho_e$ when i and j are group mates, and $R_{ij} = 0$ when i and j are in different groups.

This results in a block-diagonal structure for the correlation matrix of residuals, **R**. For two groups of four individuals, for example, the correlation matrix of residuals equals

$$\mathbf{R} = \begin{bmatrix} 1 & \rho_e & \rho_e & \rho_e & 0 & 0 & 0 & 0 \\ \rho_e & 1 & \rho_e & \rho_e & 0 & 0 & 0 & 0 \\ \rho_e & \rho_e & 1 & \rho_e & 0 & 0 & 0 & 0 \\ \rho_e & \rho_e & \rho_e & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & \rho_e & \rho_e & \rho_e \\ 0 & 0 & 0 & 0 & \rho_e & 1 & \rho_e & \rho_e \\ 0 & 0 & 0 & 0 & \rho_e & \rho_e & 1 & \rho_e \\ 0 & 0 & 0 & 0 & \rho_e & \rho_e & \rho_e & 1 \end{bmatrix}$$

The correlation between residuals of group mates equals $\rho_e = \left[2\sigma_{E_{DS}} + (n-2)\sigma^2_{E_S}\right] / \left[\sigma^2_{E_D} + (n-1)\sigma^2_{E_S}\right]$. This structure can be fitted in ASREML using the CORU statement in the description of the R-structure [94].

When groups are reasonably large, the residual correlation is likely to be positive because the $(n-2)\sigma^2_{E_S}$ becomes the dominant term in Eq. 53. In that case, one can account for residual correlations by adding a random group effect to the model and fitting independent residuals [15, 34, 39]. Fitting a random group effect is computationally easier than fitting correlated residuals and is therefore preferable when the correlation between residuals of group mates is positive. This leads to the model

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S + \mathbf{Z}_g\mathbf{g} + \mathbf{e}, \tag{55}$$

where **g** is a vector of random group effects, with incidence matrix $\mathbf{Z}_g$ linking phenotypes of individuals to their group, $\mathrm{Var}(\mathbf{g}) = \mathbf{I}\sigma^2_g$, $\mathrm{Var}(\mathbf{e}) = \mathbf{I}\sigma^2_e$, **I** denoting an identity matrix, σ^2_g the between-group non-genetic variance and σ^2_e the residual variance. With Eq. 55,

$$\sigma^2_g = 2\sigma_{E_{DS}} + (n-2)\sigma^2_{E_S}, \text{ and} \tag{56a}$$

$$\sigma^2_e = \sigma^2_{E_D} - 2\sigma_{E_{DS}} + \sigma^2_{E_S} \tag{56b}$$

Note that, in contrast to Eq. 41, the residual variance of Eq. 55 is independent of group size, whereas the between-group variance depends on group size. Hence, when group size varies in the data, it may be appropriate to fit a different group variance for each group size. (But see the section "Accounting for Variation in Group Size" below.)

Ignoring non-genetic associative effects, e.g., by omitting a group effect and fitting a simple residual, may inflate the estimated genetic variance components by as much as 200–300% [39, 40, 92]. In this case, the covariance between trait values of group mates, which equals $2\sigma_{P_{DS}} + (n-2)\sigma^2_{P_S}$, is attributed entirely to the genetic components because the model implies that $\mathrm{Cov}_{\text{group mates}} = 2\sigma_{A_{DS}} + (n-2)\sigma^2_{A_S}$.

Accounting for Variation in Group Size The above has ignored variation in group size within a population, which will be very common in practice. When group sizes vary in the population, merely fitting a fixed effect for group size may not be sufficient because variance components may also differ between group sizes. For example, interactions among individuals may be less intense in large groups, meaning that the associative genetic variance decreases with group size.

When the underlying parameters, $\sigma^2_{A_D}$, $\sigma_{A_{DS}}$, $\sigma^2_{A_S}$, $\sigma^2_{E_D}$,$\sigma_{E_{DS}}$ and $\sigma^2_{E_S}$ are assumed to remain constant, variation in group size requires only modifying the variance structure of the non-genetic part of the model. In the genetic part of the model (Eq. 51), $\mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_S\mathbf{a}_S$, variation in group size is automatically accounted for in $\mathbf{Z}_S$. Consequences for the non-genetic part of the model will depend on the model. When fitting a model with correlated residuals, both the residual variance and the residual within-group correlation will vary among group sizes (Eqs. 52–54). When fitting a model with random group effects, the group variance will be heterogeneous among group sizes while the residual variance is homogeneous

(56 and Eqs. 57). Hence, when the underlying variance components are assumed constant, a model including a random group effect is more easily adapted to account for varying group size.

However, assuming that the underlying parameters remain constant with varying group size is a strong a priori assumption. In many cases, associative effects may become smaller in larger groups because they are distributed over more group mates, a phenomenon known as "dilution" [25, 42, 43]. In principle, one could estimate separate genetic parameters for each group size. However, this would be a waste of information, and require large data sets for each group size. A simpler solution is to scale the magnitude of associative effects by a factor depending on the inverse of the number of group mates (Eq. 11),

$$A_{S,i,n} = \left(\frac{\bar{n}-1}{n-1}\right)^{d} A_{S,i,\bar{n}},$$

where $A_{S,i,n}$ represents the associative effect of individual i when expressed in a group of n members, $A_{S,i,\bar{n}}$ the associative effect when expressed in a group of the average size, and d the degree of dilution. Further details on dilution of associative effects are summarized above in the section "The Effect of Group Size on Heritable Variance".

The degree of dilution can be estimated from data containing variation in group size by using a mixed model with Restricted Maximum Likelihood and evaluating the likelihood for different fixed values of d [25, 38]. Estimates of A_S, $\sigma^2_{A_S}$ and $\sigma_{A_{DS}}$ referring to the average group size may be obtained from the following mixed model [38, 43],

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_{S(d)}\mathbf{a}_{S,\bar{n}} + \mathbf{Z}_g\mathbf{g} + \mathbf{e}, \tag{57}$$

where $\mathbf{y}$ is the vector of observations, $\mathbf{Xb}$ are the usual fixed effects, $\mathbf{Z}_D\mathbf{a}_D$ are the direct genetic effects, $\mathbf{Z}_g\mathbf{g}$ are random group-effects, and $\mathbf{e}$ is a vector of residuals. The $\mathbf{a}_{S,\bar{n}}$ is a vector of associative effects referring to the average group size, and $\mathbf{Z}_{S(d)}$ is the incidence matrix for associative effects, which depends on the degree of dilution. Elements of $\mathbf{Z}_{S(d)}$ are

$$\mathbf{Z}_{S(d)}(i,j) = \left(\frac{\bar{n}-1}{n_i-1}\right)^{d} \text{ when } j \text{ is a group member of } i,$$
$$\mathbf{Z}_{S(d)}(i,j) = 0 \text{ otherwise,} \tag{58}$$

where $\bar{n}$ denotes the average group size, and n_i the size of the group of individual i. This model yields estimates of genetic parameters and breeding values referring to the average group size because the $[(\bar{n}-1)/(n_i-1)]^d = 1$ when $n_i = \bar{n}$.

When non-genetic associative effects depend on group size in the same manner, also the group and residual variance for the model in Eq. 57 will depend on group size [43],

$$\sigma^2_{g,n} = 2\left(\frac{\bar{n}-1}{n-1}\right)^{d}\sigma_{E_{DS},\bar{n}} + (n-2)\left(\frac{\bar{n}-1}{n-1}\right)^{2d}\sigma^2_{E_S,\bar{n}} \tag{59a}$$

$$\sigma^2_{e,n} = \sigma^2_{E_D} - 2\left(\frac{\bar{n}-1}{n-1}\right)^{d}\sigma_{E_{DS},\bar{n}} + \left(\frac{\bar{n}-1}{n-1}\right)^{2d}\sigma^2_{E_S,\bar{n}} \tag{59b}$$

Hence, to obtain unbiased estimates of the genetic parameters and d, it may be required to fit a separate group and residual variance for each group size. An alternative solution is to explicitly model E_S. When group size is constant, the non-genetic parameters cannot be estimated, and a model including both E_S and a residual is overspecified (See below Eq. 53). When group size varies, however, $\sigma^2_{E_D}$, $\sigma_{E_{DS}}$ and $\sigma^2_{E_S}$ may be identifiable (unless $d = 1$), and it may be possible to fit the model

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_D\mathbf{a}_D + \mathbf{Z}_{S(d)}\mathbf{a}_{S,\bar{n}} + \mathbf{Z}_{S(d)}\mathbf{e}_{S,\bar{n}} + \mathbf{e},$$

with $\mathrm{Var}\begin{bmatrix}\mathbf{e}_{S,\bar{n}}\\ \mathbf{e}\end{bmatrix} = \mathbf{E}\otimes\mathbf{I}$, where $\mathbf{E} = \begin{bmatrix}\sigma^2_{E_S,\bar{n}} & \sigma(E_D,E_{S,\bar{n}})\\ \sigma(E_D,E_{S,\bar{n}}) & \sigma^2_{E_D}\end{bmatrix}$.

However, it may not be possible to implement the nonzero $\mathrm{Cov}(\mathbf{e}_{S,\bar{n}},\mathbf{e})$ of this model in standard software.

Canario et al. [38] present estimates for the degree of dilution of both associative genetic effects and associative common-litter effects in fattening pigs, with group sizes varying from 5 through 15 individuals. Their results suggest that both effects are fully diluted with group size ($\hat{d} = 1$). Their results also indicate that the estimate of the associative variance may depend on whether or not the analysis accounts for dilution.

Hadfield and Wilson [42] presented an alternative approach to account for the relationship between associative effects and group size. They fit an additional

associative genetic effect that is proportional to the inverse of the number of group mates. Bijma [43] compares their model to Eq. 57, and concludes that it has greater flexibility, but is also less tractable.

Identifiability of the Associative Genetic Variance

Not all data structures are equally suited for estimating the genetic variance of associative effects. A particularly critical aspect is group composition. When groups are composed of equally related individuals, for example, full or half-sib families, then direct and associative effects are fully confounded and cannot be estimated [34, 92, 95]. Also the use of specific distributions of families over groups may cause non-identifiability of the associative genetic variance [92]. Genetic parameters are identifiable when groups are composed at random with respect to family, but the standard error of $\hat{\sigma}^2_{A_S}$ may be large. Groups composed of members of two distinct families yield more precise estimates of $\sigma^2_{A_S}$ (See section "Statistical Power and Optimum Designs" below).

Moreover, the associative genetic variance is not identifiable when a fixed group-effect is fitted [96]. When fitting group as a random effect, however, $\sigma^2_{A_S}$ is identifiable and simulations with normally distributed group effects indicate that estimates are unbiased [92]. Nevertheless, the non-identifiability of $\sigma^2_{A_S}$ when using fixed group effects indicates that the information for estimating $\sigma^2_{A_S}$ is closely linked to variation in the group means, suggesting that estimates may be sensitive to the distribution of group effects. The importance of the group agrees with the observation of Bijma [97], who found that number of groups, rather than number of individuals, is the main determinant of the standard error of $\hat{\sigma}^2_{A_S}$. Cantet and Cappa [96] present methods to investigate whether variance components are identifiable from a particular data set.

Statistical Power and Optimum Designs

Standard Errors of Estimated Variance Components

Optimization of experimental designs and evaluation of the power of experiments aiming to estimate genetic parameters for socially affected traits requires knowledge of the factors determining the standard errors (SE) of estimated parameters. Accurate prediction equations for those SEs are available for balanced designs containing N_f families with n_f members each,

Socially Affected Traits, Inheritance and Genetic Improvement. Table 4 Example of the design with groups composed of two distinct families

	Family B				
Fam. A	2	3	4	5	6
1	2/2	2/2	2/2	2/2	2/2
2		2/2	2/2	2/2	2/2
3			2/2	2/2	2/2
4				2/2	2/2
5					2/2

Group size equals n= 4. Each group consists of members of two distinct families, each family contributing two individuals. Family size equals n_f = 10, so that each family can be combined with 10/(½ × 4) = 5 other families. Thus a block consists of six families, each being combined with each of the five other families in that block. Hence, there are 5*6/2 = 15 groups per block, 15*4 = 60 individuals per block, and $N_t/60$ blocks, N_t denoting the total number of individuals in the experiment.

yielding a total of $N_t = N_f\, n_f$ individuals in the experiment [97]. Two group compositions have been investigated; groups composed at random with respect to family, and groups composed of two families. In the latter design, each group is composed of members of two distinct families, each family contributing ½n individuals. This design leads to a block structure (Table 4), where each of the families within a block is combined with each of the other families in that same block, each combination occurring precisely once.

For both designs, the SEs of estimated genetic variances follows from

$$\mathrm{SE}(\hat{\sigma}^2_A) \approx \frac{1}{r}\sqrt{\frac{2}{N_f-1}\left[\sigma^4_f + \frac{2\sigma^2_f\sigma^2_e}{m} + \frac{\sigma^4_e}{m(m-1)}\right]}, \tag{60}$$

where N_f is the number of families, m the effective number of records per family, σ^2_f the between-family variance, and σ^2_e the residual variance,

$$\sigma^2_e = \sigma^2_z - \sigma^2_f, \tag{61}$$

where σ^2_z is the variance of an "effective record" [97]. Equations 60 and 61 allow prediction of $\mathrm{SE}(\hat{\sigma}^2_{A_D})$, $\mathrm{SE}(\hat{\sigma}^2_{A_S})$ and $\mathrm{SE}(\hat{\sigma}^2_{A_T})$. Application of those Equations requires knowledge of m, σ^2_z and σ^2_f, which

Socially Affected Traits, Inheritance and Genetic Improvement. Table 5 Components of SEs[a] with random group composition

Parameter	Expression	
All VC	$m = n_f$	
	$\sigma_P^2 = \sigma_{P_D}^2 + (n-1)\,\sigma_{P_S}^2$	
	$\mathrm{Cov}_\omega = 2\sigma_{P_{DS}} + (n-2)\,\sigma_{P_S}^2$	
$\hat{\sigma}_{A_D}^2$	$\sigma_z^2 = \sigma_P^2$	$\sigma_f^2 = r\sigma_{A_D}^2$
$\hat{\sigma}_{A_S}^2$	$\sigma_z^2 = \frac{\sigma_P^2 + (n-2)\mathrm{Cov}_\omega}{n-1}$	$\sigma_f^2 = r\sigma_{A_S}^2$
$\hat{\sigma}_{A_T}^2$	$\sigma_z^2 = n\left[\sigma_P^2 + (n-1)\mathrm{Cov}_\omega\right]$	$\sigma_f^2 = r\sigma_{A_T}^2$

[a]SEs for the estimated genetic variance of interest follow from substituting σ_z^2 and σ_f^2 for that parameter into Eq. 60.

depend on the parameter and experimental design of interest, and are given in Table 5 for schemes with groups composed at random, and in Table 6 for schemes with groups composed of two distinct families.

The SE of the estimated genetic covariance, $\mathrm{SE}(\hat{\sigma}_{A_{DS}})$ follows from

$$\mathrm{SE}(\hat{\sigma}_{A_{DS}}) = \sqrt{\frac{\mathrm{SE}(\hat{\sigma}_{A_D}^2)\ \mathrm{SE}(\hat{\sigma}_{A_S}^2)}{2} + \frac{\sigma_{A_{DS}}^2}{N_f - 1}}, \qquad (62)$$

where $\mathrm{SE}(\hat{\sigma}_{A_D}^2)$ and $\mathrm{SE}(\hat{\sigma}_{A_S}^2)$ follow from Eq. 60.

The SE of the ratio of total heritable variance over phenotypic variance follows from

$$\mathrm{SE}(\hat{T}^2) \approx \frac{\mathrm{SE}(\hat{\sigma}_{A_T}^2)}{\sigma_P^2}, \qquad (63)$$

where $\mathrm{SE}(\hat{\sigma}_{A_T}^2)$ follows from Eq. 60. An accurate prediction equation for the SE of the estimated genetic correlation between direct and associative effects is available only for cases where the true value of this correlation is near zero. Designs with two families per group yield lower $\mathrm{SE}(\hat{\sigma}_{A_S}^2)$ than schemes with groups composed at random with respect to family, except when group size equals two individuals, in which case both designs are nearly equivalent [97].

For designs with group composed at random, the optimum family size for estimating a genetic variance is

$$n_{f\,\mathrm{opt},\sigma^2,\mathrm{random}} \approx \sigma_z^2/\sigma_f^2 \qquad (64a)$$

For designs with group composed of two families, the optimum family size for estimating a genetic variance is

$$n_{f\,\mathrm{opt},\sigma^2,2\mathrm{fam}} \approx \frac{1}{2}\,n\,\sigma_z^2/\sigma_f^2 \qquad (64b)$$

These expressions can be applied for the genetic variance of interest by using the appropriate σ_z^2 and σ_f^2 from Tables 5 or 6. Results of Eqs. 64a and b show that optimum family size for estimating $\sigma_{A_S}^2$ may be very large when group are composed at random and may differ considerably from optimum family size for estimating $\sigma_{A_D}^2$. When groups are composed of two families, optimum family size for estimating $\sigma_{A_S}^2$ is smaller, and more similar to that for $\sigma_{A_D}^2$. Thus the scheme with two families per group may be a good compromise to estimate both the direct and associative genetic variance [97].

Expressions for optimum group sizes for estimating $\sigma_{A_S}^2$ are not available. Numerically obtained results in [97] indicate that optimum group sizes are small ($\leq$3–4) in most cases. Optimum group sizes are large only for designs with two families per group, and only when the number of groups, rather than the number of individuals, is the limiting factor in the experiment. An R-package named SE.IGE is available, which calculates SEs and optimum family and group sizes, and can be downloaded from the repository of R-packages, CRAN, at http://cran.r-project.org/package=SE.IGE, following the usual method to install R-packages.

Future Directions

This chapter has summarized the quantitative genetic theory of traits affected by social interactions among individuals and reviewed the existing empirical evidence for such effects. The theoretical framework is now well developed, both in the field of artificial selection and in evolutionary biology [18, 56, 57]. The theory of genetic variance, inheritance, and response to selection has been developed [17, 31], and statistical methodology to estimate associative variance components has become available [17, 22, 25, 39, 92, 98]. Selection experiments and data analysis provide convincing evidence of substantial associative effects on survival time in cannibalistic laying hens and quail [17, 19, 21, 24, 36, 67, 93]. The evidence is less strong for other livestock species, such as pigs and beef cattle,

Socially Affected Traits, Inheritance and Genetic Improvement. Table 6 Components of SEs[a] with group composed of two distinct families (See Table 4)

Parameter	Expression	
All VC	$m = 2n_f/n$	
	$\sigma_P^2 = \sigma_{P_D}^2 + (n-1)\sigma_{P_S}^2 + 2r\left(\frac{1}{2}n-1\right)\left[\sigma_{A_{DS}} + \left(\frac{1}{2}n-1\right)\sigma_{A_S}^2\right]$	
	$\mathrm{Cov}_{\omega,\mathrm{fam}} = 2\sigma_{P_{DS}} + (n-2)\sigma_{P_S}^2 + r\left[\sigma_{A_D}^2 + 2(\frac{1}{2}n-2)\sigma_{A_{DS}} + (\frac{1}{2}n^2-2n+3)\sigma_{A_S}^2\right]$	
	$\mathrm{Cov}_{\omega,\mathrm{nonfam}} = 2\sigma_{P_{DS}} + (n-2)\sigma_{P_S}^2 + 2\,r(\frac{1}{2}n-1)\left[\sigma_{A_{DS}} + (\frac{1}{2}n-1)\sigma_{A_S}^2\right]$	
	$\mathrm{Var}(\bar{P}_{\mathrm{fam}}) = \dfrac{\sigma_P^2 + (\frac{1}{2}n-1)\mathrm{Cov}_{\omega,\mathrm{fam}}}{\frac{1}{2}n}$	
$\hat{\sigma}_{A_D}^2$	$\sigma_z^2 = (1+\varphi^2)\,\mathrm{Var}(\bar{P}_{\mathrm{fam}}) - 2\varphi\,\mathrm{Cov}_{\omega,\mathrm{nonfam}}\varphi = \dfrac{\frac{1}{2}n-1}{\frac{1}{2}n}$	$\sigma_f^2 = r\sigma_{A_D}^2$
$\hat{\sigma}_{A_S}^2$	$\sigma_z^2 = \frac{4\,\mathrm{Var}(\bar{P}_{\mathrm{fam}})}{n^2}$	$\sigma_f^2 = r\sigma_{A_S}^2$
$\hat{\sigma}_{A_T}^2$	$\sigma_z^2 = \frac{4}{n}\left[\sigma_P^2 + (\frac{1}{2}n-1)\mathrm{Cov}_{\omega,\mathrm{fam}} + \frac{1}{2}n\mathrm{Cov}_{\omega,\mathrm{nonfam}}\right]$	$\sigma_f^2 = r\sigma_{A_T}^2$

[a] SEs for the estimated genetic variance of interest follow from substituting σ_z^2 and σ_f^2 for that parameter into Eq. 60.

where some studies have found large effects [15], but others none or only small effects [25, 95]. Further research is required on those species. Associative effects appear to be very relevant for aquaculture populations as well (see below), but estimates of the associative genetic variance are almost completely lacking at present.

Genetic improvement of associative effects has the potential to contribute significantly to animal welfare because a significant proportion of welfare issues is related to mutual behaviors [99]. Well-known examples are feather pecking and cannibalism in laying hens [100], and fighting after mixing and tail biting in pigs [14]. In the past, genetic improvement of behaviors has been hampered by the cost and labor involved in routine collection of behavioral data. As a consequence, breeding for improved behavior is rare in livestock genetic improvement. The use of associative-effect models offers the potential to solve this problem because they estimate the associative effect from the resulting phenotype without the need to observe the causative behavior. Biscarini et al. [93], for example, identified 81 QTL for associative effect on plumage condition, without observing feather pecking behavior.

Not all welfare problems are related to behavioral interactions among animals. Welfare is also threatened by metabolic stress related to high efficiency of production [4]. Results in quail [17] and in Medaka [101, 102] suggest that a genetic reduction of agonistic behaviors may produce strains that waste less resources on competitive behaviors. This suggests that genetic improvement of associative effects may have the potential to increase the efficiency of production without increasing metabolic stress. Whether this phenomenon is widespread awaits further empirical testing.

Though the statistical methodology to estimate associative variance components is available, application is practice has many pitfalls, particularly in pigs, and careful model comparison is required to assess the stability of the estimates [15, 34, 39, 91]. The omission of random group effects from the statistical model, for example, may yield a strongly overestimated associative genetic variance. Interpretation of early results has been hampered by the lack of a proper theoretical framework to judge the relevance of the estimated associative genetic variance, which is often very small compared to phenotypic variance, but can nevertheless contribute substantially to heritable variance. The definition of

a total heritable variance facilitates interpreting the relevance of associative effects for livestock genetic improvement [31]. If current estimates of the associative genetic variance in pigs and laying hens reflect the true parameters, then selection for associative effects can increase rates of genetic improvement considerably. Particularly in pigs and aquaculture, there is a need for validation by means of selection experiments.

A challenging problem is the improvement of associative effects when populations are kept in large groups, which is increasingly the case. When groups are large, the number of groups is usually small, which hampers the estimation of genetic parameters. Investigation of optimum designs to estimate genetic parameters shows that the number of groups, rather than the number of individuals, is the primary determinant of statistical power [97]. Moreover, in large groups, the intensity of the interactions is probably less, as found in pigs [38], which reduces the accuracy of estimated genetic parameters and breeding values. This issue requires further investigation.

Optimum designs for maximizing response to selection suggest a trade-off between improvement of associative effects and rates of inbreeding. The key factor to increase response to selection is the use of related group members (see above). However, such designs will probably increase the correlation between EBVs of relatives, which in turn increases rates of inbreeding when selection is based on EBVs. Rates of inbreeding can be restricted by using flexible selection algorithms, such as optimum contribution selection [103–105]. Hence, high rates of inbreeding can be avoided, but this may be at the expense of response to selection. This issue has not yet been investigated; it probably becomes less relevant when breeders start using genomic selection (see below and in [106]).

Particularly interesting is the relationship between competition and variability. Breeders have long been interested in increasing uniformity. However, though animal breeders have successfully increased the average performance of livestock, genetic improvement of uniformity has proven difficult. In the classical model, where $P = A + E$, opportunities for genetic changes in variability are very limited [107]. At best, breeders can achieve $\sigma_A^2 \approx 0$, which reduces phenotypic standard deviation by only ~16% when heritability equals 0.3. There is, however, increasing evidence that the environmental variance is under direct genetic control [e.g., 108, 109], and theoretical models for inherited variability have been developed [110–112]. Results suggest a substantial genetic coefficient of variation in the environmental variance, but also a difficulty to obtain reliable estimated breeding values for environmental variance [112].

The mechanisms underlying such inherited variability are largely unknown at present. In aquaculture, competition for feed is believed to inflate size variation among individuals. In carp, for example, Moav and Wohlfarth [16] found greater variability in ponds of mixed genetic strains than in ponds of a single genetic strain. Results in Medaka [101, 102] suggest that behavioral consequences of selection for growth depend on whether or not there is competition for feed in the selection environment. To limit size variation among individuals, regular grading of fish is common practice in aquaculture. Because group selection has the potential to reduce competition among individuals (see above), it seems to be a promising tool for reduction of variability in aquaculture. At present, however, there appear to be no studies on the consequences of group selection for variability in aquaculture. Current models of associative effects (see above) cannot explain a relationship between competition and variability because the phenotypic variance is independent of the average social breeding value. Hence, extension of current models is required to theoretically link variability and competition. The link between competition and variability may, however, largely be an empirical, rather than theoretical, issue.

At present, the use of genome wide marker information is rapidly increasing in livestock genetic improvement. A method know as Genomic Selection [6], which estimates effects of markers covering the entire genome, has become a routine tool in dairy cattle breeding and will soon be adapted by breeders of other livestock species. Genomic selection extends readily to socially affected traits, which seems particularly useful for crossbreeding schemes. A comparison of estimated genetic parameters for direct and associative effects for survival in cannibalistic laying hens shows large differences between purebred parental lines [24] and their crossbred offspring [36]. The genetic correlation between direct and social effects appears to be strongly negative in crossbreds, while around zero in purebreds.

Moreover, estimated parameters differ between reciprocal crosses. Those results indicate considerable "GxE-interaction" between purebreds and crossbreds. Genomic selection based on phenotypes recorded on crossbreds can be used to predict breeding values of nucleus individuals for direct and social effects referring to crossbred performance. Hence, combining genomic selection with associative-effect models seems promising to reduce mortality due to cannibalism in commercial crossbred herds.

Bibliography

1. Hill WG (2008) Estimation, effectiveness and opportunities of long term genetic improvement in animals and maize. Lohmann Inf 43(1):3–20
2. Havenstein GB, Ferket PR, Qureshi MA (2003a) Growth, liveability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1500–1508
3. Havenstein GB, Ferket PR, Qureshi MA (2003b) Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult Sci 82:1509–1518
4. Rauw WM, Kanis E, Noordhuizen-Stassen EN, Grommers FJ (1998) Undesirable side effects of selection for high production efficiency in farm animals: a review. Livest Prod Sci 56:15–33
5. Ducrocq V, Sölkner J (1994) "The Survival Kit", a Fortran package for the analysis of survival data. In: Proceedings of the 5th world congress on genetics applied to livestock production, University of Guelph, Guelph, Canada, vol 22, pp 51–52
6. Meuwissen THE, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. Genet Sel Evol 157(4):1819–1829
7. De Jong G (1993) Covariances between traits deriving from successive allocation of resources. Funct Ecol 7:75–83
8. Van der Waaij EH (2004) A resource allocation model describing consequences of artificial selection under metabolic stress. J Anim Sci 82:973–981
9. Smith C, James JW, Brascamp EW (1986) On the derivation of economic weights in livestock improvement. Anim Prod 43:545–551
10. Denison RF, Kiers ET, West SA (2003) Darwinian agriculture: when can humans find solutions beyond the reach of natural selection. Q Rev Biol 78:145–167
11. Griffing B (1967) Selection in reference to biological groups. I. Individual and group selection applied to populations of unordered groups. Aust J Biol Sci 20:127–142
12. Moore AJ, Brodie ED III, Wolf JB (1997) Interacting phenotypes and the evolutionary process: I. Direct and indirect genetic effects of social interactions. Evolution 51:1352–1362
13. Muir WM, Aggrey SE (2003) Poultry genetics, breeding and biotechnology. CABI, Wallingford
14. Turner SP, D'Eath RB, Roehe R, Lawrence AB (2010) Selection against aggressiveness in pigs at re-grouping: Practical application and implications for long-term behavioural patterns. Anim Welfare 19(suppl 1):123–132
15. Bergsma R, Kanis E, Knol EF, Bijma P (2008) The contribution of social effects to heritable variation in finishing traits of domestic pigs (*Sus scrofa*). Genetics 178:1559–1570
16. Moav R, Wohlfarth GW (1974) Magnification through competition of genetic differences in yield capacity in carp. Heredity 33:181–202
17. Muir WM (2005) Incorporation of competitive effects in forest tree or animal breeding programs. Genetics 170:1247–1259
18. Bijma P, Wade MJ (2008) The joint effects of kin, multilevel selection and indirect genetic effects on response to genetic selection. J Evol Biol 21:1175–1188
19. Muir WM (1996) Group selection for adaptation to multiple-hen cages: selection program and direct responses. Poult Sci 75:447–458
20. Craig JV, Muir WM (1996) Group selection for adaptation to multiple-hen cages: behavioral responses. Poult Sci 75(10):1145–1155
21. Craig JV, Muir WM (1996) Group selection for adaptation to multiple-hen cages: beak-related mortality, feathering, and body weight responses. Poult Sci 75:294–302
22. Muir WM, Schinkel A (2002) Incorporation of competitive effects in breeding programs to improve productivity and animal well being. In: Proceedings of the 7th world congress on genetics applied to livestock production, Montpellier, France, communication no. 14–07
23. Willham RL (1972) The role of maternal effects in animal breeding III. Biometrical aspects of maternal effects in animal. J Anim Sci 35:1288–1292
24. Ellen ED, Visscher J, Van Arendonk JAM, Bijma P (2008) Survival of laying hens: genetic parameters for direct and associative effects in three purebred layer Lines. Poult Sci 87:233–239
25. Arango J, Misztal I, Tsuruta S, Cubertson M, Herring W (2005) Estimation of variance components including competitive effects of Large White growing gilts. J Anim Sci 83:1241–1246
26. Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Pearson Education, Harlow
27. Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates, Sunderland
28. Fisher RA (1918) The correlation between relatives on the supposition of Mendelian inheritance. Trans R Soc Edinb 52:399–433
29. Fisher RA (1941) Average excess and average effect of a gene substitution. Ann Eugen 11:53–63
30. Wolf JB, Brodie ED III, Cheverud AJ, Moore AJ, Wade MJ (1998) Evolutionary consequences of indirect genetic effects. Trends Ecol Evol 13:64–69
31. Bijma P, Muir WM, Van Arendonk JAM (2007a) Multilevel selection 1: Quantitative genetics of inheritance and response to selection. Genetics 175:277–288

32. Bijma P (2010) Fisher's fundamental theorem of inclusive fitness and the change in fitness due to natural selection when conspecifics interact. J Evol Biol 23:194–206
33. Griffing B (1976) Selection in reference to biological groups. VI. Analysis of full sib groups. Genetics 82:723–731
34. Van Vleck LD, Cundiff LV, Koch RM (2007) Effect of competition on gain in feedlot bulls from Hereford selection lines. J Anim Sci 85:1625–1633. doi:10.2527/jas.2007-0067
35. Monsen BB, Ødegård J, Nielsen HM, Arnesen KR, Toften H, Damsgård B, Bijma P, Olesen I (2010) Genetics of social interactions in Atlantic cod (*Gadus morhua*). In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, August 2010. Book of Abstracts page 100; Paper ID 910
36. Peeters K, Eppink T, Ellen E, Visscher J, Bijma P (2010) Survival in laying hens: Genetic parameters for direct and associative effects in the reciprocal crosses of two purebred layer lines. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, August 2010. Book of Abstracts page 54; Paper ID 575
37. Brichette I, Reyero MI, Garcı'a C (2001) A genetic analysis of intraspecific competition for growth in mussel cultures. Aquaculture 192:155–169
38. Canario L, Lundeheim N, Bijma P (2010) Pig growth is affected by social genetic effects and social litter effects that depend on group size. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, August 2010 (in press). Book of Abstracts page 87; Paper ID 170
39. Chen CY, Kachman SD, Johnson RK, Newman S, Van Vleck LD (2008) Estimation of genetic parameters for average daily gain using models with competition effects. J Anim Sci 86:2525–2530
40. Hsu WL, Johnson RK, VanVleck LD (2010) Effect of pen mates on growth, backfat depth, and longissimus muscle area of swine. J Anim Sci 88:895–902
41. Bouwman AC, Bergsma R, Duijvesteijn N, Bijma P (2010) Maternal and social genetic effects on average daily gain of piglets from birth till weaning. J Anim Sci 88:2883–2892. doi:10.2527/jas.2009-2494
42. Hadfield JD, Wilson AJ (2007) Multilevel Selection 3: Modeling the effects of interacting individuals as a function of group size. Genetics 177:667–668
43. Bijma P (2010) Multilevel selection 4: Modeling the relationship of indirect genetic effects and group size. Genetics 186:1029–1031
44. Griffing B (1977) Selection for populations of interacting genotypes. In: Proceedings of the international conference on quantitative genetics, Iowa State University Press, Ames, pp 413–434
45. Griffing B (1981a) A theory of natural-selection incorporating interaction among individuals. 1. The modeling process. J Theor Biol 89:635–658
46. Griffing B (1981b) A theory of natural-selection incorporating interaction among individuals. 2. Use of related groups. J Theor Biol 89:659–677
47. Bourdon RM (2000) Understanding animal breeding, 2nd edn. Prentice Hall, Upper Saddle River
48. Ellen ED, Muir WM, Teuscher F, Bijma P (2007) Genetic improvement of traits affected by interactions among individuals: Sib selection schemes. Genetics 176:489–499
49. Eaglen SAE, Bijma P (2009) Genetic parameters of direct and maternal effects for calving ease in Dutch Holstein-Friesian cattle. J Dairy Sci 92:2229–2237
50. Price GR (1970) Selection and covariance. Nature 227:529–531
51. Hazel LN (1943) The genetic basis for constructing selection indexes. Genetics 28:476–490
52. Hazel LN, Dickerson GE, Freeman AE (1994) The selection index – Then, now and for the future. J Dairy Sci 77:3236–3251
53. Henderson CR (1975) Best linear unbiased estimation and prediction under a selection model. Biometrics 31: 423–447
54. Hamilton WD (1964) The genetical evolution of social behavior. J Theor Biol 7:1–52
55. Wade MJ (1985) Hard selection, soft selection, kin selection, and group selection. Am Nat 125:61–73
56. McGlothlin JW, Brodie ED III (2009) How to measure indirect genetic effects: the congruence of trait-based and variance-partitioning approaches. Evolution 63:1785–1795
57. McGlothlin JW, Moore AJ, Wolf JB, Brodie ED III (2010) Interacting phenotypes and the evolutionary process. III. Social evolution. Evolution 64(9):2558–2574
58. Wade MJ (1976) Group selection among laboratory populations of Tribolium. Proc Natl Acad Sci USA 73: 4604–4607
59. Wade MJ (1977) An experimental study of group selection. Evolution 31:134–153
60. Bijma P, Aanen DK (2010) Assortment, Hamilton's rule and multilevel selection. Proc R Soc B 2010(277):673–675. doi:10.1098/rspb.2009.1093
61. Frank SA (2007) All of life is social. Curr Biol 17(16):R650
62. Wade MJ, Bijma P, Ellen ED, Muir WM (2010) Group selection and social evolution in domesticated animals. Evol Appl 3:453–465. doi:10.1111/j.1752-4571.2010.00147.x, ISSN 1752-4571
63. Cameron ND (1997) Selection indices and prediction of genetic merit in animal breeding. CABI, Wallingford
64. Mrode RA, Thompson R (2005) Linear models for the prediction of animal breeding values. CABI, Wallingford
65. Wray NR, Hill WG (1989) Asymptotic rates of response from index selection. Anim Prod 49:217–227
66. Villanueva B, Wray NR, Thompson R (1993) Prediction of asymptotic rates of response from selection on multiple traits using univariate and multivariate best linear unbiased predictors. Anim Prod 57:1–13
67. Muir WM, Bijma P, Chen CY, Misztal I, Wade MJ (2010) Advances in selection theory including experimental demonstrations: The theory of selection with social competition. In: Proceedings of the 9th world congress on genetics applied to livestock production, Leipzig, August 2010. Book of Abstracts page 141; Paper ID 16

68. Dickerson GE (1947) Composition of hog carcasses as influenced by heritable differences in rate and economy of gain. Iowa Agric Exp Stn Res Bull 354:492–524
69. Willham RL (1963) The covariance between relatives for characters composed of components contributed by related individuals. Biometrics 19:18–27
70. Manfredi E, Ducrocq V, Foulley JL (1991) Genetic analysis of calving ease in dairy cattle. J Dairy Sci 74:1715–1723
71. Sawalha RM, Conington J, Brotherstone S, Villanueva B (2007) Analyses of lamb survival of Scottish Blackface sheep. Animal 1:151–157
72. Koch RM, Cundiff LV, Gregory KE (1994) Cumulative selection and genetic change for weaning or yearling weight or for yearling weight plus muscle score in Hereford cattle. J Anim Sci 72:864–885
73. Luo MF, Boettcher PJ, Schaeffer LR, Dekkers JCM (2002) Estimation of genetic parameters of calving ease in first and second parities of Canadian Holsteins using Bayesian methods. Livest Prod Sci 74:175–184
74. Meyer K (1992) Bias and sampling covariances of estimates of variance components due to maternal effects. Genet Sel Evol 24:487–509
75. Robinson DL (1996) Model which might explain negative correlation between direct and maternal genetic effects. Livest Prod Sci 45:111–122
76. Koerhuis ANM, Thompson R (1997) Models to estimate maternal effects for juvenile body weight in broiler chickens. Genet Sel Evol 29:225–249
77. Quaas RL, Pollak EJ (1980) Mixed model methodology for farm and ranch beef cattle testing programs. J Anim Sci 51:1277–1287
78. Bijma P (2006) Estimating maternal genetic effects in livestock. J Anim Sci 84:800–806
79. Muir WM, Liggett DL (1995) Group selection for adaptation to multiple-hen cages comparisons with a control and commercial lines. Poult Sci 74(suppl 1):101, Abstr
80. Muir WM, Craig JV (1998) Improving animal well-being through genetic selection. Poult Sci 77:1781–1788
81. Cheng HW, Freire R, Pajor EA (2004) Endotoxin stress responses in chickens from different genetic lines 1. sickness, behavioral, and physical responses. Poult Sci 83:707–715
82. Cheng HW, Muir WM (2005) The effects of genetic selection for survivability and productivity on chicken physiological homeostasis. World's Poult Sci J 61:383–397
83. Cheng HW, Freire R, Pajor EA, Chen Y, Muir WM (2001) Different regulation of physiological and behavioral response to social stress in two genetically selected lines of laying hens. In: Garner JP, Mench JA, Heekin SP (eds) Proceedings of the 35th international congress of the ISAE, Davis, pp 128
84. Cheng HW, Muir WM (2004) Chronic social stress differentially regulates neuroendocrine responses in laying hens II. Genetic basis of adrenal responses under three different social conditions. Psychoneuroendocrinology 29:961–971
85. Rodenburg TB, Komen H, Ellen ED, Uitdehaag KA, van Arendonk JAM (2008) Selection method and early-life history affect behavioural development, feather pecking and cannibalism in laying hens: A review. Appl Anim Behav Sci 110:217–228
86. Rodenburg TB, Bolhuis JE, Koopmanschap RE, Ellen ED, Decuypere E (2009a) Maternal care and selection for low mortality affect post-stress corticosterone and peripheral serotonin in laying hens. Physiol Behav 98:519–523
87. Rodenburg TB, Uitdehaag KA, Ellen ED, Komen J (2009b) The effects of selection on low mortality and brooding by a mother hen on open-field response, feather pecking and cannibalism in laying hens. Anim Welf 18:427–432
88. Bolhuis JE, Ellen ED, Van Reenen CG, De Groot J, Ten Napel J, Koopmanschap R, De Vries GR, Uitdehaag KA, Kemp B, Rodenburg TB (2009) Effects of genetic group selection against mortality on behaviour and peripheral serotonin in domestic laying hens with trimmed and intact beaks. Physiol Behav 97:470–475
89. Goodnight CJ (2005) Multilevel selection: the evolution of cooperation in non-kin groups. Popul Ecol 47:3–12. doi:10.1007/s10144-005-0207-2
90. Griffin AS, West SA, Buckling A (2004) Cooperation and competition in pathogenic bacteria. Nature 430:1024–1027
91. Van Vleck LD, Cassady JP (2005) Unexpected estimates of variance components with a true model containing genetic competition effects. J Anim Sci 83:68–74
92. Bijma P, Muir WM, Ellen ED, Wolf JB, Van Arendonk JAM (2007b) Multilevel selection 2: Estimating the genetic parameters determining inheritance and response to selection. Genetics 175:289–299
93. Biscarini F, Bovenhuis H, van Arendonk JAM, Parmentier HK, Jungerius AP, Van Der Poel JJ (2010) Across-line SNP association study of innate and adaptive immune response in laying hens. Anim Genet 41:26–38
94. Gilmour AR, Gogel BJ, Cullis BR, Thompson R (2006) ASReml User Guide, Release 2.0. VSN International Ltd, Hemel Hempstead
95. Cheng J, Janssens S, Buys N (2009) Full sib pens of pigs are not suitable to identify variance component of associative effect: a simulation study using Gibbs Sampling. BMC Genet 10:9
96. Cantet RJC, Cappa EP (2008) On identifiability of (co)variance components in animal models with competition effects. J Anim Breed Genet 125:371–381
97. Bijma P (2010) Estimating indirect genetic effects: precision of estimates and optimum designs. Genetics 186:1013–1028
98. Cappa EP, Cantet RJC (2008) Direct and competition additive effects in tree breeding: Bayesian estimation from an individual mixed tree model. Silvae Genet 57:45–57
99. Rodenburg TB, Bijma P, Ellen ED, Bergsma R, de Vries S, Bolhuis JE, Kemp B, van Arendonk JAM (2010) Breeding amiable animals? Improving farm animal welfare by including social effects in breeding programmes. Anim Welf 19:S77–S82
100. Kjaer JB, Sørensen P (1997) Feather pecking behaviour in White Leghorns, a genetic study. Br Poult Sci 38:333–341

101. Ruzzante DE, Doyle RW (1991) Rapid behavioural changes in Medaka (*Oryzias latipes*) caused by selection for competitive and noncompetitive growth. Evolution 45:1936–1946
102. Ruzzante DE, Doyle RW (1993) Evolution of social behaviour in a resource-rich, structured environment: selection experiments with Medaka (*Oryzias latipes*). Evolution 47:456–470
103. Wray NR, Goddard MG (1994) Increasing long-term response to selection. Genet Sel Evol 26:431–451
104. Meuwissen THE (1997) Maximizing response of selection at a predefined rate of inbreeding. J Anim Sci 75:934–940
105. Grundy B, Villaneuva B, Woolliams JA (1998) Dynamic selection procedures for constrained inbreeding and their consequences for pedigree development. Genet Res Camb 72:159–168
106. Daetwyler HD, Villanueva B, Bijma P, Woolliams JA (2007) Inbreeding in genome-wide selection. J Anim Breed Genet 124(6):369–376
107. Hohenboken WD (1985) The manipulation of variation in quantitative traits: a review of possible genetic strategies. J Anim Sci 60:101–110
108. Rowe SJ, White IMS, Avendano S, Hill WG (2006) Genetic heterogeneity of residual variance in broiler chickens. Genet Sel Evol 38:617–635
109. Ibanez-Escriche N, Sorensen D, Waagepetersen R, Blasco A (2008) Selection for environmental variation: a statistical analysis and power calculations to detect response. Genetics 180:2209–2226
110. Sancristobal-Gaudy M, Elsen JM, Bodin L, Chevalet C (1998) Prediction of the response to a selection for canalisation of a continuous trait in animal breeding. Genet Sel Evol 30:423–451
111. Hill WG, Zhang XS (2004) Effects of phenotypic variability of directional selection arising through genetic differences in residual variability. Genet Res Camb 83:121–132
112. Mulder HA, Bijma P, Hill WG (2007) Prediction of breeding values and selection responses with genetic heterogeneity of environmental variance. Genetics 175:1895–1910

Spatial Crop Structure in Agricultural Systems

Emilio H. Satorre
Cátedra de Cerealicultura, University of Buenos Aires, Buenos Aires, Argentina
CONICET (National Research Council), University of Buenos Aires, Buenos Aires, Argentina
AACREA, Buenos Aires, Argentina

Article Outline

Glossary

Competition Refers to the process whereby plants share resources (e.g., mineral nutrients, water, and light) which are in insufficient supply for their joint requirements [71].

Crop structure Refers to the spatial, temporal, and genetic arrangement of a particular crop species or genotype within a sown area.

Density Is the number of individuals of a plant species in a unit of area within a crop.

Facilitation Is the process whereby one crop species provides some sort of benefit for another species when in a polyculture. Usually, when facilitation occurs, at least one crop may positively alter the environment for the other crop [77].

Policulture (also intercrop) Refers to crop arrangements that include more than one crop species or genotypes grown together partially or totally during the growth period of a particular area.

Potential yield Is the crop yield obtained when available resource use is maximized in a particular environmental condition and limiting factors, such as soil nutrients, or reduction factors, such as insect and plant pests and diseases, are absent [41, 78].

Rectangularity Is a measure used to describe the spatial distribution of individual plants in crops sown in rows. It is the ratio of the mean distance between rows and the distance between individual plants within a row.

Resource complementarity Is a measure of the extent to which the crop species components in a polyculture share common limiting resources, i.e., plant components in a crop that show complete resource complementarity do not compete [71].

Spatial arrangement Refers to the way that crop plants are distributed in a field. For example, they may be randomly distributed, when seeds or propagules are randomly broadcast or they may be sown in rows, in a regular pattern when drillers are used.

Definition of the Subject

Crop yields mostly depend on the growth rate experienced by the plant during particular critical periods. The amount of the resources captured and the resource use efficiency determine the growth rate of the crop plants at those crucial stages for yield determination. Since plants stand still in the land, the way they are distributed greatly influence the ability of a crop to capture and use environmental resources (radiation, water, and nutrients), which are necessary for growth and yield. The spatial arrangement of plants and the temporal development of their structures (mainly leaves and roots) define the crop structure. Crop structure may be then analyzed and described in many ways. However, most effort has been concentrated on describing the size and distribution of leaves, which capture the radiant energy, since they are aboveground and easy to measure. The study of yield response to crop structure has conformed to the scientific basis for important technical management decisions and technologies involving crop density, and distance between rows and rectangularity in both mono- and polycultures in various agricultural systems of the world. Crop structure, as a research topic, is one of the few examples in which science dynamically contributes to develop crop management strategies and to guide genetic improvement among crop species. At present, the manipulation of crop structure is not only explored in controlled, semi-controlled, and field

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

research experiments, but also in mathematical modeling, which evidences the complexity of the interactions that need to be explored and, at the same time, the maturity of the knowledge reached in this area.

Introduction

Crop structure is one of the major determinants of the ability of a crop to capture resources and to produce high yields. It has received much attention from producers, agronomists, and researchers because it is under fairly close control by the farmer in most crop systems of the world. Crop structure is a complex crop attribute, determined by the crop genotype, its sowing date, density (i.e., the number of plants per unit area), and the spatial plant arrangement. Therefore, crop structure in general has a genetic, temporal, and spatial component. However, the spatial component determined by the plant density and spatial arrangement of the plants are largely considered the main modifier of crop resource capture and use and, therefore, a strong determinant of crop yield. For this reason, just for simplicity, in this entry, attention will be centered on the spatial structure of crops, since it would be very difficult to cover all the effects and interactions from other factors, such as temporal ones, in a single entry.

The structural component of a crop may be seen as determining its potential yield in any particular region. Decisions affecting the structure of the crop and so its ability to capture light and other resources may modify the potential yield of the crop. The potential yield is the yield attainable by the crop when its growth is only limited by the radiation and carbon dioxide available, and so is only affected by the temperature of the site and the crop structure. Crop structure factors may directly (for example, in the case of density) or indirectly (for example, in the case of the genotype sown, due to its length or appropriate sowing date) modify the potential yield of a crop. Although actual (real) yields harvested may usually be lower than potential yields due to the effect of limiting (i.e., water or nutrients) and reducing (i.e., diseases, pests, and weeds) factors [78], the interaction of crop structure with environment and other crop management decisions are crucial to produce high yields efficiently.

Most crops in mechanized agriculture are sown in monocultures, i.e., single-species stands are grown every growing period in the same region. However, in most subsistence and few mechanized modern agricultural systems, crops are sown in polycultures, i.e., more than one species are grown in the same area during each growing period. Crop structure characteristics are crucial in determining crop yield responses both in single species and polycultures. Usually, the interest was concentrated in defining the relationships between density and crop yield quantitatively in order to establish optimum crop populations and maximum attainable yields under various situations. As a result, the effect of density on crop productivity in mono- and polycultures has been deeply studied since mid last century [22, 34, 82]. However, due to the high contribution of single-species crops to grain crop production, the study of spatial crop structure determinants has called the attention of researchers all over the world, particularly under this modern and technologically advanced crop production system.

Crops in monocultures are usually heavily selected for uniformity so that most individuals are genetically and phenotypically similar or even identical because of the uniformity of seed size and the fact that sown seeds tend to germinate synchronously. In such crops stands, intraspecific competition (competition between plants of the same species) may be intense and is largely the process commanding crop responses and yield. When in polycultures, intra- and interspecific competition together with resource complementarities or facilitation processes determines yield of each crop species in the stand. For most of the modern agricultural systems, plant competition is therefore the ecological process largely determining the yield response to spatial crop structure arrangements.

Crop density largely determines interplant competition causing a reduction in survival, dry-matter growth, and grain yield of individual plants. Despite the fact that interplant competition causes a negative effect in the individual plants, the management of crop competition through density selection may allow maximum yields per unit area to be achieved. Identifying and understanding crops yield-density response allow researchers to predict the effect of crop management practices on yield and help farmers, agronomists, and consultants to properly design crop production structure under various ecological conditions. Spatial arrangement also determines not only interplant

competition at any density (since it may modify the competition between plants within a row with respect to plants in different rows) but also resource use efficiency. Therefore, crop-plants arrangement or crop structure affects intraspecific competition and resource use efficiency, allowing a full or partial use of available resources and how they are transformed during the various stages of crop growth. In this entry, I will discuss how density and spatial arrangement affects crop functioning and how some environmental conditions or crop management decisions may affect those relationships. Most of the information comes from crops in monocultures, but the performance of these spatial components of crop structure in polycultures will be also briefly addressed.

Ecophysiological Basis of Response to Crop Structure

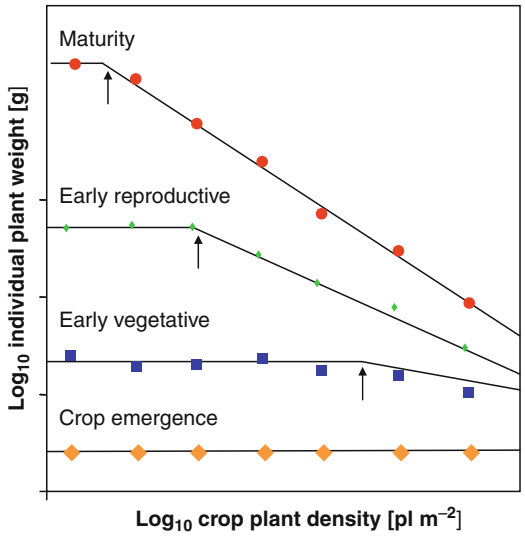
Resource Use and Dry-Matter Production

Crop dry-matter production under potential conditions is determined by the intercepted solar radiation and the radiation use efficiency of the crop canopy. From both aspects, crop density mainly affects the ability of the crop to intercept radiation (or to capture the light resource, from an ecological perspective) since there is little evidence on the effects of density on resource use efficiency. However, spatial arrangement may modify both the capture and use efficiency of resources [42, 83].

It is known that much of the incident solar radiation is not available for annual crop species growth during the early stages, due to the fact that low leaf area expansion determines low light interception. At early crop stages, increasing density may contribute to increase Leaf Area Index (LAI) of the crop (i.e., the green leaf surface per unit of area) and the amount of light intercepted. For this reason, there is a strong positive, although nonlinear, relationship between density, LAI, and the proportion of the incident light that is intercepted in early stages of crop development. When the crop grows, light resource capture increases as leaves expand and LAI increases, and a nonlinear relationship establishes among both variables. Although density may modify the number of leaves, particularly at early crop stages, the relationship between density and leaf area becomes weak when the crop advances in its development. Improving plants spatial distribution (for example, by changing the distance between rows) may also help to intercept more light at early stages of crop growth, when Leaf Area Index (LAI) values are low. To illustrate these points, early results from Puckridge and Donald in wheat [56] clearly showed that density manipulation in the range 1.4–1078 pl/m^2 could successfully increase light interception of wheat crops under the Western Australia conditions. The greater proportion of intercepted light in high-density crops explained most of the differences in early crop growth rates calculated from the results of that research; i.e., early crop growth rates were greater in crops sown at the higher densities. Similar results were also obtained with other crops, such as those reported in recent experiments for maize (e.g., [79], soybean, and sunflower [76]. There are experimental evidences that increasing density not only improve light capture early in the season but also the uptake of soil resources such as water [75]). For example, in a Mediterranean environment, high crop-sowing densities contributed to reduce soil evaporation and to increase biomass production and water use in the early phases of wheat growth [16].

During the early stages of development, competition among small crop plants may be evident only at very high densities; therefore, young individual plants tend to have similar dry-matter production, while crop productivity per unit area tends to increase linearly with density. Plotting the logarithm of plant dry matter against the logarithm of density as suggested by Kira et al. [39] helps to show the way individual plants and crops would tend to respond as they grow (Fig. 1). It is clear from the figure that, as time goes by and crop development progresses, plants increase in size and leaf area, and the onset of competition is evident even at low densities; i.e., individual plant size is continuously reduced as density increases above a density threshold (Fig. 1, see arrows). As plant size increases, crop growth rate may depend more on resource availability than on plant density; therefore, crop growth rates may be only influenced by density if plant number is below the competition threshold. This density threshold varies according to the crop or genotype characteristics and the environmental conditions.

For any crop, when crop stands are above the threshold density, LAI tends to allow full light interception and



Spatial Crop Structure in Agricultural Systems. Figure 1 Influence of density on individual plant weight at various stages of crop development, following Kira et al. [39] proposal. *Arrows* indicate competition thresholds at the various crop stages (see text for details)

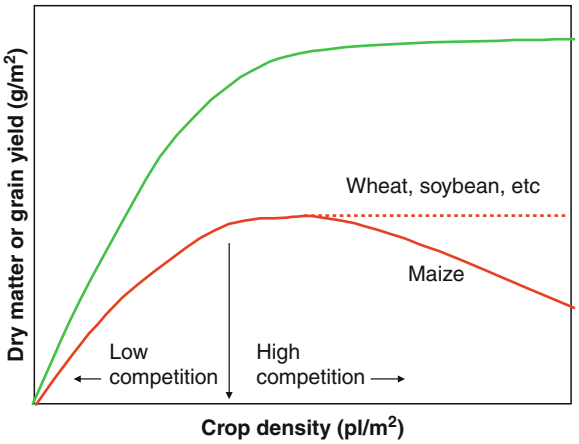
use of the available resources; therefore, crop growth rate will be maximum for the set of environmental conditions explored by the crop. At this stage, any crop density above the competition threshold (Fig. 1) would maximize crop growth rate per unit area. Sustaining maximum crop growth rates is important for high yields to be obtained, especially when that occurs during the critical period for yield determination of the crop.

The phenological period that is critical for yield determination varies among species; i.e., the period when reductions in crop growth rate significantly reduce grain number or yield depends on the species considered. For example, for wheat, it extends from the beginning of ear growth to the beginning of grain growth [29, 68], but for maize, it is considered to be centered 15 days before and after crop silking [3]. High crop yield per unit area would then be expected when crop structure, density, and plant arrangement have been suitable for the crop to reach 95% light interception, which allows maximum crop growth rate when non-limiting or reduction factors are present, before the beginning of the critical period and during it. Environmental and management practices such as fertilizer application or disease control could alter the crop yield-density response, altering the density above which crop growth rate is maximum.

In general, crop structures are aimed to use above- and belowground resources completely, allowing the crop to maximize its growth rate during critical stages. However, it has to be mentioned that increasing early resource capture through great plant densities may not necessarily maximize growth rate at critical crop stages. For example, large quantities of water use by a crop in the early phases of growth may reduce soil-water availability late in the season, leading to biomass yields similar to those obtained with lower plant densities [20, 51]. In temperate subhumid areas, this pattern of crop water use may led to reduce crop sowing rates, as in wheat in the southern pampas of Argentina [32] or the semiarid Brown soil zone of Canada during dry years [45].

Due to the relationship described in Fig. 1, the total shoot weight of a crop per unit area of land usually increases asymptotically as density increases (Fig. 2). Therefore, the performance of crops sown at low densities may depend on their ability to compensate for low plant populations by producing more tillers, branches of bigger leaves. Tillers offer vegetative plasticity in the case of barley or wheat, while branches do so in the case of soybean. Crops that do not produce tillers or branches may express some vegetative plasticity by altering leaf size or height as in the case of maize or sunflower. Moreover, in some crops, the dry-matter asymptote in Fig. 2 extends over a wide range of densities, due mainly to the large plasticity of individual plant size which determines that mean plant weight declines to exactly compensate for increases in density; i.e., proportionate reductions in plant weight occur as densities increase above the normal sown density [22, 35, 69]. Examples of such phenotypic plasticity may be found in crop species, such as wheat, soybean, or barley. Forty-fold differences in plant weight have been reported to occur between widely spaced wheat plants and plants growing in stands at normal seed rates [9, 56].

The supply of limiting resources and the genetic ability to capture and use them largely determine the maximum total biomass yield per unit area of any particular crop in a particular environment [82]. Environmental factors such as temperature or daylength, which may affect the length of the growing season, can also



Spatial Crop Structure in Agricultural Systems. Figure 2 Influence of density on crop dry matter (*green line*) and grain yield (*red line*). Low and high competition range is indicated, together with the expected response of yield to density in maize (*solid red*) and wheat or soybean (*dotted red*)

affect maximum shoot weight (Potential yield) by modifying the ability of the crop to capture resources. For example, in the case of wheat, a crop with large vegetative plasticity (see Fig. 2), long days at high latitudes reduce wheat tillering, which determines that sowing rates of 500–700 pl/m^2 are commonly used for spring wheat crops in places such as Finland [55] in contrast with the 200–300 pl/m^2 used in temperate areas. In some cases, the relationship between density and total shoot weight per unit area is better described by a parabolic model; i.e., there is a distinct maximum yield at a particular density, and shoot yield declines as density increases above this point. In these cases, it is expected that crowding reduces the efficiency of the crop to capture or use environmental resources [22].

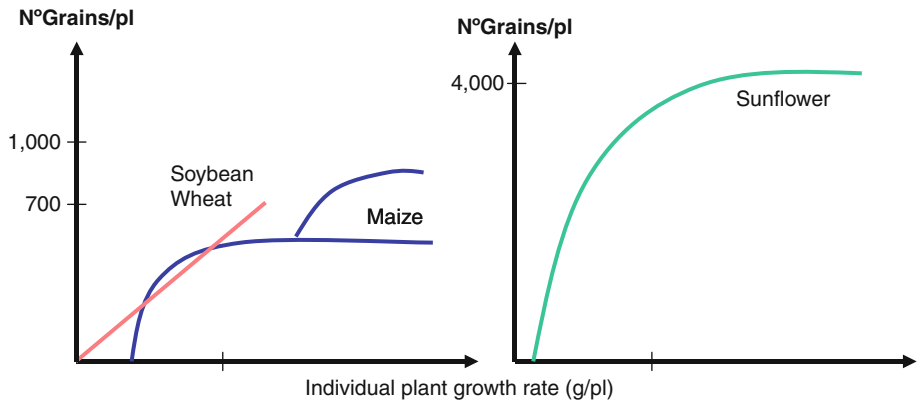
Grain Yield Production

Grain yield increases as density increase up to a value which determines maximum use of available resources (i.e., at suboptimal densities). However, performance of yield at supraoptimal densities may vary among species. It has generally been accepted that the response to density of harvest or storage organs (e.g., grains) is better described by a parabolic model [35]; i.e., grain yield decreases at higher or supraoptimal plant densities due to the fact that the allocation of resources to storage organs or grains is greatly altered by competition [34]. In general, the proportion of the total biomass allocated in the grains, i.e., harvest index (HI), declines progressively with increasing density [23]. Despite the fact that parabolic responses are generally accepted for grain yield–density relationships, some studies indicate that yield–density relationships for grain may sometimes be better described by asymptotic models (e.g., [38, 43]). In this case, the range of densities that maximize grain yield could be wide and strongly dependent upon crop genotype and environmental characteristics.

When crop plants are under severe competition due to relatively high crop densities, they show a reduction in individual growth rate. This will markedly affect grain number determination of any grain crop, particularly if the per plant low growth rates occur during its critical period. Therefore, shoot growth may become less affected than grain yield, causing a reduction in harvest index. Crops such as maize, with low reproductive plasticity, may experience severe grain yield reductions at high crop densities, mainly under limiting environments, such as with low water availability. Grain yield reductions at supraoptimal densities depend on how crops determine yield. In most grain crops, the number of grains per plant, firstly, and per unit area, lately, is strongly related to yield. However, the relationship differs between crop species (Fig. 3).

In the case of maize and sunflower, a minimum plant growth rate is necessary to fix grains, and the relationship is not linear, while in the case of soybean or wheat, it is linear, and the minimum plant growth rate necessary to fix grains is very close to zero (Fig. 3; [5, 79]). It is clear from much data that the number of grains fixed per unit area is the main factor controlling the grain yield-density response of grain crops and, since number of grains determination varies between species, density rules and decisions have to be carefully explored in any particular case. In high-yielding environments or cropping systems (i.e., with irrigation and high fertilizer rates), it appears that large plant populations will determine large number of grains per unit area and high yields.

Low seeding rates or low crop stands may be compensated by the contribution of tillers or branches. Therefore, in low-density crops, either tillering, branching, or the reproductive plasticity of the species is an



Spatial Crop Structure in Agricultural Systems. Figure 3
Expected relationship between the individual grain number and growth rate of four grain crops

essential component in determining the number of grains per unit area at harvest. However, tillering and branching have been recognized as complex phenomenon, controlled by endogenous (genetically) and environmental factors [60]. It has been accepted that a large number of tillers or branches are produced by plants when the availability of resources is high. However, recent studies have found that light quality may affect tiller or branch responses. Light composition with low Red/Far Red ratio in a crop canopy reduces tillering or branching of individual plants [13]. These results indicate that, within a wide range of resource availability, photomorphogenic reactions mediated by light quality may also affect responses to various crop structures. Therefore, density and plant arrangement responses will ultimately be the result of the interaction between resource-based and non-resource-based environmental signals.

High density in crops may produce adverse agronomic effects, such as lodging or higher susceptibility to pest or disease damage. Although different plant densities may have little effect on carbon photoassimilation in top plant structures, including reproductive structures, high densities may tend to reduce partitioning to the stem internodes and to increase leaf senescence among other effects [61, 80, 81]. For example, when stems export photoassimilates due to high competition pressure by crop plants, the rate of leaf senescence is increased and basal nodes weaken, which contributes to increase lodge susceptibility and to reduce grain yields under high sowing rates.

Crop Structure Responses in Monocultures

The Effect of Soil Resources

As mentioned above, the supply of limiting resources, i.e., water, light, and nutrients may affect the form and parameters of the biomass and yield–density relationships, largely through their effect on the maximum yield per unit area that can be achieved at very high densities. Resources have little effect on the yield of widely spaced plants, i.e., yield per plant at very low densities, since in that case, biomass and yield productivity is limited by the possibility to capture resources due to the low-density crop structure. For example, nitrogen fertilizer applications tended to increase maximum wheat yields per unit area under field conditions, but only slightly affect the maximum yield per plant when plants were sown widely spaced [9]. However, in most agricultural conditions, the response to density is dependent upon the supply of limiting resources since it is competition for limiting resources the driving process in crop stands. Generally, increasing the availability of soil resources through fertilizer applications (nutrients) of irrigation (water) will relax or reduce the interplant competition so that the environment may support a higher number of plants. This will be so if the addition of a limiting factor does not affect the competition or availability of other competition factor; i.e., increasing the availability of soil resources, such as water or nitrogen, may reduce competition for soil factors, but it may increase competition for aboveground resources, such as light [22].

Nitrogen, a major soil limiting factor, strongly interacts with density in most grain crop species [70]. The optimum density and, therefore, the maximum grain yield tend to be greater by increasing nitrogen applications if nitrogen is a yield limiting factor. For example, optimum maize crop density tends to be higher as nitrogen [47, 49] or water [3] availability increases. When supplies of the major limiting resource (either water or nitrogen) are made, the crop system might be enabled to sustain a higher crop density and hence a greater grain yield. Since individual plant growth is reduced as density increases within the range of crop densities that allow maximum grain yields, the overall demand of growth resources per unit of area will tend to be similar; therefore, the nitrogen application rate or water provision required will tend to be similar between the lowest and highest density that allow maximum yields [59].

Nitrogen or water availability have a great impact on early vegetative growth by promoting plant tillering or branching, or by increasing leaf area and its activity; However, while this increases the shoot weight of the crop plants in most species, it may reduce the harvest index, particularly at high crop density (see above) if resources during the critical period of yield determination are scarce.

Crop species and genotypes within species may present different responses to density at various soil resource conditions. Differences are usually associated to the way resources are partitioned between vegetative and reproductive structures within the plant, to the ability to produce tillers or branches, and to the morphological and geometrical characteristics of their canopies. Sowing date and plant arrangements may modify the performance of the plants under various crop structures by altering the phenotypic plasticity of the crops.

The Effect of Plant Arrangement

Among the management factors that determine crop structure and that may affect crop yield–density relationships, the planting arrangement of the crop is the most important. Plant spatial arrangement, usually the distance between rows, is under close control by farmers. At any given crop density, sowing patterns can be considered random, clumped, or regular; extensive and mechanized grain crops are normally sown in rows, i.e., in a clumped arrangement, although in some cases regular patterns may be found. Historically, planting arrangement in a crop was determined to allow mechanical weed control or other labor, i.e., to let the tractor, the animal, and the tool to do its labor on the weeds between rows of the crop. However, in various parts of the world, herbicide technology and the tolerance of modern genotypes to herbicides have allowed modifying planting arrangements since no secondary, mechanical weed control labors are necessary. The planting arrangement of crops is often described by its "rectangularity," i.e., the ratio of the distance between rows to the distance between plants within a row. In general, crop yield tends to be the greatest, at any density, if the plants are arranged regularly, i.e., the rectangularity is 1 [10, 30, 33, 36, 53]. However, the effect of planting arrangement is often not significant if densities are at or above those required to achieve maximum use of resources and yield [3, 10, 50]. The extent to which rectangularity affects the yield of a crop is dependent on the plasticity of the individual plants of any particular genotype, variety, or hybrid and the environmental conditions. Grain crops yield patterns are not consistent through literature when rectangularity is analyzed. In maize crop, [17] found that contrasting crop genotypes regularly sown in narrow rows (38 cm between rows) produced significant higher yields than crops in a more rectangular pattern (70 cm between rows) in 2 years. They observed that crop growth rate was higher early, and light interception was greater at the critical period of maize (flowering) when crops were sown regularly spaced. However, Cirilo [20] found no significant differences between maize crops sown at 52 vs 70 cm between rows, since at the critical period of yield determination (see above), crop growth rate was similar among cropping patterns. Similarly, in the case of wheat crops, Holliday [36] reported yield increases between 8% and 33% due to reducing row spacing from 20 to 10 cm. In the case of wheat crop, most research worldwide has shown that closer row spacing (15–18 cm) gave higher grain yields than wider row spacing (usually greater than 23 cm; [32, 54, 73, 52]), though a few experiments showed that wider row spacing did not result in wheat yield losses [40]. Differences in cycle length among varieties or sowing dates may help to explain such results, as in the case of soybean in the

Southern Pampas. When long-cycle soybean varieties are sown, no yield differences between various row widths may be found (i.e., 52 vs 35 cm between rows). However, when short-cycle soybean varieties are sown, yields tend to increase consistently as distance between rows is reduced. Similarly, consistent yield increases are found when soybean varieties are sown late in the season at narrow rows, while no differences may be found, particularly in long cycle varieties, at optimum sowing dates among various row distances.

Planting arrangement may be used in crops to overcome water plant stress. For example, in the case of wheat, in some subhumid areas with late water stress periods, wide cropping patterns (for example, 52 cm between rows) proved to be more stable and productive than narrow ones, mainly due to a better water use efficiency in those environments. This sowing pattern is presently used in the low-producing areas of the Northwest of Argentina under a monsoonal climate. However, also in wheat crops, several experiments reported that there was a consistent yield depression at low sowing rates as rectangularity was increased [10, 26]. In some other cases, wheat crop yields per unit area gradually declined as rectangularity increases either by increasing plant density or increasing row width, though yield declines at high density were greater than at low-density crops [82]. When crops have enough phenotypic and reproductive plasticity, results are consistent in showing that there are better possibilities to equal high-density crop yields if low-density crops are sown in a square pattern; i.e., a better plant spatial distribution may promote grain yield advantages when crops are sown at low density rather than at high density.

The reported evidence suggests that any advantage derived from spatial arrangement is brought about by improving crops ability to exploit available resources (e.g., [84]). Low rectangularity crops increase yield due to the its effect on (1) improving the capture of incident radiation, particularly in early crop stages; (2) allowing the crop to reach 95% light interception during the critical period of yield determination (see above and [4]); (3) improving resource use efficiency, for example, water use efficiency by reducing water-soil losses through direct evaporation; and (4) improving weed control or reducing weed competition. Therefore, it may be also inferred that positive effects of improving plant arrangement by reducing rectangularity is particularly important when productive conditions may reduce plant growth due to limiting factors (i.e., low-resource availability, late sown crops under short growth periods, short cycle varieties, etc.) and when environmental or management conditions may affect crop resource capture during the critical periods.

Rectangular, more clumped, arrangements possibly cause an early reduction of crop growth rate, which in some cases may delayed or even avoid use of distant resources. On the other hand, as mentioned above, reducing the distance between rows and rectangularity and increasing plant density contributes to rapidly exploit resources by intercepting more light or capturing more water or soil resources which in most crops enhance the competitiveness of the crops against weeds [8, 31, 58, 65, 66, 72]. For example, Solie et al. [72] concluded that wheat yield increases of 18% could be obtained when row spacing was reduced from 23 to 7.5 cm when cheat (Bromus secalinus)-free and cheat-infested fields were evaluated. Similarly, [14] found that weed yield reduction was lower when soybean crops were sown at narrow rows than when they were sown widely spaced. Conversely, weed production increases with increasing weed density, or by increasing crop rectangularity or by reducing crop density. For example, Felton [28] found that increasing the row spacing in soybeans from 50 to 100 cm increased weed yield 3.5 times.

Mathematical Relationships Between Plant Yield and Density

Since density is a main determinant of crop structure responses, mathematical models have been proposed for interpreting and predicting the effect of density on yield. Interest in quantitative relationships between crop yield and density was largely stimulated by the need to clearly define optimum sowing densities for crop production. Such relationships should, ideally, take account of environmental factors, such as limiting resources, or spatial arrangement (rectangularity) which may affect the response to density. Willey & Heath [82], Mead [46], and Ratkowsky [57] among others made a comprehensive review of the various models used to describe yield–density relationships pointing out the advantages and disadvantages of the

various mathematical functions proposed. Several types of equations have been described, but only the so-called reciprocal equations for yield per plant have proved to be satisfactory for a wide range of crops and conditions. Some forms of the reciprocal equation that have been proposed are:

$$\text{Yield} = \text{density}/(\text{a} + \text{b}^{*}\text{density}) \quad (1)$$

In Eq. 1, yield is the shoot or grain yield per unit area and density is number of crop plants per unit area, and "a" and "b" are parameters of the equation. This is the simplest equation and describes only the asymptotic model; i.e., yield increases asymptotically toward a maximum yield as density increases. A basic form of Eq. 1 was first proposed by Shinozaki and Kira [69], and its beauty relies on the fact that parameters ("a" & "b") have biological meaning [82]. In this equation, parameter "a" is the reciprocal of the yield per plant (w^{-1}) when a crop genotype is sown at an infinitely low density, i.e., it is an estimation of the yield of wide-spaced plants, in competition-free conditions. This may be regarded as a measure of the "genetic potential" of individual plants of the crop. The fitted value of "a" indeed largely depends on the genetic characteristics of a particular genotype. If the yield–density relationship is truly asymptotic, parameter "b" is inversely related to the value of the asymptote, i.e., the maximum yield per unit area at very high densities; Willey & Heath [82] regarded this parameter as a measure of the "environmental potential," i.e., as an estimator of the maximum crop yield that can be attained in a particular environment and crop production system.

Other reciprocal equations incorporate a third parameter so that they can also describe parabolic yield–density relationships, i.e., the possibility of a reduction of shoot or grain yield at high densities. For example, Holliday [35] proposed the expression:

$$\text{Yield} = \text{density}/\left(\text{a} + \text{b}^{*}\text{density} + \text{c}^{*}\text{density}^{2}\right) \quad (2)$$

In Eq. 2, the quadratic term accounts for parabolic relationships; if the parameter "c" is equal to 0.0, then the equation reduces to that of Shinozaki & Kira [69]. Similarly, Farazdaghi and Harris [25] proposed the expression:

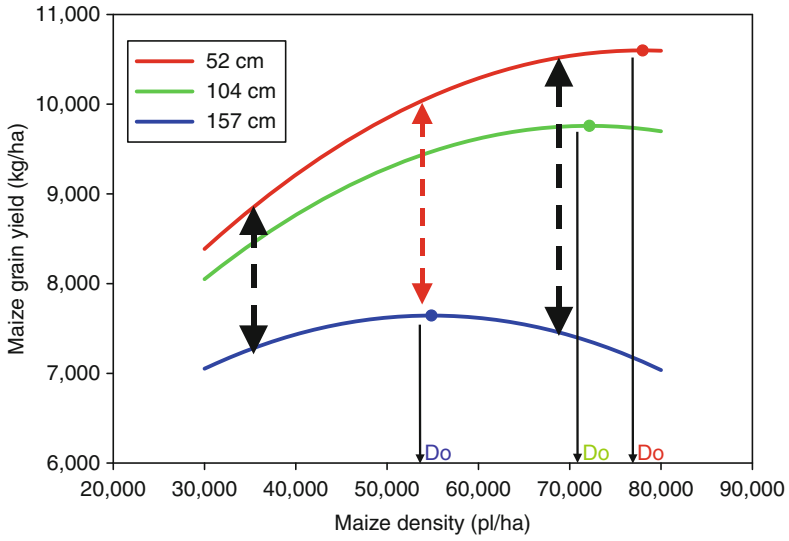
$$\text{Yield} = \text{density}/(\text{a} + \text{b}^{*}\text{density}^{z}) \quad (3)$$

Like in Eq. 2, this becomes identical to Eq. 1 when $z = 1$.

In spite of the good fit provided in several cases by these mathematical approaches [57], the reciprocal equations have some statistical limitations. However, novel statistical techniques, using data transformation and nonlinear procedures, have improved the statistical treatment of these models [57].

For modeling purposes, the response of crops to plant density should be also defined in terms of the spatial arrangement of the plants. Few researchers have intended to distinguish between density and plant arrangement (rectangularity) when modelling crop response, since usually responses to different densities have been studied at a constant row width. Although the extent to which spatial arrangement may affect the yield of crops is strongly dependent on the plasticity of the genotype [82] and the availability of environmental resources (see above), it appears to be important to consider the effect of rectangularity on the yield-density equations, particularly when crops may be sown at various densities and distances between rows. In these cases, equations should be able to describe the effects of density as well as those of rectangularity. For this purpose, it has been proposed to include intrarow and interrow spacing (rectangularity) as variables in Eqs. 1–3 [82]. However, there has been insufficient research on the effect of rectangularity on yield-density responses, and the yield-density-arrangement equations have been only tested on wheat and maize crops in few cases. Recently, Satorre [64] suggested that optimum density decreases as rectangularity increases in maize crops sown at 53, 104, and 157 cm between rows (Fig. 4). Moreover, and more important, the authors found that the magnitude of the yield increase obtained due to improving plant arrangement, by reducing rectangularity or the distance between rows, was greater in high-yielding environments than in low yielding, since vegetative growth at early crop stages was unaffected.

The reciprocal equations have proved to be robust and provide a tool to describe asymptotic or parabolic yield–density relationships. However, despite these efforts to describe the effect of crop structure on crop yield, there is still a huge work to be done since, also for modeling purposes, the response of crops to plant density should be also defined in terms of the planting



Spatial Crop Structure in Agricultural Systems. Figure 4
Yield density response in maize crops sown at 52, 104, 157 cm between rows. Optimum density (Do) at each planting arrangement is indicated by solid vertical rows. Optimum density (Do) decreases 214 plants/ha for every centimeter of increase in the distance between rows above 52 cm. Nonsolid vertical *arrows* indicate the magnitude of resource reduction found between extreme plant arrangements due to plant rectangularity increment

arrangement and management, and environmental conditions that may modify them. In this case, the effect of environmental conditions on the performance of the yield-density model parameters also needs to be described.

Crop Structure Responses in Polycultures

Crops, in developed countries, are normally grown in single-species stands or monocultures. As was discussed above, in such crop structures, there is little or no interspecific competition, but usually intense intraspecific competition. However, in the tropics and at present in some temperate areas, mixed crops are very common [7]. The concept of mixed crop or polycultures refers to the practice of growing more than one specie or genotype in the same land at the same time [6]. In such polycultures, competition occurs between plants of different species or genotypes, as well as between plants of a single genotype. In this general concept, the space and time component of the mixture allows the recognition of a whole gradient of intensity of interactions among component crops. For example, crops may be sown in sequence, i.e., a second crop is sown in the growing period immediately after a first crop is harvested, or partially or totally overlapped (i.e., two or more crops are sown and grown simultaneously in the same field). Similarly, crops may be sown in strips or in alternate rows. As the overlap of time or space between individuals of the various crop species increases, the opportunity to capture positive interactions among crop components also increases if they complement or facilitate the use of environmental resources. By this means, mixed crops or polycultures have showed some evidence of greater yield, greater stability of yield, and lower pest and disease damage than monocultures [1, 2]. Particularly, mixes of legumes and non-legumes species have shown greater yield when sown together than when they are sown alone [74].

Mixed crops are far less common in temperate areas under mechanized agricultural systems than in tropical subsistence farming [37]. However, recent biotechnological advances which helped to improve weed control with the use of previously nonselective herbicides in crops such as soybean, maize, and sunflower has opened new alternatives for the development of polycultures in extensive grain crops. For example, this increasing

interest in exploring the performance of mixtures of cultivars and species in modern, mechanized grain-cropping systems has been slowly expanding in some regions of the Pampas. In the Argentinean Pampas, one of the most productive areas of the world, mixed grain crops may be found in various patterns depending on the component crops species, the degree of temporal overlap among species, and on the spatial arrangement and relative density of the plants [62, 85].

Within the concept of polycultures, successive crops is defined when two or more species are grown in the same land in the same cropping or calendar year but without any overlapping; i.e., a second crop is sown immediately after harvesting the previous crop. In this plant arrangement, the use of resources is intensified per unit of time when compared to a single crop per year, but the interaction between crop species is reduced to the interference produced by the use of resources by one species on the resources available to the following crop [6]. As mentioned before, in the Pampas, nearly three million hectares are sown every year in a successive crops planting pattern. Wheat/double-cropped soybean, maize, or sorghum, and Barley/double-cropped soybean are examples of such extensive crops planting arrangements. In such systems, wheat crop structure (variety, density, distance between rows, and sowing dates) remains unaffected, while the soybean structure is usually modified by reducing the distance between rows and by increasing the plant density [11, 19, 67]. Wheat/double-cropped soybean, the more extensively used successive multiple-crop pattern, has proved to be more productive in terms of total yield and income per hectare and less variable than either sole wheat or sole soybean crops in the same unit of land.

As previously mentioned, the term intercrop is applied when two or more species or genotypes are grown simultaneously during part or the whole crop growing period in the same land. Intercrops are also used in the Pampas in a wheat/soybean mixture when the soybean growing period left by the wheat is short enough to affect its productivity, i.e., the yield of the second crop. In the case of such intercrops, the wheat (winter crop) and the soybean (summer crop) crop structures are both modified. The soybean is sown in spaces previously unsown with wheat, usually after wheat flowering and well before it is harvested. In wheat/soybean intercrop, wheat crop is sown in rows widely apart (for example, at 52 cm between rows) or in a variable spatial pattern, some rows are left unsown, and others follow the usual planting pattern for the region (i.e., 17.5 or 19 cm between rows; see Pictures 1 and 2). In intercrops, the soybean varieties are frequently sown at 52, 38, 35, or 19 cm between rows. Therefore, one row of wheat remains unsown in every three rows when at 17 cm between rows (i.e., 66% wheat, 100% soybean), or 1 every other row is sown when the wheat is at 19 cm between rows (i.e., 50% wheat, 100% soybean). Since the wheat plants tend to compensate with a higher vegetative and reproductive growth in the absence of crop plants, wheat yield is reduced by only 20–25% when compared to 100% monocultures (e.g., [24]). Successive and partially overlapped winter–summer intercrops increase the radiation use efficiency [86] and the weed and soil erosion control. However, it increases management complexities and requires well-trained people to conduct mechanized operations.

More recently, summer–summer crop species intercrops have been also evaluated in mechanized temperate agriculture. Intercrops of soybean in maize crops or soybean in sunflower crops have been evaluated at commercial fields in various regions of the Pampas in Argentina [18, 19]. The release of transgenic glyphosate-resistant soybean and maize has been crucial to explore this planting arrangement at commercial fields. Similarly, biotechnology and the release of imidazolinone resistant sunflower have been crucial to explore sunflower–soybean intercrops. A less complex intercrop may be obtained when each crop species is sown in wide strips in the field. Some yield and pest control advantages have been reported in maize–soybean strip patterns, although they are dependent on the width of the strip and its orientation, among other factors [48]. It is well documented that yield advantages (greater grain yield and yield stability) were often found when, particularly, grass and legume crops were mixed [74] mainly due to the complementary use of belowground resources. In such cases, it has been proposed that legume plants may use both soil and atmospheric nitrogen, while grass species may only use soil nitrogen; in such circumstances, both species complementary use nitrogen. However, it has also been proposed that in some cases, atmospheric nitrogen

Spatial Crop Structure in Agricultural Systems. Picture 1
Interplanting soybean in a wheat crop

Spatial Crop Structure in Agricultural Systems. Picture 2
Harvesting wheat in a wheat/soybean intercrop. See soybean plants growing in the harvested crop

previously fixed by the legume species may be transferred to the belowground environment of the grass species. In such cases, growth of grasses in the presence of legumes tends to be greater than when they are alone. In this case, facilitation tends to be the dominant process. It has been also found that the incidence of pest and diseases have sometimes been reduced when species or genotypes are grown in mixtures [12, 27, 77]. Such advantages may support the interest and promote the increase of area sown with more complex crop structures.

Future Directions

Attaining high yields per unit area requires that individual crop plants may usually experiences a competitive stress. Therefore, highly productive crops depend on crop structure by controlling the amount of resources used and their use efficiency which will determine the extent of competition experienced by the crop plants. In this respect, the discussion in this entry has deliberately concentrated on the effect of density and plant arrangement on crop growth and yield determination, and it has avoided any discussion on the effect of crop structure on the mortality of plants since this hardly occurs, at least as a density dependant process, in most crop production conditions. Interpreting the response of crops to crop structure from an ecophysiological and ecological approach was aimed to help construct a framework in which the temporal and spatial aspects of crop structure may be understood. Plant functional responses, determining individual species' requirements and critical periods for yield determination, and competitive interactions are responsible of the effects of density and plant arrangement on yield under various environments and crop management situations. The conceptual framework built may provide a rationale for predicting and manipulating crop structure under various conditions since modifying crop structure (density, plant arrangement, genotype) may be seen as a way to change crop spatial and temporal structure and, by this means, the use of crop resources. Although high densities for any particular environment should provide a greater use of early available environmental resources, yield-density responses still rely strongly on the way resources are used during the critical periods of yield components determination of the various crops. Taking this into account, density or plant arrangement manipulation should be effective only when it assures that the crop must be able to achieve high plant growth rates during those periods. Moreover, since genetic improvement has successfully widened the adaptation of crop species and genotypes to various production conditions, genotype specific interactions and characteristics need to be individually considered within the framework presented here to develop effective crop structures.

Although interspecific plant interactions, such as crop–weed interactions, are frequent in extensive grain crops. Polycultures increase the complexity of crop structures by adding the influence of crop plant–crop plant interactions and new crop management complexities to the result of the cropping system. Analysis of the "ecological combining ability" of crop species and genotypes in modern agriculture opens a whole new world to explore more productive and sustainable cropping practices, partially resembling the complexity and functioning of natural systems. The evaluation of the yield advantages of mixtures or polycultures compare to pure-stand yields or monocultures is also crucial. It has for long been based upon the absolute or relative yield differences between mixtures and polycultures [34, 44], but other criteria including resource use efficiency and environment health will surely need to be considered. In any case, the design and management of crop structure remains one, perhaps the most, important decision that farmers and agronomists face every time they plan to grow a grain crop.

Bibliography

Primary Literature

1. Alexander HL, Roelfs AP, Cobbs G (1986) Effects of disease and plant competition on yield in monocultures and mixtures of two wheat cultivars. Plant Pathol 35:457–465
2. Allard RW, Adams J (1969) Population studies in predominantly self-pollinating species. XIII Intergenotypic competition and population structure in barley and wheat. The American Naturalist 103:621–645
3. Andrade F, Cirilo A, Uhart S, Otegui M (1996) Ecofisiología del cultivo de maíz. Editorial La Barrosa & Dekalb Press, Argentina
4. Andrade FH, Calviño P, Cirilo A, Barbieri P (2002) Yield responses to narrow rows depends on increased radiation interception. Agron J 94:975–980
5. Andrade HF, Otegui ME, Vega C (2000) Intercepted radiation at flowering and kernel number in maize. Agron J 92:92–97

6. Andrews DJ, Kassam AH (1976) Importance of multiple cropping in increasing world food supplies. In: Sánchez A, Triplett GB (eds) Multiple cropping. American Society of Agronomy (ASA) Spec. Pub. 27, Madison, pp 1–10
7. Francis CA (1986) Multiple cropping: practices and potentials. Macmillan, New York
8. Arias S, Satorre EH (1991) Competencia entre trigo (*Triticum aestivum*) y Brassica sp. El efecto de la densidad del cultivo y la maleza sobre el rendimiento de grano. Actas de la XII Reunión Argentina sobre la maleza y su control (Mar del Plata, Argentina) 2:5–10
9. Arias S, Satorre EH, Guglielmini A (1994) Competencia entre trigo (*Triticum aestivum* L.) y Brassica sp. El efecto de la densidad del cultivo y la maleza en dos niveles de fertilización nitrogenada. Actas III Congreso Nacional de Trigo (Bahía Blanca, Argentina) 1:193–194
10. Auld BA, Kemp DR, Medd RW (1983) The influence of spatial arrangement on grain yield of wheat. Aust J Agric Res 34:99–108
11. Baigorrí HE (2008) Elección de variedades. In: Satorre EH (ed) Producción de Soja. AACREA Unidad de Comunicación y Marketing, Buenos Aires, Argentina, pp 33–42
12. Baker RJ, Briggs KG (1982) Effects of plant density on the performance of 10 barley cultivars. Crop Sci 22:1164–1167
13. Barnes C, Bugbee B (1991) Morphological responses of wheat to changes in phytocrome photoequilibrium. Plant Physiol 97:359–365
14. Bedmar F, Eyherabide JJ, Satorre EH (2000) Bases para el manejo de malezas. In: Andrade y FH, Sadras VO (eds) Bases para el manejo del maíz, el girasol y la soja. Editorial Médica Panamericana SA, Argentina. ISBN 987-521-016-1, pp 269–307; 443 pp
15. Bleasdale JKA (1966) Plant growth and crop yield. Ann Appl Biol 57:173–182
16. van den Boogaard R, Veneklass EJ, Peacock JM, Lambers H (1996) Yield and water use of wheat (*Triticum aestivum*) in a mediterranean environment: cultivar differences and sowing density effects. Plant Soil 181(2):251–262
17. Bullock D, Nielsen R, Nyquist W (1988) A growth analysis of corn grown in conventional and equidistant plant spacing. Crop Sci 28:254–258
18. Calviño P (2007) Intensificación de Sistemas de producción de granos y el manejo del riesgo en agricultura. In: Actas del Congreso Mundo Soja 2007. SEMA, Buenos Aires, Argentina, pp 75–76
19. Calviño P (2008) El cultivo de Soja de Segunda. In: Satorre EH (ed) Producción de Soja. AACREA, Unidad de Comunicación y Marketing, Buenos Aires, Argentina, pp 33–42, pp 93–98
20. Cirilo A (2002) Respuesta a la densidad y distribución de plantas del cultivo de Maíz. In: Satorre EH (ed) Maíz. Cuaderno de Actualización técnica Nº 65, AACREA, Buenos Aires, Argentina, pp 62–71
21. Darwinkel A (1978) Patterns of tillering and grain production of winter wheat at a wide range of plant densities. Netherland J Agric Sci 26:383–398
22. Donald CM (1963) Competition among crop and pasture plants. Adv Agron 15:1–118
23. Donald CM, Hamblin J (1976) The biological yield and harvest index of cereals as agronomic and plant breeding criteria. Adv Agron 28:361–405
24. Dodds P, Slafer GA, Satorre EH (1990) Sistemas de producción de trigo y soja en el norte de Buenos Aires. Evaluación del cultivo de Trigo. Actas II Congreso Nacional de Trigo, vol 1. Pergamino, Argentina, pp 198–208
25. Farazdaghi H, Harris PM (1968) Plant competition and crop yield. Nature 217:289–290
26. Fawcett RG (1964) Effect of certain conditions on yield of crop plants. Nature 204:858–860
27. Federer WT, Conningale JC, Rutger JN, Wijesinha A (1982) Statistical analysis of yields from uniblends and biblends of eight dry bean cultivars. Crop Sci 22:111–115
28. Felton WL (1976) The influence of row spacing and plant population on the effect of weed competition in soybeans (*Glycine max*). Aust J Exp Agric Anim Husb 16:926–931
29. Fischer RA (1984) Wheat. In: Smith WH, Barta SJ (eds) Symposium on potential productivity of field crops under different environments. Philippines, IRRI, Los Baños, pp 129–153
30. Fischer RA, Miles RE (1973) The role of spatial pattern in the competition between crop plants and weeds. A theoretical analysis. Math Biosci 18:335–350
31. Flemming GF, Young FL, Ogg AC (1988) Competitive relationships among winter wheat (*Triticum aestivum*), jointed goatgrass (*Aegilop scylindrica*), and downy brome (*Bromus tectorum*). Weed Sci 36:479–486
32. Gallez LM, Mockel FE, Cantamutto MA, Gullace GD, Vallati AR (1986) Densidad de siembra y separación entre hileras: Su influencia sobre el rendimiento de trigo en la región pampeana semiárida. Actas I Congreso Nacional de Trigo (Pergamino, Argentina) 3:167–177
33. Goodall DW (1960) Quantitative effects of intraspecific competition: an experiment with marigolds. Bull Res Council Israel 8:181–194
34. Harper JL (1977) Population biology of plants. Academic, London
35. Holliday R (1960) Plant population and crop yield. Field Crop Abstr 13(3):159–167
36. Holliday R (1963) The effect of row width on the yield of cereals. Field Crop Abstr 16:71–81
37. Horwith B (1985) A role for intercropping in modern agriculture. Bioscience 35(5):286
38. Kantolic A (2008) Fecha y densidad de siembra en soja. In: Satorre E (ed) Producción de Soja. AACREA, Unidad de Comunicación y Marketing, Buenos Aires, pp 23–32
39. Kira T, Ogawa H, Shinozaki K (1953) Intraespecific competition among higher plants. 1- Competition-density-yield interrelationships in regularly dispersed populations. J Inst Polytech Osaka City Univ 4:1–16
40. Lafond GP, Domitruk D, Bailey KL, Derksen D (1996) Effects of row spacing, seeding rate and seed-placed phosphorus on

wheat and barley in the canadian prairies. Better Crop Plant Food 80(4):20–22
41. Loomis RS, Connor DJ (1992) Crop ecology: productivity and management in agricultural systems. Cambridge University Press, Cambridge
42. Maddonni GA, Cirilo AG, Otegui ME (2006) Row width and maize grain yield. Agron J 98:1532–1543
43. Martin MPLD, Field RJ (1987) Competition between vegetative plants of wild oat (*A. fatua*) and wheat (*Triticum aestivum* L.). Weed Res 27:119–124
44. McGilchrist CA, Trenbath BR (1971) A revised analysis of plant competition experiments. Biometrics 27:659–671
45. McLeod JG, Campbell CA, Gan Y, Dyck FB, Vera CL (1996) Seeding depth, rate and row spacing for winter wheat grown on stubble and chemical fallow in the semiarid prairies. Can J Plant Sci 76(2):207–214
46. Mead R (1979) Competition experiments. Biometrics 35:41–54
47. Mitchell R (1970) Crop growth and culture. Iowa State University Press, Ames, p 349
48. Monzon JP, Carrozza TJ, Calviño P, Andrade FH (2005) Efectos del Intercultivo en franjas de maíz y soja sobre el rendimiento- In: Actas del VIII Congreso Nacional de Maíz. AIANBA, Rosario, Argentina, pp 64–66
49. Nafziger E (1998) Corn. Chapter 2. In: Agronomy handbook, Illinois. 1997–1998. University of Illinois at Urbana-Champaign. College of Agricultural, Consumer and Environmental Sciences Department of crop Sciences. Cooperative Extension Service. Circular 1344, pp 16–26
50. Nerson H (1980) Effects of population density and number of ears on wheat yield and its components. Field Crop Res 3:225–234
51. Otegui ME, Ruiz R (1993) Performance of six maize hybrids sown at three population densities in Argentina. Test of agrochemicals and cultivars. Ann Appl Biol 122(Suppl): 172–173
52. Panda SC, Pattanaik A, Rath BS, Tripathy RK, Behera B (1996) Response of wheat (*Triticum aestivum*) to crop geometry and weed management. Indian J Agron 41(4):553–557
53. Panda SC, Pattanaik A, Rath BS, Tripathy RK, Behera BP, Pant MM (1979) Dependence of plant yield on density and planting pattern. Ann Bot 44:513–516
54. Panwar RS, Malik RK, Balyan RS, Singh DP (1995) Effect of isoproturon, sowing method and seed rate on weeds and yield of wheat (*Triticum aestivum*). Indian J Agric Sci 65(2):109–111
55. Peltonen J, Peltonen-Sainio P (1997) Breaking uniculm growth habit of spring cereals at high latitudes by crop management. II Tillering, grain yield and yield components. J Agron Crop Sci 178(2):87–95
56. Puckridge DW, Donald CM (1967) Competition among wheat plants sown at a wide range of densities. Aust J Agric Res 18:193–211
57. Ratkowsky DA (1983) Nonlinear regression modelling. A unified practical approach. Marcel Dekker, New York and Basel
58. Ross MA, Harper JL (1972) Occupation of biological space during seedling establishment. J Ecol 60:77–88
59. Salazar GM, Moreno RO, Salazar GR, Carrillo ML (1996) Wheat production as affected by seeding rate x fertilization interaction. Cereal Res Commun 24(2):231–237
60. Sánchez RA, Casal JJ, Ballaré CL, Scopel AL (1993) Plant responses to canopy density mediated by photomorphogenic processes. In: Buxton DR, Shibles R, Forsberg RA, Blad BL, Asay KH, Paulsen GM, Wilson RF (eds) International crop science I. Crop Science Society of America, Madison, pp 779–786
61. Sarandon SJ, Chidichimo HO (1985) Efecto de la densidad de siembra sobre la acumulación y redistribución del nitrógeno en tres cultivares de T. aestivum L. Revista de la Facultad de Agronomía (La Plata) 61–62:105–122
62. Sarandon SJ, Chamorro AM (2003) Policultivos en los sistemas de producción de granos. In: Satorre EH, Arnold B, Slafer GA, de La Fuente EB, Miralles DJ, Otegui ME, Savin R (eds) Producción de Granos. Bases funcionales para su manejo. Ed. Facultad de Agronomía, University of Buenos Aires, Argentina, pp 353–376
63. Satorre EH (1988) The competitive ability of spring cereals. Ph.D. thesis, University of Reading, Reading
64. Satorre EH (2008) Estructura especial: respuesta del cultivo a la densidad, distancia entre hileras y uniformidad. In: Satorre EH (ed) Producción de Maíz. AACREA, Unidad de Comunicación y Marketing, Buenos Aires, pp 25–32
65. Satorre EH, Arias SP (1990) Competencia entre trigo (*Triticum aestivum*) y malezas. III El efecto de la densidad del cultivo y la maleza. Actas II Congreso Nacional de Trigo. Pergamino, Argentina, vol 4, pp 1–10
66. Satorre EH, Ghersa CM (1987) Relationship between canopy structure and weed biomass. Field Crop Res 17:37–43
67. Satorre EH, Slafer GA (1999) Wheat production systems of the Pampas. In: Satorre EH, Slafer GA (eds) Wheat. Ecology and physiology of yield determination. Food products Press, New York, pp 333–350
68. Savin R, Slafer GA (1991) Shading effects on yield of an Argentinian wheat cultivar. J Agric Sci Camb 116:1–7
69. Shinozaki K, Kira T (1956) Intraspecific competition among higher plants VII logistic theory of the C-D effect. J Inst Polytech Osaka City Univ 7:35–72
70. Snaydon RW (1984) Plant demography in an agricultural context. In: Dirzo R, Sarukhan J (eds) Perspectives on plant population biology. Sinauer Associates, Sunderland, pp 389–408
71. Snaydon RW, Satorre EH (1989) Modifications and interpretations of bivariate diagrams for plant competition data. J Appl Ecol 26:1043–1057
72. Solie JB, Solomon SG Jr, Self KP, Peeper TF, Koscelny JA (1991) Reduced row spacing for improved wheat yields in weed-free and weed-infested fields. Trans ASAE 34(4):1654–1660
73. Thakur SS, Pandey IB, Singh SJ, Mishra SS (1996) Effect of seed rate and row spacing on late sown wheat in alluvial calcareous soil. J Res Birsa Agric Univ 8(2):123–125

74. Trenbath BR (1974) Biomass productivity of mixtures. Adv Agron 26:177–210
75. Trenbath BR (1976) Plant interactions in mixed crop communities. In: Papendick RI, Sánchez PA, Triplett GB (eds) Multiple cropping. ASA special pub. N° 27 (ASA), pp 129–170
76. Valentinuz O (1996) Crecimiento y rendimiento comparados de girasol, maíz y soja ante cambios en la densidad de plantas. M.Sc. thesis, Fac. de Ciencias Agrarias, Universidad de Mar del Plata, Argentina, p 45
77. Vandermeer J (1992) The ecology of intercropping. Cambridge University Press, Cambridge
78. Van Ittersum MK, Rabbinge R (1997) Concepts in production ecology for analysis and quantification of agricultural input-output combinations. Field Crop Res 52:197–208
79. Vega CRC, Andrade FH, Sadras VO, Uhart SA, Valentinuz OR (2001) Seed number as a function of growth. A comparative study in soybean, sunflower, and maize. Crop Sci 41:748–754
80. Verona CA, Loffer CM, Fernández ON (1980) Efecto de la densidad de plantas sobre el rendimiento y la distribución de N en T. durum Def. Revista de Investigaciones Agropecuarias. INTA 15(1):75–95
81. Lin WZ, Min FJ, Rong HM, Ping YY, Min CH (1997) Planting density effects on assimilation and partitioning of photosynthates during grain filling in the late sown wheat. Photosynthetica 33(2):199–204
82. Willey RW, Heath SB (1969) The quantitative relationships between plant population and crop yield. Adv Agron 21:281–321
83. Yunusa IAM (1989) Effects of planting density and plant arrangement pattern on growth and yields of maize (*Zea mays* L.) and soya bean (Glycine max L. Merr.) grown in mixtures. J Agric Sci Cambridge 112:1–8
84. Kemp DR, Auld BA, Medd RW (1983) Does optimizing plant arrangement reduce interference or improve the utilization of space? Agric Syst 12:31–36
85. Satorre EH (1998) Aumentar los rendimientos en forma sustentable en la Pampa Argentina: Aspectos Generales. In: Solbrig, Vainesman (eds) Hacia una agricultura productiva y sostenible en la Pampa. Orientación Gráfica Editora, Buenos Aires, Argentina, pp 72–98
86. Ghersa CM, Martinez-Ghersa MA (1989) Cambios ecológicos en los agroecosistemas de la pampa ondulada. Efectos de la introducción de la soja. Ecología 2:182–188

Sustainable Ecological Aquaculture

Stephen F. Cross
Coastal Aquaculture Research & Training (CART) Network, Department of Geography, University of Victoria, Victoria, BC, Canada
SEA-Vision Group of Companies, Courtenay, BC, Canada

Article Outline

Glossary

Alternative energy Is a generic term that refers to any source of usable energy intended to replace fuel sources without the undesired consequences of the replaced fuels. It is typically used to describe renewable forms of energy that can be used in place of fossil fuels and thereby reducing, mitigating, or eliminating the negative environmental consequences associated with the traditionally used fuels.

Aquaculture Is the farming of aquatic organisms and represents a controlled approach to the production of marine or freshwater species – differentiating it from fishing, which is the harvest of wild organisms.

Integrated multi-trophic aquaculture (IMTA) An ecologically designed recycling process in which the organic wastes generated from one species become inputs (food or nutrients) for another. A fed aquaculture species, such as fish or shrimp, are combined with inorganic extractive (e.g., seaweed) and organic extractive (e.g., shellfish) aquaculture to create an ecologically "balanced" food production system.

Ocean ranching A seafood production approach that combines aquaculture techniques with traditional fishery methods. Juveniles are produced in hatchery and controlled rearing facilities, and then released to the ocean environment where they grow under natural (no containment) conditions to harvest size and then captured (fished) using standard industry methods (e.g., trolling, seining, divers, etc.).

Polyculture An agriculture or aquaculture approach that uses multiple crops (or species) in the same space, typically in an effort to avoid the production issues (e.g., disease susceptibility) associated with single-species, or monoculture production. Differentiated from IMTA, which selects species based upon ecosystem function (trophic relationships), polyculture simply supports species diversification within the production model.

Sustainability Meeting the environmental, social, and economic needs of the present generation without compromising the ability of future generations to meet their needs.

Definition of the Subject and Its Importance

An increasing global demand for seafood has resulted in considerable and increasing pressure on world fisheries, with a number having already collapsed and others in serious decline [1]. Satisfying the growing seafood demand has seen aquaculture production increase significantly over the past few decades, with the current contribution of aquaculture to the global seafood supply equal to or exceeding that of the wild fisheries. However, commensurate with this controlled, although often intensive, approach to seafood production are a number of environmental consequences that have called into question, as with the pressured growth in the wild fisheries, the ultimate sustainability of these approaches.

Sustainable ecological aquaculture (SEA) systems endeavor to address the socioeconomic and environmental facets of sustainability by integrating ecological and engineering system design with comprehensive best management practices. Operational efficiency is considered a keystone element in such designs, and one which will ultimately demonstrate that an industry focus on sustainability can also be profitable – a critical argument in encouraging innovation and facilitating long-term change within the corporate environment of global food production.

Introduction

The United Nations Food and Agriculture Organization (FAO) defines sustainability as "*Meeting the needs of the present without compromising the ability of future generations to meet their needs*" [2]. This definition

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

requires that mankind keeps population densities below the carrying capacity of a region, facilitates the *renewal* of renewable resources, conserves and establishes priorities for the use of nonrenewable resources, and keeps environmental impact below the level required to allow affected systems to recover and continue to evolve.

In a simple, yet technical-based definition, sustainability – in reference to natural resource management – has also been described as the "*long-term maintenance of ecosystem components and functions for future generations*" [3]. Linking economic, social, and environmental aspects of human society this concept is intended to configure civilization and human activities so that society, its members and its economies are able to meet their needs and express their greatest potential in the present, while preserving biodiversity and ecosystem integrity, and planning and acting for the ability to maintain these ideals indefinitely [4].

Although sustainability values may vary greatly among cultures, there is a fundamental commonality among these details that is best described as a "*parallel care and respect for the ecosystem and for the people within*." From these common values emerges the goal of sustainability – to concurrently achieve human and ecosystem well-being. It thus follows that the measure of sustainability for any activity (including aquaculture) be the achievement of, or the contribution to, human and ecosystem well-being together. In this way, the concept of sustainability can be considered a positive concept that has as much to do with achieving well-being for people and ecosystems as it has to do strictly (as is often the focus) with environmental protection.

In general, society has been moving steadily toward sustainable industrial activities for a number of decades, recovering "waste" in an effort to recycle and reuse such materials [5]. Industry and government have both recognized that these waste streams are in fact a valuable resource, and society now expects the use of recoverable materials and the integration of natural design into most product development. These ideals are further promulgated through initiatives such as eco-labeling, eco-certification, eco-design, etc. – all of which are becoming more prevalent in the seafood production industry [6].

Consumers now use their purchasing power to ensure that the products they acquire meet these ever-changing social criteria. A growing awareness of sustainability issues has placed pressure on business (industry) to shift or to modify their production processes to accommodate the eco-ethical consumer needs [7]. This has become increasingly critical to both the wild fisheries and the aquaculture industry sectors as demand for a high quality, and sustainable supply of seafood continues to rise.

While it is indisputable that the achievement of sustainability for coastal aquaculture – embracing social, economic and environmental objectives – is necessary, the motivation for fundamental change in current industry production approaches, where required, has not yet been clearly established. The development of sustainable ecological aquaculture (SEA) systems represents a movement toward this goal, and commercialization efforts are now in place to provide working models and the basis for future innovations [8]. The following sections provide an example of SEA-System development in coastal British Columbia, a region renowned for the social conflicts associated with open netcage (finfish) aquaculture. The environmental issues associated with this approach to finfish aquaculture are summarized and then used to exemplify how a sustainable ecological aquaculture (SEA) system approach can be developed to resolve these issues, and further to illustrate how a comprehensive approach to SEA system design and operation can satisfy the environmental, economic, and social aspects of aquatic food production sustainability.

Sustainability Issues in Coastal Aquaculture Production

The development of coastal aquaculture, and the subsequent increase in its global role for seafood production, has not been without its environmental and socioeconomic risks and negative impacts [9]. The rapid development in Asian shrimp production through pond culture, for example, stimulated significant socioeconomic activity in traditionally poor, rural coastal communities – resulting, as envisioned, in increased livelihood opportunities in the wake of declining subsistence fisheries. Although successful in

addressing a number of social issues associated with the coastal fisheries situation, the inherent value in shrimp production and the local and regional wealth it generated facilitated the tendency to further increase production, despite exceeding environmental sustainability criteria, to support further wealth creation, with a predictable result – catastrophic production collapses with serious socioeconomic and environmental consequences [10]. The three pillars of sustainability – environmental, social, and economic – are truly in balance, and ignoring one can have serious consequences to this integral relationship.

In the global salmon aquaculture industry, with farm production originating in Norway, Chile, United Kingdom, Canada, United States, Australia, and New Zealand, a standardized production approach, including infrastructure configuration and general management practices, has resulted in a common suite of sustainability issues associated with the industry. The net-cage aquaculture operational procedures that will ultimately influence whether this approach is considered environmentally sustainable will also form the basis upon which social and economic criteria for sustainability are determined. As with many new coastal activities, social "acceptance" of open net-cage aquaculture has, in many jurisdictions, waned as inherent environmental issues have been identified during the early stages of its development. Issues that currently jeopardize the sustainability of this form of coastal aquaculture include the following.

Species selection is typically seen as a serious risk when exotic (non-indigenous) species are used [11]. Should escapes occur, the potential for disruption of ecosystem structure through displacement of indigenous species, or the potential for interbreeding that could weaken the natural, resident populations are associated with use of such species – despite their selection and obvious benefits as farm stock (disease resistance, faster growth rates, etc.). Farming of high densities of animals in proximity to wild populations of similar phylogeny may also pose threats of disease and/or parasite transfer both to and from the farm facility.

Stock escapes have occurred both incidentally, as a result of human error during the husbandry process, or catastrophically due to containment system failure during extreme weather episodes, and marine mammal interactions causing severe subsurface net damage. The risks associated with stock escapes include interactions with wild populations – genetic dilution – as well as those associated with the escapes of farm stock comprised of non-indigenous species (referenced above) [12, 13].

Chemotherapeutic compounds represent a wide range of complex chemical and antimicrobial compounds that have been developed to manage farm stock disease and parasite issues. Their use is considered a site management activity, and is recognized as a stressor due to the periodic release of the respective compounds (and residues) into the environment [14]. The environmental effects of this stressor could manifest itself in a number of ways – changes to contaminant concentrations (partitioned in various ways within the ecosystem), and through a suite of potential effects determined by the bioavailability of these residues to nontarget organisms within the ecosystem.

Chemicals used in net-cage aquaculture comprise a variety of compounds and include the micronutrients of fish feed (trace metals), disinfectants, anaesthetics, and antifoulants. All are released to the marine environment, albeit typically in very low concentrations and at differing release rates. The fate and effects of these residues within the environment, like that of the chemotherapeutic compounds, pose a risk to a variety of ecosystem components through environmental partitioning, accumulation and persistence, and the bioavailability and effects to nontarget species [15].

Organic fish wastes are a natural product comprised of fecal material and incidental wasted feed. These materials are released from the fish and enter the environment where they can have localized impacts to benthic habitat, changing biophysical conditions and displacing or disrupting species composition of these communities [16, 17]. While localized in nature, these impacts are of considerable concern when considered with respect to aquaculture expansion and the potential for broader, cumulative effects.

Inorganic fish wastes, comprise of dissolved nutrients (e.g., nitrogen, nitrate/nitrites, ammonium, phosphorus) are excreted as a result of fish metabolism and primarily released to the environment through the urine [18–20]. While localized effects of nutrient release are often associated with enrichment effects, nutrient loading from large or multi-farm operations have been implicated in potential eutrophication

processes, with speculation that harmful algal blooms can be generated and sustained as a result of the cumulative nutrient enrichment (far-field) effects from such aquaculture facilities.

Energy consumption, and particularly that component generated from hydrocarbon-based (fossil) fuels, is currently a globally recognized sustainability issue – regardless of the associated activity [21]. Salmon, and other temperate finish aquaculture, currently operate in remote coastal areas and as a result, rely upon off-grid source of power for much of the required operational needs. Expansion of these industry sectors to satisfy increasing seafood supply will likely result in further use of such coastal areas and thereby increase the use and hence the broader scale effects associated with this use.

The significance of these open net-cage aquaculture issues, in terms of impact upon environmental and socioeconomic sustainability, varies among the farm operational regions and reflects local concerns, values, and hence how, where, and what priority research effort is focused. Many of these sustainability issues are addressed independently, through a variety of ongoing private sector and/or academic-based research initiatives, and usually in an effort to continually improve environmental and economic performance of the present production model [22].

While a continual improvement paradigm is a recognized and essential component to maintain sustainability, typically characterized with ongoing and relatively minor changes to operational procedures or infrastructure design (e.g., materials use), a more dramatic step in the development of sustainable aquaculture will be required in order to supply increasing demands for seafood. The evolution toward sustainable ecological aquaculture (SEA) systems considers the array of stressor-impact concerns associated within current production models with the objective of designing an approach that incorporates innovative infrastructure and proactive operational controls [23].

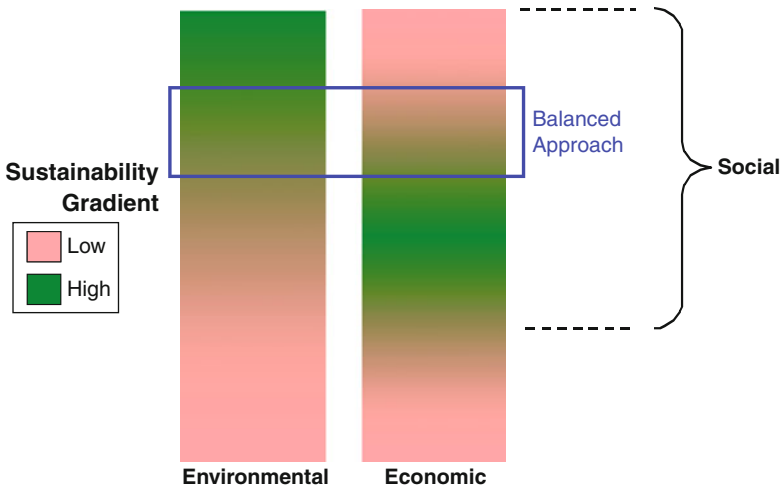
Design and Operational Framework for SEA Systems: Considerations for Socioeconomic Sustainability

A sustainable ecological aquaculture (SEA) system can be defined as *a holistic aquaculture approach that uses the principles of ecology to achieve the goal of sustainability*. It is essentially a blend of two developmental pathways for aquaculture systems, whether referring to a freshwater or to a marine environment scenario, i.e., sustainable aquaculture and ecological aquaculture. In terms of the *sustainable* aquaculture component, a sustainable ecological aquaculture system endeavors to satisfy the perpetual needs of society (e.g., ensuring food security, maintaining livelihoods) while protecting the environment – and do so in a manner that is efficient and hence economically viable [22].

Figure 1 illustrates the relationship among the three facets of sustainability. While the range of Social acceptance may vary considerably among communities, and/or given a specific form of aquaculture, the balance between environmental and economic sustainability is much more objective in nature with their concurrent evaluation typically resulting in a compromise that may in fact represent a narrow range of opportunity. If, for example, all of the criteria considered necessary to achieve environmental sustainability (top portion of the left gradient) is the goal, then the associated economic costs may increase and actually exceed the ability to accomplish the environmental objectives (illustrated with a pink portion at the top of the right gradient). Hence, to achieve economic sustainability, a sacrifice along the environmental gradient of sustainability may be required in the short-term. As system innovation addresses operational efficiencies and costs, then the balanced approach shown with the blue box (Fig. 1) will move toward the top of both gradients – improving environmental performance while remaining, and potentially increasing, social acceptance.

Environmental Sustainability: The Ecological Design of SEA Systems

The configuration and operational design of many current aquaculture systems are, or could with minor modifications, be considered environmentally sustainable. Minor ongoing operational improvements, typically introduced as efficiencies in husbandry practices, have and will continue to result in the reduction of environmental effects. For example, the development and introduction of effective vaccines has



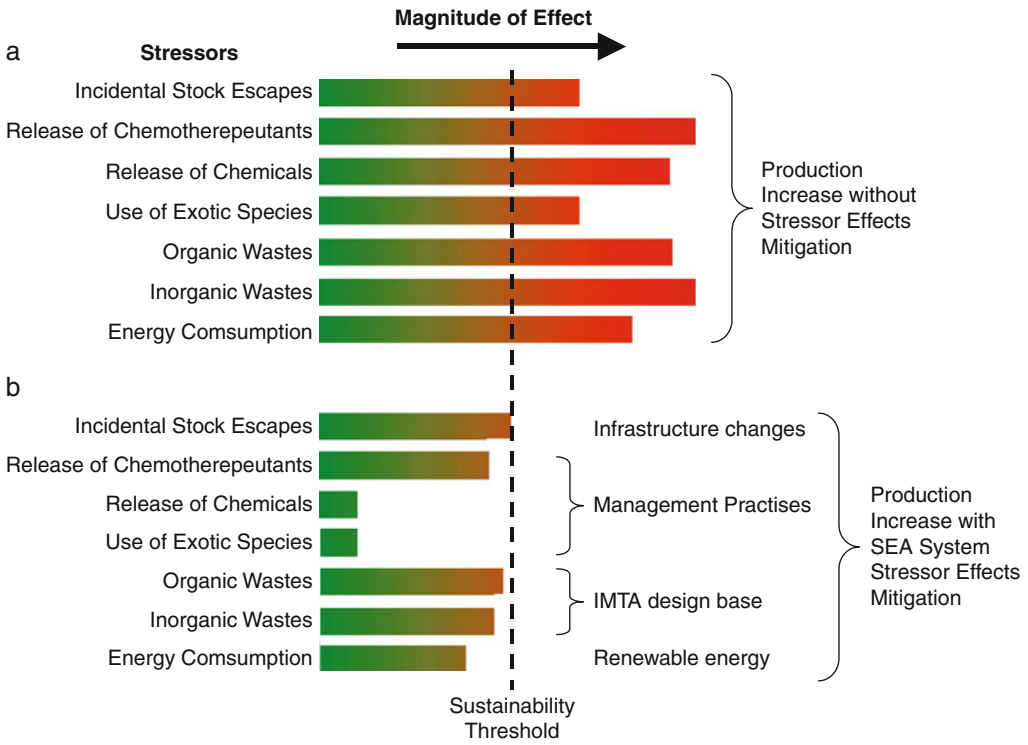
Sustainable Ecological Aquaculture. Figure 1
The inherent balance between environmental and economic sustainability gradients, based upon a comprehensive suite of sustainability factors/indicators, the associated need for compromise, and the relationship to social acceptance

dramatically reduced the use of antibiotics, the residues from which are released to the environment and presumably neutralized through natural assimilative processes. The engineering and introduction of new structural materials (e.g., netting), with greater breaking strengths and increased antifouling properties, prevent negative interactions with predators and reduce maintenance (net washing) costs while optimizing water quality and flows through the cages.

However, as the demand pressures on current seafood supply continues to rise, the scale of coastal production (individual farm configuration and/or regional allocation of farming units) will need to increase at a rate faster than minor adaptive changes to existing system design will be able to occur to remain environmentally sustainable. Figure 2 illustrates the hypothetical impacts of increased production on sustainability using seven environmental stressors – stock escapes (and use of exotic species), release of therapeutic and chemical residues, discharge of organic and inorganic wastes, and energy use.

Scenario I in Fig. 2 (upper portion) represents the projected effects of present farm operational models given a dramatic increase in production with a limited adaptive response to mitigate the associated environmental impacts. At present, the magnitude of independent environmental effects resulting from each of these seven stressor effects is likely to the left of the vertical dashed line, which represents the sustainability threshold for each stressor effect. As production is increased, through increased farm size and/or the number of farms within an operational area (density), the magnitude of stressor effects of any number of these (and other) operational factors will exceed the environmental, and eventually the social and economic sustainability thresholds.

Scenario II in Fig. 2 (lower portion) suggests how a sustainable ecological aquaculture approach, representing a significant shift in fundamental system design and operational considerations, would proactively mitigate (or eliminate) the impacts of the example stressor effects and thereby accommodate increased production needs. First – use of an *integrated multitrophic aquaculture (IMTA)* production model provides an inherent increase in system productivity (and product diversification), yet represents a design and change in *infrastructure configuration* that facilitates interception and use of the organic and inorganic wastes, generated and released from the fed (fish) component, by extractive species such as shellfish, echinoderms and seaweeds/kelps [24–27]. Second – revised *management practices* that comprise protocols committing to best (e.g., *organic)* practices/standards result in strict control over all operational



Sustainable Ecological Aquaculture. Figure 2
Anticipated sustainability impacts of increased farm/regional production based on two developmental scenarios: (**a**) magnitude of individual environmental stressor effects without the introduction of appropriate mitigation measures; and (**b**) potential mitigation of these stressor effects using a sustainable ecological aquaculture approach to production

inputs, including elimination of therapeutics and other process chemicals, restricted introduction/use of non-indigenous species, and sourcing of feeds. Third – integration of renewable energy into system operations provides a significant and ongoing reduction in the use of fossil fuels and hence the carbon foot-print of the operation.

Example: Commercialization of the SEA-System Approach

The principles of sustainable ecological aquaculture are currently being applied by the SEA-Vision Group of companies of western Canada (www.SEAvisiongroup.ca) in the development and commercialization of its own SEAfood System. This unique coastal aquaculture production system is based on the predominant open netcage approach used in finfish aquaculture but incorporates engineering and ecological design components that facilitate a scalable transition from single to multiple species production. In the development of this new system, the SEA-Vision Group has considered the social, environmental, and economic pillars of sustainability – resulting in an environmentally efficient, cost-effective production system that meets the scrutiny of both the local community and the consumer. A description of the key socioeconomic and environment design aspects of the SEAfood System are provided in the following sections.

Social Sustainability Considerations Social sustainability is based on *acceptance* and on *involvement*. Introduction and commercialization of the SEAfood System in coastal British Columbia considered three levels of social engagement – community design input, operational involvement, and satisfaction of consumer demand.

Community design input was considered essential in establishing the conceptual framework upon which the local coastal community would be willing to accept and support the introduction of a new commercial-scale production system/operation – one intended to address the ongoing controversies associated with coastal aquaculture in the region. In British Columbia, the aquaculture industry comprises two independent sectors – shellfish and finfish. Shellfish, which primarily encompasses self-feeding extractive species such as oysters, scallops, mussels, and intertidal clams, also includes (and refers to) other extractive invertebrates that have had their historical use as a harvest fishery and have recently garnered attention as potential culture candidates, for example, urchins, sea cucumbers, geoduck clams, and abalone. The finfish sector was developed with salmon (indigenous and introduced species) but has begun to diversify with the introduction of marine species such as sablefish and rock cod.

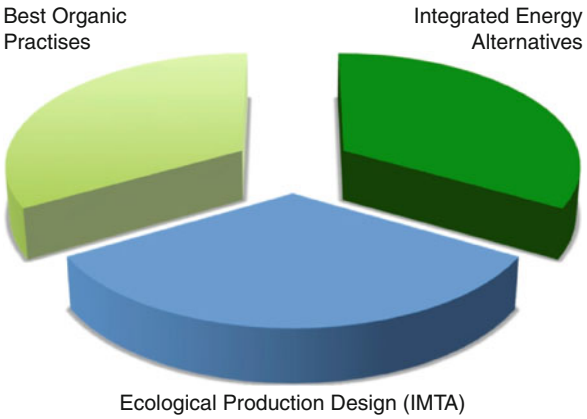
Sites best suited to aquaculture in British Columbia are generally located in remote coastal areas, substantially removed from urban centers and populations. These areas remain resource-based and under joint management control of Provincial/Federal governments and the local aboriginal (First Nation) peoples that maintain claim over their traditional coastal territories. In soliciting *community design input* for the new SEA-Vision Group production model, the integration of traditional ecological knowledge (TEK) of the residing coastal first nation community (the Ka:'yu:'k't'h'/Che:k:tles7et'h' First Nation = KCFN) was a critical social engagement step [28] in the commercialization process. Discussion of the SEAfood System conceptual design with the KCFN allowed input as to species selection that satisfied the ecological design criteria of the system yet met the needs and concerns of the local community. The KCFN conveyed their specific concerns regarding coastal aquaculture, identified a suite of indigenous species candidates for the system design, and provided valuable input into which of these species would be of most value to their people's tradition. A formal agreement as to species use, restrictions, and other operational criteria was prepared and has formed the basis for local social acceptance of the SEA-system approach in this case – one that also supports the social sustainability issues of the community.

Operational involvement has also been an important social sustainability factor for the SEAfood System development, providing and supporting direct and indirect coastal community livelihoods – for example, farm staff, contract services (divers, harvest vessels), investment. Integration of local workers into the business, including those from the KCFN, further supports the social acceptance of the approach, allowing ongoing input into system operational improvements. A continuing working partnership with the local community is critical to the success of current operations and the potential for future operational expansion and corporate growth.

Consumer demand for seafood sustainability was also an important consideration of the social engagement process. Many of the environmental issues associated with open netcage aquaculture have had a direct influence on consumer acceptance of the product (farmed salmon), and with an associated and growing demand for wholesome, sustainably produced seafood, the design and operational attributes of the SEAfood System deliberately focused on these consumer criteria to provide a supply of seafood that would satisfy these demands. By systematically addressing this range of environmental concerns, and by continually and immediately addressing new issues/concerns as they are realized, the sustainable ecological approach allows the consumer to be integrated into the continual improvement paradigm of the approach – and thereby further supporting the sustainability of the SEAfood System.

Environmental Sustainability Attributes In order to address, and facilitate improvement among all of the socio-environmental issues of open netcage aquaculture in the region, the SEA-Vision Group system focuses on three design components – its production model, energy needs, and operational practices (Fig. 3). This combination effectively encompasses ecological design (multispecies, IMTA approach) to reduce the waste/nutrient foot-print, integrated energy alternatives to reduce the carbon foot-print; and implementation of best, organic practices to mitigate the potential effects of antibiotics and chemical use.

Production Model The ecological production design of the SEAfood System is based on that of an Integrated



Sustainable Ecological Aquaculture. Figure 3
The SEAfood System™ addresses the majority of socio-environmental issues of open netcage aquaculture using an effective combination of ecological (multispecies, IMTA) system design, integrated energy alternatives, and a suite of best organic practices

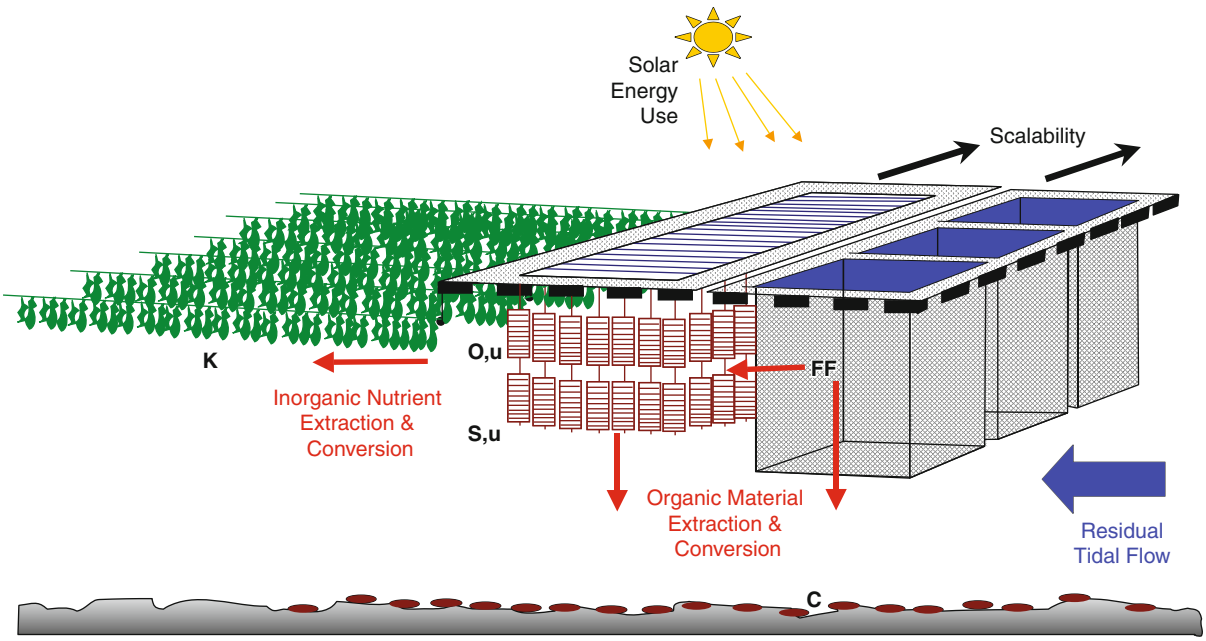
Multi-Trophic Aquaculture (IMTA) model [29]. This multispecies approach expands upon the monoculture model for fed (fish) production by integrating a number of extractive species to intercept and remove the organic and inorganic wastes generated by the fish. Two simple criteria are used in the selection of species for the SEAfood System: (a) each species must fill an ecological role in the removal of organic and/or inorganic wastes; and (b) each must have economic value – a marketable and profitable product [30, 31].

The SEAfood System model developed in British Columbia combines a single fed species – sablefish (*Anoplopoma fimbria*) or Chinook salmon (*Oncorhynchus tshawytscha*), with a variety of extractive species positioned deliberately to facilitate interception and removal of the organic and inorganic fish wastes that are released into the environment as particulates and dissolved nutrients. Downstream of the fish component is a shellfish (bivalve mollusk) system that is situated and configured to maximize the extraction of the fine particulate fraction of the organic waste streams [32]. Further downstream is a series of kelp/seaweed grids (*Saccharina lattisima* or *Porphyra sp.*) that are positioned to intercept and capitalize on the enriched dissolved nitrogenous wastes. Beneath the entire floating infrastructure of the farm, sea cucumbers (*Parastichopus californicus*) are grown in an ocean-ranching style – released as juveniles, these animals consume the rich settleable organic material that would otherwise accumulate over the seafloor.

Figure 4 illustrates the infrastructure configuration and spatial relationship of the SEAfood System species. The various extractive components of the SEAfood System are situated in such a manner so as to maximize receipt of the organic and inorganic wastes originating from the fish. SEAfood System sites – SEAfarms – are configured with the extractive species downstream and to one side of the fish component to allow for operational logistics around the cages (e.g., vessels used for net changes, harvesting, etc.). Site selection and subsequent system orientation thus requires a thorough physical oceanographic assessment to ensure that system component placement is in the direction of the residual tidal flow (downstream).

The total length and production capacity of a SEAfarm is scalable and is determined by the finfish production component. In the initial model each SEAfarm is 200 m in length and includes twelve 15-m square cages or six 30-m cages. This small operational foot-print for the fish production component (by current industry standards) is considered an additional environmental benefit to the SEAfood System design, allowing for better water flow, waste transfer, and overall assimilative efficiencies among the species components.

Infrastructure compatibility between the extractive components and that of the base finfish production system is considered key to SEAfood System production capacity and operational efficiencies. A new suspended shellfish aquaculture system was therefore designed and engineered to meet the unique needs of multispecies production. The system, shown in Fig. 5, uses similar construction materials to that of the finfish cage (e.g., galvanized steel, high-density polyethylene flotation billets), provides a production platform with a number of benefits: (a) strong connection points for securing to the finfish cage system, (b) a very high floatation capacity that supports considerable shellfish production – both in surface area and in depth, (c) a moving *SEA-Tram* system that supports multiple, wind/solar-powered winch systems for on-system handling of shellfish product, (d) adjustable cross-beams to allow changes to spacing of the shellfish droplines,



Sustainable Ecological Aquaculture. Figure 4

Infrastructure configuration, orientation with respect to tidal flow, and the spatial relationship of the SEAfood System™ species. The integrated multi-trophic aquaculture (IMTA) design aspects of the system support a single-fed component (**FF** – sablefish or salmon), a 2-tiered shellfish component (**O** – oysters; **S** – scallops) to extract fine organic particulates, a deposit feeding component comprised of sea cucumbers (**C**) to remove settleable organics, and a large kelp/seaweed component to assimilate dissolved nutrients. Urchins (**u**) are integrated with the shellfish to reduce biofouling and solar energy is used to power operational components of the system

and (e) standardized outboard attachment points for kelp/seaweed lines.

The shellfish component of the first SEAfood System production model, shown in Fig. 5, comprises Pacific oysters (*Crassostrea gigas*) in the surface layer (1–4 m) and Pacific scallops (*Patinopecten yessoensis*) in the lower (5–10 m) portion of the water column. With 1.0 m spacing between vertical lines of shellfish trays/nets, the overall 2-tiered shellfish system comprises a biofiltration component that is 15 m wide (immediately downstream of the fish), 10 m deep, and extends parallel to the finfish cages and the entire length of the farm (200 m). A SEAfarm of this size thus has the capacity to physically support approximately 2,600 vertical shellfish lines with each line comprised of 8 oyster trays and 12 scallop nets – a total of 52,000 shellfish production units.

Kelp lines are attached to the downstream side of the shellfish component, with a 1.0-m spacing, which in the current British Columbia SEAfarm model results in a total of 200 kelp/seaweed production lines. These lines run perpendicular to the shellfish system with the production grid extending 80 m downstream of the shellfish structure (although length is only limited by available space).

The sea cucumber component of the SEAfood System is managed using an ocean-ranching approach that makes it independent of the floating aquaculture production units that support the finfish, shellfish, and kelp/seaweed. Cucumber seed (small juveniles: 2–3 cm) are sowed over the ocean floor (5–10 m^2) and allowed to move freely in the vicinity of the farm structures, taking advantage of the continual input of organics originating from the overlying species assemblage – fed and extractive components. Once achieving market size these animals are harvested using divers, and although a somewhat labor-intensive method, this extensive approach allows for waste management

Sustainable Ecological Aquaculture. Figure 5
Left photograph: a segment of the SEAfood System's shellfish component showing the powered SEA-Tram and gantry for hoisting shellfish droplines. *Right photograph*: SEA-Tram gantry showing author lifting dropline of scallop nets using one of the winch components; these six winches have subsequently been changed to electric units with a solar panel system mounted at the top of the gantry. Battery storage is at the base of the gantry

across the entire farm rather than just below the source of the settleable organics (the finfish).

Energy Needs Widespread recognition and growing concern over fossil fuel consumption trends, associated carbon emissions, and the global/regional climatalogical effects of these atmospheric loads are also reflected at the aquaculture farm scale, representing a significant environmental as well as economic (business) impact. For example, in remote coastal aquaculture operations, the reliance on diesel fuel to power boats and associated equipment (hydraulic pumps, winches) represents a significant impact on operations. In an analysis of the current shellfish aquaculture industry, it has been estimated that to produce a dozen [12] oysters using a suspended (raft) culture approach requires 0.24 l of diesel fuel over the entire production cycle – this includes on-farm activities only and does not account for seed and harvest transport to/from the farm.

Integration of sustainable energy alternatives (SEA-Power) within the SEAfood System design was envisioned as another approach to improve upon environmental performance (reducing the carbon footprint) while addressing this inherent and increasing operational cost of aquaculture production. As alternative energy systems continue to increase in efficiency, their unit size, weight, materials use, and life cycle characteristics now lends itself to uses other than satisfying the energy needs of large facilities (e.g., homes, commercial buildings).

Following a detailed site-specific meteorological assessment, selection, and integration of a solar energy system has been used within the first SEAfood System operation to operate the winch systems within the shellfish production component. Panels at the top of the SEA-Tram collect solar-derived electrical energy to maintain a battery storage unit at the base. The 2.0-kW system for the first SEAfarm site has been designed for independent operation of six electric winches, each capable of lifting a maximum of 240 kg (shellfish droplines). The energy use pattern for the SEA-Tram is unique, in terms of typical alternative energy system uses, in that operation of the winch units occur over a 10–12-h working day, and comprise a series of long-lift (entire dropline) retrieval as well as short lifts (vertical moves from one layer of shellfish to the next for product handling).

Each proposed SEAfarm site will have different alternative energy sources and will require different

energy extraction and storage configurations. The shift between small solar and wind power units, or using a combination of the two, may be needed to maintain operational energy needs for this intended use within a SEAfarm. The size of the farm may also dictate the need for additional SEA-Tram (and associated energy units) in order to efficiently manage shellfish production levels within this SEAfood System component. The current model considers a SEA-Tram for each 100-m length of farm – a total of two for the one discussed in this example (one operating on each end of the SEAfarm).

Operational Practices Application of organic operational standards is considered an effective approach by which many of the other environmental issues associated with coastal aquaculture, including food formulations, use of antibiotics and other chemicals, animal welfare (stocking density), etc., can be addressed [33]. While certified organic standards are currently under development in North America, it is anticipated that these operational protocols will be similar to Standards that are currently in practice in other jurisdictions (e.g., *Naturland organic standards* in the EU).

Organic standards include strict operational requirements for all aspects of the food production. In aquaculture, the standards dictate specifications for environmental management, prohibitions on use of antibiotics and chemicals, animal welfare (stocking densities, humane forms of dispatch), feed composition and sources, product handling (husbandry, harvesting, transport), biosecurity, emergency protocols, auditing requirements, staff training, branding, sales, etc.

Integrating these rigorous practices within the ecologically designed SEAfood System further supports the goal of achieving environmental as well as socio-economic sustainability. As a third-party-verified (audited) operational program, a certified organic aspect provides an important linkage between environmental sustainability practices and the inherent and ongoing need for social acceptability – a level of assurance to the consumer and to other coastal stakeholders. The implementation of a certified organic approach is also beneficial from a business/economic perspective, in that the approach will allow for further product differentiation and market access.

Economic Sustainability: The Business Model As discussed above, the ecological design and operational practices associated with the SEAfood System address many of the environmental issues/stressors associated with current methods of coastal aquaculture production, and in doing provides an additional measure of social acceptability (and sustainability) for this growing seafood producing sector. However, the sustainable ecological aquaculture (SEA) system approach is also inherently more complex than that of traditional production approaches – and with increased complexity comes increased operational (and business) risk. A cost-benefit analysis of the SEAfood System production model, implemented during the early design stage of system development, is thus an essential exercise in terms of achieving economic (and business) sustainability [30, 31]. Basic criteria that have been incorporated into this assessment include the following: (a) species selection, value and production capacity, (b) operational efficiencies; (c) economy of scale; and (d) vertical integration.

Species Selection, Value, and Production Capacity The choice of species used in the SEAfood System developed for British Columbia considered social sustainability, ecological function within the production model, and market value (profitability). First, all species used in the system are native (or long established through introductions), and thereby satisfy the social issues and potential ecological risks of using exotic species – particularly in regard to the fed (finfish) component of the system. Sablefish and Pacific salmon, although slightly more difficult to culture than the Atlantic salmon, receive a significantly higher farm-gate value given the comparatively lower regional supply but increasing demand for these particular species. This inherent price premium, coupled with the *green* nature of the SEAfood System approach, offsets the cost of implementing and maintaining the operational standards required for *organic* certification – adding additional value to the fish and offering a variety of new market segment opportunities.

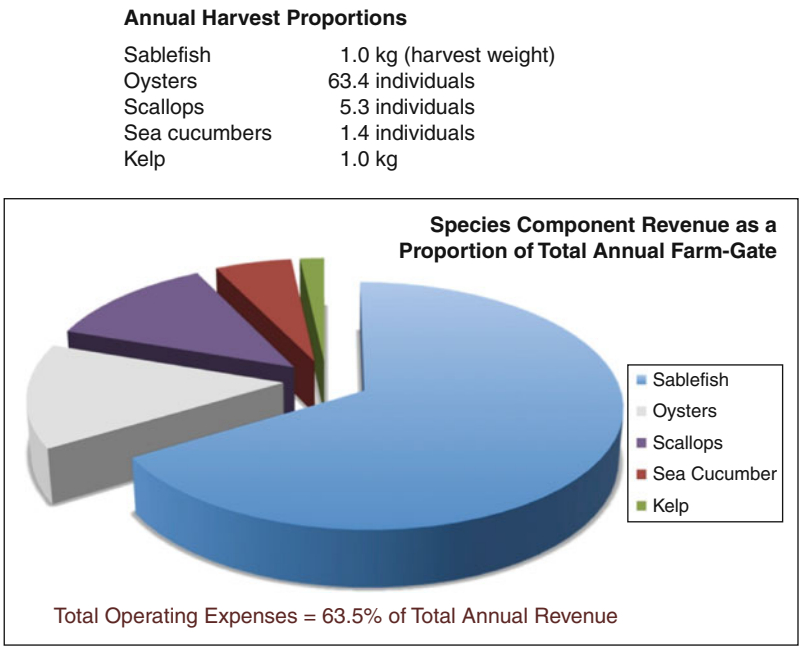
The selection of the organic extractive species is a much more difficult task given the number of species available to satisfy this role within the SEAfood System [34–37]. Choice of appropriate species ultimately requires a compromise between extractive efficiency

and product value. For example, although blue mussels (*Mytilus edulis*) are known to be the most efficient shellfish species in terms of filtration capacity, their commercial value is much lower than that of other shellfish species candidates. Therefore, to maximize the profitability of the shellfish extractive component, a 2-tiered approach was introduced into the design – oysters in the surface layer (replacing a mussel) and scallops in the lower portion of the water column. The combination of the two extractive species not only increases the operational efficiency for this component but also provides a significantly higher farm-gate value to the overall seafood production model.

The business decision to adapt a much more complex, multispecies production system, and ultimately the economic sustainability of this approach, will be determined not only by the values of the individual species but also by the level of combined production that can be achieved for the additional, extractive species, the cost of production (CoP) for these integrated components, and continued consumer demand for the products.

Assuming that consumer demand remains constant (although it will likely continue to grow), Fig. 6 illustrates the relative proportions of annual production (harvest volumes) among the system components, as well as the farm-gate value for the various SEAfood System species in the first SEAfood System operation established on the Canadian west coast. For each kilogram of sablefish produced from the system, approximately 5 dozen half-shell oysters, 5–6 scallops, 1–2 sea cucumbers, and a kilogram of kelp are harvested. It should be noted that this proportion of species represents a starting commercial balance for the SEAfood System and one that will be refined over time to ensure extractive efficiency of the system. These values also represent the proportion of annually harvested stock, and the SEAfood System maintains multiple year classes of each species.

The pie chart in Fig. 6 illustrates the proportion (percent) of farm-gate revenue generated annually from each of the component species. These values were generated from total annual revenue projected for four regional SEAfarms, and averaged to provide standardized estimate of farm-gate sales for a single facility. The fed component of the system (e.g., sablefish) remains the highest value (66.4%), with oysters



Sustainable Ecological Aquaculture. Figure 6
Annual harvest proportions and the individual species component revenues represented as a proportion of her total annual farm-gate sales

contributing 13.9%, scallops 12.5%, sea cucumbers 5.5%, and kelp 1.8% of the total annual sales revenue. With operating expenses of 63.5% of the total sales, the Net Revenue (Total Expenses – Total Gross Revenue) is 36.5%. This analysis indicates that from a business/economic perspective the SEAfood System is potentially quite profitable, and given these margins would not only be considered sustainable but would also support future growth.

Operational Efficiencies The SEAfood System maintains multiple-year classes for each component species, ensuring an annual balance between seed entry and product removal (harvest) for each of these species. Although intuitively a much more complex management challenge than a process involving a single species/product, there are inherent operational efficiencies – and economic (profitability) gains – that can be realized within this type of operational system.

The infrastructure design and configuration of this multispecies SEAfood System represents an adaptation of the galvanized steel netcage array used in the finfish aquaculture industry. The majority of species components (fish, shellfish, urchins, and attachment points for the kelp/seaweed) are directly accessible from the consolidated SEAfood System infrastructure components and thus do not require transportation of staff, materials, and vessels among what might otherwise comprise independently moored structures. The operational efficiencies gained through this design are significant, and are associated primarily with savings in labor and the requirement for dedicated working vessels (with fuel and equipment costs). Development of unique infrastructure (e.g., the shellfish SEA-Tram component) has further reduced the Cost of Production (CoP) within the consolidated multispecies SEAfood System model. By introducing alternative energy components to power shellfish winches, the saving in fuel (estimated from traditional shellfish production methods) is projected at over 43,000 l annually for each SEAfarm (based on 0.23 l/dozen shellfish produced). Fuel costs have continued to burden CoP over the past decade, and the present and future projections for the cost of fuel is a continual increase. The shift from fossil fuels to wind/solar energy is therefore readily justified, and in fact, represents a significant reduction in CoP and in business (economic) risk.

A shared labor pool will also result in a significant reduction in overall CoP. While each farm will require staff for finfish husbandry (feeding, net maintenance, grading, harvesting, etc.) as well as for the additional, extractive species component (shellfish grading, net maintenance, harvesting, etc.), in a consolidated multispecies system, there is opportunity for staff to assist in all tasks and thereby eliminate traditional slow periods in workloads. It is estimated that the SEAfood System will require 75–80% of the staff that would be cumulatively needed to independently operate the various production components.

While these inherent operational efficiencies are evident within the single SEAfarm operation, further CoP reductions can be realized through economy of scale – the addition of multiple SEAfarm sites.

Economy of Scale Compatibility and scalability were the key design criteria in developing the SEAfood System. Engineering of unique extractive species infrastructure, using comparable materials and assembly lengths, makes the system compatible with most of the current finfish cage system designs. Further, and as illustrated in Fig. 4, this manufacturing flexibility allows scalability in each SEAfarm. The configuration described in this chapter, for example, is based on a single array of 12 steel fish cages – approximately 200 m in length – with the extractive system components paralleling these structures. Other farms may choose to use large fish cages, perhaps with a fewer number, while others may establish very long farms with greater numbers of fish cages.

Economy of scale for the present *SEA*food System production operation is further realized through the development of a vertically integrated *SEA*farm Cluster – an operational aggregate of 4–5 production SEAfarm sites and an affiliated seed (hatchery) facility (see following section). Each of the production sites is configured identically as per the first *SEA*farm – the operational template – including infrastructure configuration and species composition and stocking densities. The joint management/operation of the *SEA*farm Cluster facilities allow increased production with a commensurate reduction in the cost of operations resulting regional efficiencies. Spatial distribution of a number of smaller SEAfarm units, rather than establishing large farm facilities, is considered an additional

design feature in reducing the environmental footprint of the commercial production system.

Vertical Integration One of the greatest operational risks associated with a multispecies production model is the assurance of annual seed supply. While this is always a challenge for aquaculture (fish, shellfish) companies, this becomes even more difficult when the function of the ecological production system relies on an established biomass for each species component – fish, shellfish, echinoderms, and kelp/seaweed.

To optimize and fully control the *SEA*food System production model, vertical integration within the operational framework includes a small, floating *"marine species"* hatchery. The *SEA*food-Hatchery capacity is small by comparison to the current hatchery model, and is designed to accommodate seed/juvenile production for no more than 4–5 *SEA*farm sites – allowing control of seed production of all marine component species (if salmon is used, these are acquired through other commercial facilities). The floating (barge-based) design of the *SEA*food-Hatchery supports placement of this aspect of the overall aquaculture production system directly *within* a *SEA*farm cluster (4–5 farms in an operational area), facilitating use of farm infrastructure for broodstock management, juvenile rearing, and other activities that would otherwise be conducted within a land-based hatchery – saving capital infrastructure costs, operational expenses, and lowering the overall cost of production for these integrated aquaculture systems. This becomes particularly relevant in remote coastal areas where transportation expenditures for all aspects of production can be limiting.

Summary of Cost–Benefits Risk associated with any form of aquaculture, including that of the SEAfood System approach, the business risks, and hence the determinants of economic sustainability, are mitigated largely by the inherent ecological design and business framework established for this new aquaculture approach. In addition to the intuitive environmental benefits associated with the SEAfood System approach, which directly address societal concerns associated with traditional aquaculture, this new production model provides significant economic benefits/incentives for future industry commercialization efforts. Specifically, the mitigation of these risks increase profitability of this ecological approach that stem jointly from the:

- Selection and integration of high-valued commercial species to the SEAfood System, thereby ensuring a higher combined farm-gate value
- Coproduction of multiple species, providing an inherent product diversification and reducing the potential operational or financial risks associated with single species production
- Reduced Cost of Production, resulting from a shared labor pool, integrated infrastructure, and lower per-unit production expenditures for the SEA-system components
- Inherent, potentially higher margins associated with a sustainable ecological aquaculture (SEA) system production approach that is viewed by consumers as *natural*, and that follows the operational standards established for organic certification

Future Directions

The sustainability of aquatic food production systems is currently recognized as a critical issue in global seafood supply and demand. Environmental effects resulting from many aquaculture approaches may represent ecosystem stressors that will not support long-term sustainability goals given the inherent and increasing need for marine protein. With the dramatic shift from a reliance on global fisheries to the production of seafood through controlled aquaculture systems, innovation in design and operational practices will facilitate the sustainable growth of global aquatic food production.

The vision for sustainable ecological aquaculture (SEA) systems is one that will continue to evolve under a *continual improvement* paradigm. All three pillars of sustainability – social, economic, environmental – and how these will effectively be integrated into the global seafood production process will necessarily require a multi-disciplinary and collaborative approach of science. Ongoing social pressures including demand for seafood, concerns over environmental degradation, and growing competition for space by other coastal foreshore stakeholder will further dictate the need for increased production

efficiency. Modifications to SEA system design, improvements to infrastructure, and integration of new species, will all support a long-term sustainable ecological aquaculture (SEA) vision.

Bibliography

1. FAO (Food and Agriculture Organization) (2009) The state of world fisheries and aquaculture 2008. Fisheries Department, Food and Agriculture Organization of the United Nations (FAO), Rome
2. United Nations (1987) Report of the world commission on Environment and Development – our growing future. Annex to Document A/42/427
3. Munro DA (1991) Caring for the earth – a strategy for sustainable living. Published (1991-12-31) in partnership by IUCN, UNEP, WWF, 228 p
4. Holling CS (2000) Theories for sustainable futures. Conserv Ecol 4(2):7
5. Anderberg S (1998) Industrial metabolism and linkages between economics, ethics, and the environment. Ecol Econ 24:311–320
6. D'Souza CM, Taghian POLamb, Peretiatko R (2007) Green decisions: demographics and consumer understanding of environmental labels. Int J Consum Stud 31:311–376
7. Lavallee S, Plouffe S (2009) The eco-label and sustainable development. Int J Life Cycle Ass 9(6):349–354
8. Costa Pierce B (2010) Sustainable ecological aquaculture systems: the need for a new social contract for aquaculture development. Mar Technol Soc J 44:1–25
9. GESAMP (2008) (imo/fao/unesco-ioc/unido/wmo/iaea/un/unep joint group of experts on scientific aspects of marine environmental protection) assessment and communication of environmental risks in coastal aquaculture. Reports and studies GESAMP no. 76. FAO, Rome, 198 pp
10. Flaherty M, Vandergeest P, Miller P (1999) Rice paddy or shrimp pond: tough decision in rural Thailand. World Dev 27(18):2045–2060
11. Leung KMY, Dudgeon D (2008) Ecological risk assessment and management of exotic organisms associated with aquaculture activities. In: Bondad-Reantaso GG, Arthur JR, Subasinghe RP (eds) Understanding and applying risk analysis in aquaculture. FAO fisheries and aquaculture technical paper 519. FAO, Rome, pp 67–100
12. Kolar CS, Lodge DM (2002) Ecological predictions and risk assessment for alien fishes in North America. Science 298:1233–1236
13. Youngson AF, Dousat A, Saroglia M, Jordan WC (2001) Genetic interactions between escaped finfish species n European aquaculture and wild conspecifics. J Appl Ichthyol 17:153–162
14. Haya K, Sephton D, Martin J, Chopin T (2004) Monitoring of therapeutants and phycotoxins in kelps and mussels co-cultured with Atlantic salmon in an integrated multi-trophic aquaculture system. Bull Aquac Assoc Can 104(3):29–34
15. Cross SF (2004) Marine finfish and suspended shellfish aquaculture: water quality interactions and the potential for integrated aquaculture. PhD thesis, University of Stirling (UK), 368 p
16. Weise AM, Cromey CJ, Callier MD, Archambault P, Chamberlain J, McKindsey CW (2009) Shellfish – DEPMOD: modeling the biodeposition from suspended shellfish aquaculture and assessing benthic effects. Aquaculture 288(3–4):239–253
17. Cromey CJ, Nickell TD, Black KD (2002) DEPOMOD modeling the deposition and biological effects of waste solids from marine cage farms. Aquaculture 214:211–239
18. Chopin T, Robinson S, Sawhney M, Bastarache S, Belyea E, Shea R, Armstrong W, Stewart I, Fitzgerald P (2004) The AquaNet integrated multi-trophic aquaculture project: rationale of the project and development of kelp cultivation as the inorganic extractive component of the system. Bull Aquac Assoc Can 104(3):11–18
19. Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Shpigel M, Yarish C (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361–391
20. Troell M, Halling C, Neori A, Chopin T, Buschmann AH, Kautsky N, Yarish C (2003) Integrated mariculture: asking the right questions. Aquaculture 226:69–90
21. Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. Nature 424:365
22. CostaPierce BA (2002) Farming systems research and extension methods for the development of sustainable aquaculture ecosystems. In: Costa-Pierce BA (ed) Ecological aquaculture: the evolution of the blue revolution. Blackwell, Oxford, UK, pp 103–124
23. Soto D, AguilarManjarrez J, Brugère C, Angel D, Bailey C, Black K, Edwards P, Costa-Pierce B, Chopin T, Deudero S, Freeman S, Hambrey J, Hishamunda N, Knowler D, Silvert W, Marba N, Mathe S, Norambuena R, Simard F, Tett P, Troell M, Wainberg A (2008) Applying an ecosystembased approach to aquaculture: principles, scales and some management measures. In: Soto D, Aguilar-Manjarrez J, Hishamunda N (eds) Building an ecosystem approach to aquaculture. FAO/Universitat de les Illes Balears expert workshop, 7–11 May 2007, Palma de Mallorca. FAO fisheries and aquaculture proceedings, No. 14. FAO, Rome, pp 15–35
24. Chopin T, Buschmann AH, Halling C, Troell M, Kautsky N, Neori A, Kraemer GP, Zertuche-Gonzalez JA, Yarish C, Neefus C (2001) Integrating seaweeds into marine aquaculture systems: a key toward sustainability. J Phycol 37:975–986
25. Cross SF (2004) Finfish-shellfish integrated aquaculture: water quality interactions and the implication for multi-trophic aquaculture policy development. Bull Aquac Assoc Can 104–3:44–55
26. Duarte P, Meneses R, Hawkins AJS, Zhu M, Fang J, Grant J (2003) Mathematical modeling to assess the carrying capacity for multi-species culture within coastal waters. Ecol Model 168:109–143

27. Lander T, Barrington K, Robinson S, MacDonald B, Martin J (2004) Dynamics of the blue mussel as an extractive organism in an integrated multi-trophic aquaculture system. Bull Aquac Assoc Can 104(3):19–28
28. Huntington HP (2000) Using traditional ecological knowledge (TEK) in science – methods and applications. Ecol Appl 10:1270–1274
29. Neori A, Troell M, Chopin T, Yarish C, Critchley A, Buschmann AH (2007) The need for a balanced ecosystem approach to blue revolution aquaculture. Environment 49(3):36–43
30. Ridler N, Robinson B, Chopin T, Robinson S, Page F (2006) Development of integrated multi-trophic aquaculture in the Bay of Fundy, Canada: a socio-economic case study. World Aquac 37(3):43–48
31. Ridler N, Wowchuk M, Robinson B, Barrington K, Chopin T, Robinson S, Page F, Reid G, Haya K (2007) Integrated multi-trophic aquaculture (IMTA): a potential strategic choice for farmers. Aquac Econ Manage 11:99–110
32. Mazzola A, Sarà G (2001) The effect of fish farming organic waste on food availability for bivalve molluscs (Gaeta Gulf, Central Tyrrhenian, MED): stable carbon isotopic analysis. Aquaculture 192:361–379
33. Mansfield B (2006) Orgaic views of nature: the debate over organic certification of aquatic animals. Sociologia Ruralis 44(2):216–232
34. Shpigel M, Lee J, Soohoo B, Fridman R, Gordin H (1993) The use of effluent water from fish ponds as a food source for the pacific oyster Crassostrea gigas Tunberg. Aquac Fish Manage 24:529–543
35. Shpigel M, Blaylock RA (1991) The Pacific oyster, Crassostrea gigas, as a biological filter for a marine fish aquaculture pond. Aquaculture 92:187–197
36. Smaal A, van Stralen M, Schuiling E (2001) The interaction between shellfish culture and ecosystem processes. Can J Fish Aquat Sci 58:991–1002
37. Shpigel M, Fridman R (1990) Propagation of the Manila clam Tapes semidecussatus in the effluent of marine aquaculture ponds in Eilat, Israel. Aquaculture 90:113–122

Sustainable Herbicide-Resistant Crops

Jonathan Gressel
Plant Sciences Department, Weizmann Institute of Science, Rehovot, Israel

Article Outline

Glossary

Gene stacking Developing transgenic crops containing two or more transgenes, either by simultaneous transformation or by hybridizing plants with different transgenes.

Graminicide A herbicide that specifically controls weeds in the grass family (Graminae – Poaceae).

Harvest index The ratio of grain to total biomass in a crop.

Herbicide hypersensitivity The property of certain genetically engineered crops to be susceptible to herbicides to which the wild type is naturally resistant.

Herbicide resistance The ability to withstand a critical level of herbicide with little or no effect on yield. Resistance can be natural, evolved, or generated by mutagenesis as well as by using recombinant DNA technology (genetic engineering).

Selectivity The ability to differentially control weeds with little or no effect on a specific crop.

Shattering Premature dropping of seeds before they can be harvested.

Systemic herbicide A herbicide that is applied to the leaves but spreads to underground plant organs, and affects these parts of the plant as well as leaves, or is applied to the soil and also affects leaves.

Target site The specific site to which a herbicide binds, beginning the cascade leading to plant death.

Transposon A DNA element that can move from one location in the genome to another.

Volunteer weed Weeds of a plant species that had been a crop in a previous season.

Definition of the Subject

While transgenic herbicide-resistant crops have been a boon to agriculture, reducing both production costs and ecological impacts of farming, weeds have rapidly evolved resistance to the major herbicide used in transgenic crops (glyphosate) [1], rapidly rendering the technology less sustainable than had been thought [2]. While no practice in agriculture has been sustainable forever, the period of sustainability can be extended. Methods are outlined to extend both the usefulness to crops where needed as well as the sustainability of transgenic herbicide technologies such as rotations of crops and herbicides, increasing the targets of herbicide action, suppressing herbicide targets in rotation. Transgenically inducing hypersensitivity to herbicides can be considered as an option for some weeds.

Introduction

Chemical weed control with herbicides has been a major quantum step that has allowed agriculture to supply food to a rapidly growing population, decreasing the drudgery of mechanical and manual (too often fe-manual) control of weeds. It allowed the switch in the direction of crop domestication from selection of ever taller grain crops (with lower harvest index) that were more competitive with weeds, to dwarf "green revolution" high harvest index crops. This broke the cycle of weeds continuing to evolve taller forms as a consequence of farmer and breeder selecting for height in crops so that the crop would compete with weeds.

The amount of additional arable land that would have been needed to feed the present populations of India and China just does not exist in these countries, and there would have been massive famine had it not been for the green revolution dwarfing wheat and rice, and the availability and use of herbicides to control the highly competitive, taller weeds. There would have

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

been this famine even if all available natural wild ecosystems were brought under the plow. Still, the chemical industry has not been able to generate new herbicides needed for each cropping situation where they are needed. Some of the older generation herbicides were found to have environmental or toxicological issues and have been de-registered as regulatory standards change. Additionally, weeds continue to evolve, just as they had to previous efforts to deal with them. Agriculture is not statically sustainable. New strategies must continuously be developed as previous ones are lost. So far, agricultural scientists have been successful at this continuing exercise, coming up with new technologies to replace lost ones, and delaying Malthus's nineteenth century predictions that due to population increasing at a higher rate than yields, there would be starvation. Indeed, typical annual yield increases would not keep up with population increases, but in every generation or so a new technology appears, providing quantum leaps. These included the use of hybrid vigor, tractors, selective herbicides, the green revolution, etc., all leading to major jumps in yield.

Genetically engineered herbicide resistance was another quantum leap; it allows taking a desired herbicide with limited use because it is not selective with a particular crop and then expand its use by transgenically rendering the crop resistant [3]. The major herbicide being used transgenically, glyphosate, indeed has excellent properties; no mammalian toxicity in realistic amounts; little environmental toxicity, it kills most weeds, including many that are hard to control [4]. The latter include weeds that have hidden buds and/or underground organs that require that a herbicide be systemic and penetrate to all parts of a plant to prevent resprouting.

Successes of Transgenic Herbicide-Resistant Crops

It has been argued that no technology has been taken up as rapidly and as massively as transgenic herbicide resistance to the herbicide glyphosate [5, 6]. This resistance has only been released in four crops: soy, maize, cotton, and oilseed rape (canola). Over 80% of the world's soybean and most of the maize and oilseed rape are glyphosate resistant, as is most of the North American cotton [5]. In the latter case, farmers had a choice of herbicide-resistant cotton with or without a transgene encoding the Bt insecticidal gene, and more farmers cultivated transgenic cotton with herbicide resistance than with the insecticidal gene. This rapid adoption by growers occurred despite high "technology fees" for the seeds, the availability of non-transgenic varieties, and a predominantly bad public perception generated by antitechnology, anti-multinational, and antiglobalization groups. The reasons are simple; the herbicide is inexpensive and the control package costs less, a larger spectrum of weeds is controlled, and the farmers had more flexibility in application timing than with previous herbicides [3, 6]. This had a major (positive) environmental impact. Many of the previous herbicides used were applied to cultivated bare ground before planting and much herbicide washed (with soil) into rivers or percolated into underground aquifers. The rapid dissipation of glyphosate allowed farmers to apply herbicide to standing weeds before planting, without cultivation, and then plant into soil-holding stubble. Spraying requires far less energy than plowing followed by disk cultivating, with considerable saving in fossil fuels. It has been estimated that the amount of fossil fuel saved per year is equivalent to that which would be saved from removing over five million automobiles from the roads [6]. This lack of preplant cultivation (no-, minimum, or eco-tilling) reduces soil erosion by 95% [6, 7]. It was not just in industrial USA and Canada where the technology was rapidly adopted. Argentina had a problem with a mistake made generations ago; they repeated an earlier mistake of a Mr. Johnson in the USA who introduced a Middle-Eastern weed, *Sorghum halepense* to the USA as a pasture grass. This "johnsongrass" turned out to be a poor pasture grass in both countries, but especially in Argentina, an excellent weed that propagates by seed as well as by underground buds on rhizomes. Other herbicides would burn off the tops only to have it resprout; cultivation cut the rhizomes into multiple propagules, spreading the weed. Transgenic glyphosate-resistant soybeans, planted into "johnsongrass" and other weed stubble that kept the soil from overheating, became a hit [7]. This transgenic soybean spread rapidly to neighboring Brazil where it was cultivated illegally until the government changed its policies due to massive farmer civil disobedience.

Countries in Europe, though reluctant to allow farmers the choice to cultivate transgenic herbicide-resistant maize and soy, have had no choice but to import them to feed their livestock. The claim that European agriculture is sustainable without cultivation of transgenic crops is only correct when the importation of transgenic feed grains to sustain European livestock is politely ignored.

There are those who argue that transgenic crops are not needed; modern breeding using DNA marker-assisted techniques can meet all needs. This author knows only of cases where marker-assisted breeding has allowed more rapid stabilization of inherent traits, such as a naturally occurring mutation for herbicide resistance that also occurs in weeds at a high frequency. Breeding cannot bring in herbicide resistance traits that do not exist in that crop species. This can only be done with recombinant technologies. Mutation breeding is counter-indicated with crops having related weeds, as this is not even sustainable in the short term, as discussed below.

Problems with the Present Transgenic Herbicide-Resistant Crops

There has never been a rapidly adopted technology that is not without drawbacks, despite widespread use. A case in point is the Model T Ford that revolutionized personal transport, cityscapes, suburbia, and lifestyles. It was inexpensive and met unforeseen needs. Still, it could never meet present day fuel, pollution, or safety standards with its arm breaking hand crank, poor suspension, steering and brakes, and tires that rapidly blew out. Early cars had detractors who passed regulations such as to have flagmen run in front of cars so as not scare horses, not too unlike some of the irrelevant regulatory requirements for transgenics (e.g., knowing flanking sequences of insertion sites). Such detractors rarely call for the rational and much needed regulation concomitant with all technologies.

The next generations of herbicide-resistant crops need to evolve to meet new needs and higher standards. They will be more sophisticated, more efficient, and their use far more complex than the present ones. The author hopes that he will not be misconstrued from the following sections: he is clearly not against the use of transgenic herbicide-resistant crops, but there are far too many cases where they are being used injudiciously, compromising the long-term effectiveness of the technology. The agronomic, economic, environmental, and toxicological benefits discussed above should make it clear that they are needed. Just for those reasons it is discussed at length how to sustain such useful products.

There Are Not Enough Herbicide-Resistant Crops

Industry has decided for its own (arguably misdirected) reasons to both limit the crops rendered herbicide resistant, as well as the markets where they are sold. Weeds are a major problem in "truck" crops (intensively cultivated fruit and vegetable crops). There are very few herbicides registered for use in these crops and most such crops are hardly competitive with weeds (imagine un-weeded lettuce or radishes). They must be intensively mechanically and manually cultivated. Many such crops have already been transformed using resistance to the herbicide glufosinate as the selectable marker. Despite the need for these crops in the field, the company that owns patent rights to both the gene and the herbicide steadfastly refuses to register the use of their gene and herbicide in such crops.

Hand weeding is de rigueur in much of the developing world, especially Africa due to the small farm sizes, lack of capital for machinery as well as chemicals. When transgenic glyphosate-resistant maize was finally released in South Africa (and marketed to rich, large-scale farmers) adoption was rapid among resource-poor farmers. A lady farmer could spray down weeds on 2 ha with a backpack sprayer in a day, where she could only hoe weeds on 1 ha in a month, with much weeded too late. There has been no major effort to market this maize in other African countries, despite its potential use in controlling parasitic weeds [8]. Parasitic weeds that attach to roots of major crops and suck the crops dry before emerging from the soil and flower are a scourge of major crops in Africa, the Mediterranean basin, and eastern Europe. They cannot be controlled by cultivation or conventional herbicides, facilitating their spread and gain in importance. Fifteen years ago, it was demonstrated that transgenic crops with resistance to systemic herbicides with three different targets sites of action could be successfully used to

control parasitic weeds [9]. No transgenic crop has yet to be marketed with this use in mind. Non-transgenic maize [10, 11] and sunflowers [12, 13] with mutant acetolactate synthase genes have been marketed, and can be used for this purpose. A similar herbicide-resistant sorghum is also being developed for use in *Striga* control in Africa [14]. Parasitic weeds have not rapidly evolved resistance in maize because at the high local herbicide levels used with maize seed dressings, resistance is recessive [15]. At the levels used in field spraying sunflowers, both parasitic weeds and regular weeds are rapidly expected to evolve resistance.

Wheat and rice are humanity's two major food crops. Before the advent of selective herbicides, wheat fields were biodiversely multicolored red (from poppies) or white or yellow (various mustards or other weeds). These weeds severely reduced yields and grain quality. The advent of the herbicide 2,4-D after World War II rid wheat and rice fields of broad leaf weeds that made fields so pretty to city folk, but devastated farmers. No real sustainable fit resistance has evolved to 2,4-D among the weeds it so successfully controlled. Grass weeds, which could not compete with broad leaf weeds, quickly filled the ecological vacuum for weeds in wheat and rice. Industry countered by developing selective graminicides that could control the grass weeds in wheat and rice, grasses themselves. One by one grass weeds evolved resistance to the various graminicides, most often by using the same enzymes the crops uses to degrade and detoxify the herbicide. Initially, the weeds had too low a level of these enzymes and they slowly evolved upregulated levels, probably by gene duplication or modifications in controlling elements such as promoters and enhancers of gene expression [3]. Thus, wheat and rice are ideal targets for transformation with genes endowing resistance to general broad spectrum herbicides such as glyphosate. Such new transgenic wheat or rice varieties could then be treated with a single broad spectrum herbicide instead of 2,4-D (or a related herbicide) together with a graminicide, a more expensive technology. Both transgenic glyphosate- and glufosinate-resistant rice and wheat have been generated and tested, but neither has been marketed.

There is good evidence that transgenic herbicide resistance, as presently generated, may well not be sustainable, even in the short term in wheat and rice. This is rather certain for rice on a broad scale and for wheat on a more limited scale. This is due to two particular weeds, one more limited in distribution (*Aegilops cylindrica*) in wheat, and weedy rice in rice. Neither weed can be controlled by the selective herbicides in these crops, as each is too closely related to the crop, and are naturally resistant to the same herbicides. Wheat and rice are thus problematic targets for transgenic herbicide resistances that can then provide selectivity between weed and crop. Both weeds are able to introgress genes (hybridize and then internalize genes) from the respective crop. *Aegilops cylindrica* is a weed composed of two related but distinct genomes and wheat is made up of three. One genome is common to both wheat and its hard-to-control weed. When one mutant non-transgenic herbicide-resistant (Clearfield™) wheat was developed, the resistant gene quickly appeared in *Aegilops cylindrica* in the USA [16], and other herbicide resistances were shown to move from wheat to two other *Aegilops* species in Europe [17, 18]. Thus, instead of being controlled by the herbicide, these weeds could again become uncontrollable problems.

Weedy rice (also called feral rice or red rice) is a large group of varied de-domesticated rice strains, but almost all are the same species as cultivated rice [19]. The consistent trait of weedy rice is that it shatters seed; a dominant back mutation from the recessive non-shattering of the crop. Weedy rice can have other dominant mutations rendering it ever more feral and weedy, a regain of secondary dormancy such that some seeds remain dormant for a few seasons; a dominant mutation to reddish or purple seed color; long awns, a dominant mutation to height and to tillering [19]. Weedy rice had been controlled by transplanting nursery-grown non-weedy domestic rice into cultivated paddies, giving the crop a month head start. This labor intensive, backbreaking process is rapidly being replaced by direct seeding, and weedy rice is celebrating. The only chemical answer has been a non-transgenic Clearfield™ rice, and the mutant gene quickly moves into weedy rice [20]. The Clearfield™ technology quickly became "passé" in tropical Latin America where it had been used season after season even during the same years [21, 22].

Thus, it can be seen that where a crop has a related introgressing weed in the same agroecosystem,

transgene flow from crop to weed can be a real issue, especially with a trait such as herbicide resistance. Transgene flow from crop to related wild species would be an ecological nonissue as herbicides are not used in the wild, so there would be no selective advantage to resistance. One must carefully read and interpret the literature claims of gene flow from transgenic crops to "wild" relatives, (for example, [23]). A close perusal of the data quickly reveals that there was no gene flow to wild relatives, but that all the gene flow was to weeds of agroecosystems or of ruderal (human disturbed) ecosystems such as roadsides. There are many basic differences between weedy species and wild species [24].

There are many crops with weedy or feral relatives in the same agroecosystems, and these weeds are major problems in those crops. They include weedy beets, weedy radish, weedy sunflowers, and especially weedy sorghum (shattercane) [23, 25–28]. All the weeds are the same botanical species as the crop, even if botanists have tagged them with different Latin binomials. There are no known weedy forms of cotton, maize, or soybeans in the vast majority of the areas where these crops are cultivated. There are no weedy relatives of oilseed rape in the western provinces of Canada where transgenic herbicide-resistant oilseed rape was initially introduced, but there are in the eastern provinces and gene flow is a problem there. Thus, there is a Catch 22 situation with many crops: transgenic herbicide resistance is needed to control weeds related to the crop, but the transgene will quickly hybridize into the weed, abrogating the utility of the technology. As will be discussed in a later Section, the problem can be overcome by more sophisticated transgenic technologies.

The Evolution of Weed Resistance

More than 90% of the area covered by transgenic herbicide-resistant crops is devoted to one gene (CP4 type EPSP synthase) and the herbicide glyphosate (Roundup Ready™) [5, 6]. When this was just released and being rapidly adopted, the manufacturer saw no reason to institute any strategies that might delay resistance from evolving, and the company scientists claimed that the nature of the herbicide and its mode of action precluded the evolution of resistance, a view they maintained well after the first cases of evolved resistance were confirmed [2]. This was despite ample evidence that there was diversity in the level of resistance among weeds due to a multiplicity of factors, and these factors could either combine to provide resilient resistance or any given factor could intensify with the same result [29]. Nature has a way of making fools of those who claim that something cannot evolve, and has done so with a vengeance. There has been a propensity of some of the most pernicious weeds to have the variability and mutability to rapidly evolve resistance to many different herbicides.

It may be a question of numbers, these are weeds that shed thousands of seeds where one or two would be enough for replacement of parents or it could be that they are more mutable than others. Thus, weeds in the genus *Amaranthus* and *Lolium* have rapidly evolved resistance to most herbicides controlling them [3, 30], and they proceeded to evolve resistance to glyphosate in various locations around the globe [1], often with different populations evolving differing modes of resistance. Some of the weeds that evolved resistance were those where glyphosate was most needed; those requiring a systemic herbicide. Some of these weeds evolved a "phoenix" resistance. The leaves burnt off and new shoots emerged from the ashes in a manner similar to the mythical bird. In *Conyza*, they emerged from dormant buds at the base of the rosette of leaves [31]; in *Sorghum halepense* (johnsongrass) in Argentina and the USA from the underground rhizomes [30, 32, 33]. In many of these cases, glyphosate was the herbicide of last resort, the weeds had evolved resistance (or were naturally resistant) to other herbicides.

Very Few Herbicide-Resistant Genes Have Been Commercialized

The ease of use, the low price of herbicide, the broad spectrum of weed control quickly rendered glyphosate resistance the choice of farmers. The farmers either believed or wished that weeds would not evolve resistance to glyphosate. Worse yet, competitors of the producers of glyphosate believed this hype and severely cut back on research on discovery of new herbicides and on developing transgenic resistances to their own preexisting herbicides, which they thought could never compete with glyphosate, and by not bringing

new products to market self-fulfilled their predictions. The options available to farmers were further reduced when it came time for industry to reregister older herbicides. Many companies decided that they could not justify the outlay for renewed registration because they thought that other herbicides will never again compete with glyphosate. Many transgenic herbicide resistances under development that had value were never released and at least one that had been commercialized was withdrawn. When glyphosate resistance was initially released, seed companies continued to breed conventional varieties to provide farmers with choice. The less farmers chose conventional varieties, the less incentive remained to breed them.

The lack of alternatives clearly exacerbated the rate at which glyphosate resistance evolved and spread. This in turn was made worse by policies of the manufacturer who convinced many that resistance could not be an issue and no management strategies were needed that might involve competitors' products that could assist in sustaining their own technology.

Non-transgenic Strategies to Sustain Transgenic Herbicide Resistances

There are a series of strategies used to sustain the useful lifetime of conventional herbicide technologies. These can extend the useful lifetime of the transgenic herbicide only where there is a no similarity between the transgenic crop and the weed; that is, such technologies are poor at dealing with problems such as transgene flow from crop to related weed. One strategic mistake with glyphosate-resistant crops was the promotion of glyphosate for all applications, including preplant where crop herbicide resistance is not required. There are other herbicides such as paraquat and inhibitors of protoporphyrinogen oxidase that provide rapid burn down of most weeds; with the notable exception being those that require a systemic herbicide. Thus, any weeds that might have evolved glyphosate resistance in previous seasons and germinate before planting would be controlled by these preplant herbicides. At least by changing the preplant herbicide, glyphosate-resistant weeds will not have a head start on the crop.

Crop and herbicide rotations have always been excellent tools to delay herbicide resistance using conventional herbicides, and they could have delayed glyphosate resistance, had they been applied. The best herbicides for use in rotation are those that exert negative cross-resistance; that is, they are more effective at controlling resistant individuals that in controlling the wild type [34]. Still, there have been no reports about herbicides that preferentially control glyphosate-resistant weeds. Most cases of glyphosate resistance evolved following the sole use of glyphosate, season after season, with no other herbicide that controls the target weeds used in the interim.

Some herbicide mixtures have been highly effective in delaying resistance in the past. Resistance evolved to atrazine in maize where atrazine was used alone, but not where it was mixed with herbicides in the chloroacetamide group inhibiting long-chain fatty acid biosynthesis [35]. These chloroacetamide herbicides even prevented the evolution of resistance in weed species that they did not control; but they must have weakened them to the point that they were unable to compete. Not all mixtures work equally well, and there are some rule-of-thumb criteria for choosing good mixtures, such as having the same persistence, different targets of action, overlapping weed control spectra, etc. [36].

Genetic Engineering Strategies to Extend the Sustainability of Transgenic Herbicide Resistances

From the foregoing, it is clear that many of the problems with what had been and still is a most useful agricultural tool, transgenic herbicide resistance, are derived from the overreliance on a single technology. If there were more transgenic herbicides to rotate, the risk would have been spread and each would last for much longer, and each in turn would have controlled resistance to the previous one. There are herbicide chemistries that have less propensity to having herbicide resistance evolve, and should be used more often in rotations and mixtures. The chloroacetomide herbicides inhibiting long-chain fatty acid biosynthesis have been heavily used for over 4 decades. The only major case of resistance reported for this group was when a rice weed evolved resistance to one herbicide in the group [37]. The cross-resistance of the weed to herbicides of a different group suggests that resistance probably was not at the target site of herbicide action. These chloroacetamide herbicides inhibit more than one fatty

acid elongase, and resistance at all the target sites would require the exceedingly rare confluence of a few mutations appearing simultaneously. Resistance due to catabolism could continue to evolve to members of this group of herbicides, but the diversity of chemistries preclude the probability of a single metabolic system degrading them all.

Industry has dedicated most of its efforts to finding herbicides that affect single targets of action. Nature has been wiser; it evolved natural herbicides (allelochemicals) as part of one of its strategies of using chemical warfare for one plant species to compete with others. Many of these allelochemicals inhibit more than one target site [3]. For example, sorghum secretes the phytotoxin sorgoleone that inhibits two separate targets in plants, the psbA protein in photosystem II and the enzyme HPPD (4-hydroxyphenylpyruvate dioxygenase) [38], which are key targets of two separate groups of commercial herbicides. Two targets would have to simultaneously mutate for target site resistance to evolve. If the chemical industry could find such multisite inhibitors, the biotech industry could find or generate genes encoding enzymes that will degrade them to provide crops with selective transgenic herbicide resistance. Technologies such as gene shuffling allow researchers to take genes that encode enzymes that inefficiently degrade a herbicide and turn them into usable genes. This has been elegantly used to generate herbicide-resistant maize, but so far only published for generating metabolic resistance to the herbicide glyphosate [39], where additional resistant genes are redundant.

There are ways to extend useful life of the present and future transgenic herbicide resistances by stacking genes. There are also ways to deal with crops where gene flow is an issue, as well as to develop sophisticated systems using herbicide hypersensitivity to both prevent volunteer weeds and crop × weed hybrids from going feral. One can even envisage ways of overcoming weed resistance after it has evolved, all as outlined below.

Stacking Herbicide Resistances

Two companies have announced that they are stacking herbicide resistances to preclude some of the problems that at least one [2] had denied would occur. This will require the farmer to use both herbicides, effectively raising production costs. Convincing them to do so may not be easy. One company is stacking glyphosate resistance with resistance to dicamba, a herbicide similar to 2,4-D in structure and mode of action [40]. Another is stacking glyphosate resistance with resistance to acetolactate synthase inhibiting herbicides [41]. These may solve many problems, but not all. Dicamba controls only broad leaf weeds so it may be effective against the *Amaranthus* species that evolved resistance to glyphosate [30], but will have no effect on the populations of grass weeds such as johnsongrass and *Lolium* spp. that have already or will evolve resistance to glyphosate. At high rates, dicamba will kill some perennial broad leaf weeds, but a high level of transgenic resistance will be required.

The choice of acetolactate synthase inhibiting herbicides as a mixing partner for stacked-gene crops raises questions, even though these are systemic herbicide. Too many weeds that have evolved glyphosate resistance such as *Lolium* spp., the *Amaranthus* spp., *Conyza* spp., as well as the *Sorghum halepense* have already evolved resistance to these acetolactate synthase herbicides [30, 32], the most resistance-prone group of herbicides known. Even if this has not occurred in all populations, it easily can.

The best stacking partners will be with genes encoding resistance to systemic general herbicides with multiple target sites of action (mostly yet undiscovered), and/or with genes encoding resistance to graminicides, as these are cases where the needs are the greatest.

Mitigating Gene Flow with Tandemly Coupled Genes for Herbicide Resistance and Non-weediness

As discussed above, there is a major reservation to conferring transgenic herbicide resistance on major crops such as rice, wheat, sorghum, sunflowers as well as others having related weeds. This is because such herbicide resistances would be non-sustainable. The ability to control the closely related feral, weedy forms of these crops or other closely related weeds residing in the same agroecosystem as the crop, will soon be lost due to gene flow. This problem can be precluded by assuring that any hybrid and future offspring from a cross of a transgenic herbicide-resistant crop with an interbreeding weed will lose at least one weediness

trait, rendering it less fit to compete with its wild-type brethren, as well as less fit to compete with other species. In highly competitive ecosystems such as agricultural fields, where hundreds of weed seeds germinate in a small area, and competitive "self thinning" leaves a single survivor, "less fit" usually means "unfit." Thus offspring having lost a weediness trait are offspring that cannot establish as a majority of a population; at worst, these offspring will remain in an insignificant frequency in the population, at best, they will die off completely. This use of a tandem construct of a herbicide resistance gene coupled with an anti-weediness gene or genes is the basis of the concept of transgenic mitigation [42]. The mitigating genes used can be those genes causing dwarfing, genes preventing seed shattering, genes preventing secondary dormancy, and with "root" or "head" crops, genes preventing "bolting" (premature flowering) [26]. The result is offspring that are more like the crop and cannot compete with weeds. Because a tandem construct is used, the herbicide resistance and the mitigating genes are genetically linked and will not segregate from each other. Where goes resistance, so goes mitigation. That this strategy might work has been demonstrated with transgenically mitigated tobacco competing with wild-type tobacco as a model system [43, 44], and with oilseed rape in the screenhouse [45, 46] as well as in the field [47].

A Special Endogenous Mitigator for Wheat Wheat has a propensity to naturally introgress genes into wild and weedy *Aegilops* species [16–18], including those with which it has no homologous chromosomes, such as *Aegilops peregrina* [48]. It transfers such genes by the process of homoeologous recombination by which similar but not identical (homoeologous) chromosomes recombine. This recombination does not occur in the F_1 generation, only in backcrosses after some wheat chromosomes are lost, most notably chromosome 4B. Homoeologous recombination would be deadly to wheat itself; the chromosomes of its three different genomes would pair with each other during meiosis causing a spaghetti-like mess. There is a gene *ph*1 on this long arm of chromosome 4 in the B genome (4BL) that prevents homoeologous recombination, and thus this type of gene flow from wheat to related *Aegilops* species can occur only after 4BL is lost. Thus, it was suggested to insert the gene of choice (herbicide resistance) on 4BL near *ph*1 so that they will remain together, either by homologous recombination onto 4BL or by random insertions and cytologically determining which occurred onto 4BL [49]. Thus, while the F_1 and some backcrosses with *Aegilops* could be resistant to herbicides, it is unlikely that the resistance gene could ever integrate into the *Aegilops* genome by homoeologous recombination.

Mitigating Volunteer Weed Establishment Gene Flow by Engineering Hypersensitivity to Herbicides

An interesting tandem construct was demonstrated to work in rice to assure that transgenically glyphosate-resistant rice volunteers cannot survive. This was achieved by spraying the next crop of (non-glyphosate-resistant) rice with the herbicide bentazon. Bentazon is a selective herbicide that can be used in conventional rice, because rice has an enzyme that degrades bentazon to nontoxic products. The transgenic glyphosate-resistant rice contained the glyphosate-resistant gene in tandem with an antisense (reverse direction) form of the gene encoding bentazon resistance [50]. This antisense form suppresses the expression of the bentazon resistance rendering rice to be hypersensitive to bentazon. When bentazon is used, glyphosate-resistant volunteer rice and any hybrids with weedy rice die.

This strategy has been theoretically extended to other crops and other herbicide resistances to develop a seemingly complicated and sophisticated strategy [51]. Still, such strategies can easily be implemented if there is an agreement among farmer groups, chemical producers, biotech and seed companies. The strategy is to have a series of transgenic varieties with opposing metabolic herbicide resistances and hypersensitivities cultured in rotation so that volunteers and hybrids with weedy forms are killed in alternate generations, and weedy forms that did not introgress the transgene are also controlled in each generation. For example, variety 1 contains a gene encoding glyphosate metabolism (glyphosateR) in tandem with the antisense form of the gene degrading glufosinate. Thus, variety 1 is glyphosate resistant and hypersensitive to glufosinate. Variety 2 has a gene encoding glufosinate metabolism (glufosinateR) and an antisense form of the gene

encoding glyphosate resistance. Thus variety 2 is glufosinate resistant and is hypersensitive to glyphosate. Thus, when the herbicides and varieties are used in rotation, glyphosate use with variety 1 will kill volunteers of and hybrids with variety 2, as well as the weedy wild type. Glufosinate with variety 2 will kill volunteers and hybrids with variety 1 as well as the wild-type weed [51]. Other mitigator genes can be added in tandem to further preclude gene flow. This type of stacking has the advantage of requiring the use of a single herbicide during each cropping generation.

As complicated as the strategy may sound, it requires only that the herbicide and crop seed be bundled (sold together) and that only a single variety is available each cropping season. Not only does such a strategy deal with gene flow to weedy rice, it also will delay the evolution of both glyphosate and glufosinate resistances. From past experience with herbicide resistances, such rotations extend the total expected useful lifetime of both herbicides well beyond the sum of expected lifetimes of each, if each had been used as the sole herbicide without rotation.

Implementation of such sustainability extending practices is not unknown even though industry typically cites antitrust laws as preventing joining forces for such strategies. For example, Australian cotton growers joined forces with academic experts on resistance management and industry and decided which insecticides may and may not be used at different times during the growing season to delay the evolution of all resistances [52].

Overcoming Transgenic Herbicide Resistance Once It Has Evolved in Weeds (Science Fiction?)

The following are strategies proposed (for the first time) for controlling herbicide-resistant weeds once they have evolved. The first strategy is an extension of a concept suggested on how to cause insects to commit chemical suicide [53], which was modified to have parasitic *Striga hermonthica* [54], and other outcrossing weeds such as *Lolium* spp. [26] commit chemical suicide. The proposal is as follows: after an outcrossing weed has evolved herbicide resistance, either via gene flow from the crop or by natural means of evolution, the gene(s) responsible must be isolated. The gene(s) in the antisense form is/are inserted into a transposon (a DNA element that can move from one location in the genome to another) that is compatible with the weed, and multiple copies of the transposon are transformed into the weed and seeds bulked up. The weed seeds are sown into a field where resistance is a problem. For 4–6 generations, alternative crops and herbicides are used. It is rare that any herbicide kills more than 95% of weeds. The remaining weeds will interbreed. All offspring of a weed crossing with others bearing multicopies of the transposon will also contain the trait and the trait will quickly spread. If the trait were to be spread by nuclear, Mendelian genetics, only 50% of offspring would bear the trait. Transgenic traits are functionally dominant in plants, so even heterozygous plants with the transgenic override of resistance are now sensitive to the herbicide, and the herbicide again can be used. Because of the antisense, none of the transposon-bearing weeds are now resistant to the original herbicide, and the original transgenic herbicide-resistant crop can be grown again. This strategy is limited to obligate outcrossing weeds, and would be lost when/if the outcrossing species evolved self-compatibility. Transposons normally move genes around a genome, and presumably have moved some transgenes in the crops already commercialized. Thus, it might be hard to find scientific regulatory concerns about such a technology.

Alternatively, the antisense form of the gene overriding resistance can be inserted into a disarmed (no longer pathogenic) systemic virus that is specific to the weed, and the weeds inoculated by virus-coated grit applied by commercial sandblasting equipment. The virus would spread and the weeds would be functionally susceptible to the herbicide. This use of viruses was initially suggested for producing vaccines to animal diseases in fodder crops [26]. Most viruses do not reach generative (sex) cells, which means that every generation of weed would have to receive such an application, as the trait will not be inherited. There should be few regulatory biosafety concerns about the use of a virus that does not insert DNA into generative cells in seed-propagated crops, as the trait will not be inherited through seeds. If a virus can be found that does insert its nucleic acid into generative cells, a combination of the above two strategies could be considered; the antisense gene in a multicopy transposon could be transmitted by such a virus, and herbicide sensitivity would be conferred on the virus/transposon infected weed, but also on all its

offspring. Here, there may well be biosafety concerns that must be ascertained and dealt with if indeed this strategy can be determined to be worthwhile.

Future Directions

The present use of transgenic herbicide resistance has proven itself to be non-sustainable for long periods as a stand-alone technology without instituting meaningful strategies to keep these technologies in the farmers' arsenal of weed control options. Similarly and even less sustainable, is the practice of mutating crops to herbicide resistance, where the crops have interbreeding-related weeds in the same fields. Both governments and industry have failed to institute management strategies to sustain such technologies. The lack of government regulatory intervention is surprising and disturbing. The regulatory systems required management strategies to delay resistance to transgenic Bt insect-resistant crops, a small fraction of the transgenic crops released, but ignored such issues with herbicide-resistant crops, including the deregulation (i.e., permission for commercial cultivation) of transgenic herbicide-resistant oilseed rape, beets, and rice, all crops with interbreeding weeds. Governments have questionable regulatory authority over the non-transgenic herbicide resistances released in rice, wheat, sunflowers, and oilseed rape, all crops with related interbreeding weeds, and a demonstrated ability to lose sustainability. Perhaps, they should assist sustainable agriculture by obtaining such regulatory authority.

The first priority for the future is to learn from the past and rapidly institute strategies to keep the present herbicide resistances useful. Unfortunately, neither industry nor farmers have long-term considerations; this year's profitability is all that seems to matter. Long-term sustainability of profitability is more an interest of governments, and the regulatory regimes must be set up to attain sustainability through requiring innovations. Goals and milestones must be set for instituting management strategies, and then support academia and industry in testing which are the most cost-effective strategies for maintaining long-term sustainability of herbicide-resistant crops, both of transgenic and non-transgenic herbicide-resistant crops. Such crops must be available in the future and are imperative for food security.

Bibliography

Primary Literature

1. Owen M, Dixon P, Shaw D, Weller S, Young B, Wilson R, Jordan D (2010) Sustainability of glyphosate-based weed management: The benchmark study. ISB News Report (http://www.isb.vt.edu/news/2010/Aug10.pdf) August: 1-4
2. Bradshaw LD, Padgette SR, Kimball SL, Wells BH (1997) Perspectives on glyphosate resistance. Weed Technol 11:189–198
3. Gressel J (2002) Molecular biology of weed control. Taylor & Francis, London
4. Franz JE, Mao MK, Sikorski JA (1997) Glyphosate: a unique global herbicide. American Chemical Society, Washington DC
5. James C (2010) Global status of commercialized biotech/GM crops: 2009, The first fourteen years, 1996–2009. ISAAA Brief 41-2009 ISAAA Brief 41-2009 (http://www.isaaa.org/resources/publications/briefs/41/executivesummary/default.asp)
6. Brookes G, Barfoot P (2010) Global impact of biotech crops: Environmental effects, 1996–2008. AgBioForum 13 (1): #6
7. Trigo E, Chudnovsky D, Cap E, López A (2003) The adoption of transgenic crops in Argentine. International Institute for Sustainable Development, Winnepeg
8. Gressel J (2009) Crops with target-site herbicide resistance for *Orobanche* and 3 control. Pest Manag Sci 65:560–565
9. Joel DM, Kleifeld Y, Losner-Goshen D, Herzlinger G, Gressel J (1995) Transgenic crops against parasites. Nature 374:220–221
10. Kanampiu FK, Kabambe V, Massawe C, Jasi L, Friesen D, Ransom JK, Gressel J (2003) Multi-site, multi-season field tests demonstrate that herbicide seed-coating herbicide-resistance maize controls *Striga* spp. and increases yields in several African countries. Crop Prot 22:697–706
11. Kanampiu F, Karaya H, Burnet M, Gressel J (2009) Needs for and effectiveness of slow release herbicide seed treatment *Striga* control formulations for protection against early season crop phytotoxicity. Crop Prot 28:845–853
12. Pfenning M, Palfay G, Guillet T (2008) The Clearfield® technology – A new broad-spectrum post-emergence weed control system for European sunflower growers. J Plant Dis Protect 21:649–653
13. Prostko EP, Grey TL, Davis JW (2009) Imidazolinone-resistant sunflower tolerance to imazapic. Weed Technol 23:188–190
14. Tuinstra MR, Soumana S, Al-Khatib K, Kapran I, Toure A, van Ast A, Bastiaans L, Ochanda NW, Salami I, Kayentao M, Dembele S (2009) Efficacy of herbicide seed treatments for controlling *Striga* infestation of sorghum. Crop Sci 49:923–929
15. Gressel J (2005) Problems in qualifying and quantifying assumptions in plant protection models: resultant simulations can be mistaken by a factor of million. Crop Prot 24:1007–1015
16. Gaines TA, Henry WB, Byrne PF, Westra P, Nissen SJ, Shaner DL (2008) Jointed goatgrass (*Aegilops cylindrica*) by imidazolinone-resistant wheat hybridization under field conditions. Weed Sci 56:32–36
17. Loureiro I, Escorial C, Baudin JMG, Chueca MC (2008) Hybridisation between wheat and *Aegilops geniculata* and

hybrid fertility for potential herbicide resistance transfer. Weed Res 48:561–570
18. Loureiro I, Escorial C, Garcia-baudin JM, Chueca C (2009) Hybridization, fertility and herbicide resistance of hybrids between wheat and *Aegilops biuncialis*. Agron Sustain Dev 29:237–245
19. Vaughan DA, Sanchez PL, Ushiki J, Kaga A, Tomooka N (2005) Asian rice and weedy rice – evolutionary perspectives. In: Gressel J (ed) Crop ferality and volunteerism. CRC Press, Boca Raton
20. Gealy DR (2005) Gene movement between rice (*Oryza sativa*) and weedy rice (*Oryza sativa*): a U.S. temperate rice perspective. In: Gressel J (ed) Crop ferality and volunteerism. CRC Press, Boca Raton
21. Valverde BE (2005) The damage by weedy rice – can feral rice remain undetected? In: Gressel J (ed) Crop ferality and volunteerism. CRC Press, Boca Raton
22. Gressel J, Valverde BE (2009) The other, ignored HIV – Highly invasive vegetation. Food Security 1:463–478
23. Ellstrand NC (2003) Dangerous liaisons – when cultivated plants mate with their wild relatives. Johns Hopkins University Press, Baltimore
24. Warwick SI, Stewart CN Jr (2005) Crops come from wild plants: how domestication, transgenes, and linkage together shape ferality. In: Gressel J (ed) Crop ferality and volunteerism. CRC Press, Boca Raton
25. Barnaud A, Trigueros G, McKey D, Joly HI (2008) High outcrossing rates in fields with mixed sorghum landraces: how are landraces maintained? Heredity 101:445–452
26. Gressel J (2008) Genetic glass ceilings: transgenics for crop biodiversity. Johns Hopkins University Press, Baltimore
27. Tesso T, Kapran I, Grenier C, Snow A, Sweeney P, Pedersen J, Marx D, Bothma G, Ejeta G (2008) The potential for crop-to-wild gene flow in sorghum in Ethiopia and Niger: a geographic survey. Crop Sci 48:1425–1431
28. Snow AA, Culley TM, Campbell LG, Sweeney PM, Hegde SG, Ellstrand NC (2010) Long-term persistence of crop alleles in weedy populations of wild radish (*Raphanus raphanistrum*). New Phytol 186:537–548
29. Gressel J (1996) Fewer constraints than proclaimed to the evolution of glyphosate-resistant weeds. Food Secur 8(1):2–5
30. Heap IM (2010) International survey of herbicide-resistant weeds. http://www.weedscience.org/
31. Davis VM, Kruger GR, Stachler JM, Loux MM, Johnson WG (2009) Growth and seed production of horseweed (*Conyza canadensis*) populations resistant to glyphosate, ALS-inhibiting, and multiple (glyphosate plus ALS-inhibiting) herbicides. Weed Sci 57:494–504
32. Valverde BE, Gressel J (2006) Dealing with the evolution and spread of *Sorghum halepense* glyphosate resistance in Argentina. A consultancy report to SENASA available online: http://www.weedscience.org/paper/Johnsongrass%20Glyphosate%20Report.pdf
33. Vila-Aiub MM, Balbi MC, Gundel PE, Ghersa CM, Powles SB (2007) Evolution of glyphosate-resistant Johnsongrass (*Sorghum halepense*) in glyphosate-resistant soybean. Weed Sci 55:566–571
34. Gressel J, Segel L (1990) Negative cross-resistance; a possible key to atrazine resistance management: a call for whole plant data. Z Naturforsch 45c:470–473
35. Owen MDK, Gressel J (2001) Non-traditional concepts of $ynergy for evaluating integrated weed management. In: Hall JC, Hoagland R, Zablotowicz R (eds) Pesticide biotransformations in plants and microorganisms: similarities and divergences. American Chemical Society, Washington DC, pp 376–396
36. Wrubel RP, Gressel J (1994) Are herbicide mixtures useful for delaying the rapid evolution of resistance? A case study. Weed Technol 8:635–648
37. Huang B-Q, Gressel J (1997) Barnyardgrass (*Echinochloa crus-galli*) resistance to both butachlor and thiobencarb in China. Resistant Pest Mgmt 9:5–7
38. Dayan FE, Rimando AM, Pan ZQ, Baerson SR, Gimsing AL, Duke SO (2010) Sorgoleone. Phytochemistry 71:1032–1103
39. Castle LA, Siehl DL, Gorton R, Patten PA, Chen YH, Bertain S, Cho HJ, Duck N, Wong J, Liu D, Lassner MW (2004) Discovery and directed evolution of a glyphosate tolerance gene. Science 304:1151–1154
40. Anonymous (2010) Monsanto completes key regulatory submission for soybeans with dicamba herbicide tolerance trait. http://monsanto.mediaroom.com/index.php?s = 43&item = 863
41. Green JM, Hale T, Pagano MA, Andreassi JL, Gutteridge SA (2009) Response of 98140 corn with *gat4621* and *hra* transgenes to glyphosate and ALS-inhibiting herbicides. Weed Sci 57:142–148
42. Gressel J (1999) Tandem constructs: preventing the rise of superweeds. Trends Biotechnol 17:361–366
43. Al-Ahmad HI, Galili S, Gressel J (2004) Tandem constructs mitigate risks of transgene flow from crops: tobacco as a model. Mol Ecol 13:687–710
44. Al-Ahmad H, Galili S, Gressel J (2005) Poor competitive fitness of transgenically mitigated tobacco in competition with the wild type in a replacement series. Planta 272:372–385
45. Al-Ahmad H, Dwyer J, Moloney MM, Gressel J (2006) Mitigation of establishment of Brassica napus transgenes in volunteers using a tandem construct containing a selectively unfit gene. Plant Biotech J 4:7–21
46. Al-Ahmad H, Gressel J (2006) Mitigation using a tandem construct containing a selectively unfit gene precludes establishment of *Brassica napus* transgenes in hybrids and backcrosses with weedy *Brassica rapa*. Plant Biotech J 4:23–33
47. Rose CW, Millwood MHS, Rao MR, Halfhill MD, Raymer PL, Warwick SI, Al-Ahmad H, Gressel J, Stewart CNJ (2009) Genetic load and transgenic tandem mitigating genes in transgenic *Brassica rapa* (field mustard) × *Brassica napus* (oilseed rape) hybrid populations. BMC Biotechnol 9:93 http://www.biomedcentral.com/1472-6750/1479/1493
48. Weissmann S, Feldman M, Gressel J (2005) Sporadic inter–generic DNA introgression from wheat into wild *Aegilops* spp. Mol Biol Evol 22:2055–2062

49. Weissmann S, Feldman M, Gressel J (2008) Hypothesis: Transgene establishment in wild relatives of wheat can be prevented by utilizing the *Ph*1 gene as a *senso stricto* chaperon to prevent homoeologous recombination. Plant Sci 175: 410–414
50. Lin C, Jun F, Xu X, Te Z, Cheng J, Tu J, Ye G, Shen Z (2008) A built-in strategy for containment of transgenic plants: creation of selectively terminable transgenic rice. PLoS ONE 3(3): e1818
51. Gressel J, Valverde BE (2009) A strategy to provide long-term control of weedy rice while mitigating herbicide resistance transgene flow, and its potential use for other crops with related weeds. Pest Manag Sci 65:723–731
52. Forrester NW (1990) Designing, implementing and servicing an insecticide resistance management strategy. Pestic Sci 28:167–179
53. Grigliatti TA, Pfeifer TA, Meister GA (2001) TAC-TICS: transposon-based insect control systems. In: Vurro M, Gressel J, Butts T, Harman G, Pilgeram A, St-Leger R, Nuss D (eds) Enhancing biocontrol agents and handling risks. IOS Press, Amsterdam, pp 201–216
54. Gressel J, Levy A (2000) Giving *Striga hermonthica* the DT's. In: Haussmann BIG, Hess DE, Koyama ML, Grivet L, Rattunde HFW, Geiger HH (eds) Breeding for striga resistance in cereals. Margraf Verlag, Weikersheim, pp 207–224

Books and Reviews

Gressel J (2009) Evolving understanding of the evolution of herbicide resistance. Pest Manag Sci 65:1164–1173

Gressel J, Levy AA (2009) Stress, mutators, mutations and stress resistance. In: Pareek A, Sopory SK, Bohnert HJ, Govindjee (eds) Abiotic stress adaptation in plants: physiological, molecular and genomic foundations. Springer, Dordrecht

Gressel J, Segel LA (1978) The paucity of genetic adaptive resistance of plants to herbicides: possible biological reasons and implications. J Theor Biol 75:349–371

LeBaron H, Gressel J (eds) (1982) Herbicide resistance in plants. Wiley, New York, p 449

Nandula VK (ed) (2010) Glyphosate resistance in crops and weeds: history, development, and management. Wiley, New York

Powles SB, Holtum JAM (1994) Herbicide resistance in plants: biology and biochemistry. Lewis, Boca Raton

Powles SB, Shaner D (eds) (2001) Herbicide resistance in world grains. CRC Press, Boca Raton

Valverde BE (2007) Status and management of grass-weed herbicide resistance in Latin America. Weed Technol 21:310–323

Valverde BE, Riches CR, Caseley JC (2000) Prevention and management of herbicide resistant weeds in rice. Cámara de Insumos Agropecuarios, Costa Rica, Internet available: www.weedscience.org/

Sustainable Productivity, Heat Tolerance for

Anthony E. Hall
Department of Botany and Plant Sciences, University of California Riverside, Quincy, CA, USA

Article Outline

Glossary

C_3 photosynthetic system In which the enzyme rubisco is responsible for the initial fixation of carbon dioxide. All tree and vine crops, all cool-season-adapted annual crops, and most warm-season-adapted annual crops have this photosynthetic system.

C_4 photosynthetic system In which the enzyme PEP carboxylase is responsible for the initial fixation of carbon dioxide. A few tropical grasses (e.g., maize, sorghum, pearl millet, and sugarcane) and a very few warm-season-adapted herbaceous dicotyledonous crops (e.g., grain amaranth) have this photosynthetic system.

CTD Plant canopy temperature depression, the number of degrees Celsius the plant canopy is cooler than air temperature.

FACE Free-air CO_2 enrichment is a system for studying crop responses to elevated [CO_2] under natural open-air field conditions.

Harvest index The ratio of grain yield to total aboveground biomass at harvest.

Heat resistance A cultivar is heat-resistant if it has greater yields of economic product, such as the weight of grain or fruit per unit land area, than standard cultivars in hot environments.

Heat tolerance A cultivar is heat-tolerant if it has a specific process such as germination, vegetative survival, pollination, or fruit set that withstands heat better than the process does in standard cultivars.

Subtropical zones Are where the coldest month has a mean air temperature $<18°C$, there is a long period (8–12 months) when plants can actively grow (mean monthly air temperatures $>10°C$), and only occasional frosts occur. This zone is located at low elevations in latitudes between 20° and 30° [26].

Temperate zones Are where there are only 4–7 months when temperatures are high enough for plants to actively grow (mean monthly air temperatures $>10°C$), and there is a long, cold winter. This zone is located at either high latitudes or at high elevations in more equatorial latitudes [26].

Tropical zones Are where all monthly mean air temperatures are $>18°C$, and there is no frost and minimal chilling. This zone is located at low elevations between the Tropic of Cancer and the Tropic of Capricorn [26].

Definition of the Subject and Its Importance

As a consequence of global climate change, air temperatures are predicted to increase by about 4°C during the twenty-first century. Plant physiological and developmental processes respond to temperature by increasing at low temperatures, responding only slightly at optimum temperatures and decreasing at high temperatures. At high temperatures, the processes can become irreversibly damaged. Of particular concern for crop production are the substantial decreases in grain or fruit production caused by experimentally induced increases in air temperature that have been observed in field conditions. In addition, in some zones and years some crop species already are being subjected to temperatures that are above optimal. In a small number of cases, crop cultivars have been bred with developmental and physiological processes that can tolerate a few degrees higher temperature, such that they are heat-resistant and produce more economic yield than standard cultivars in hot environments. Consequently, it is important to determine the circumstances where future breeding of cultivars with heat-tolerant processes, such that they are more heat-resistant than current cultivars, can contribute to the sustainability of crop production during future global warming.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Introduction

During the 1990s, accelerating increases in atmospheric carbon dioxide concentration [CO_2] have occurred leading to the current concentration of 380 ppm and a predicted level greater than 600 ppm by the end of the twenty-first century. As a consequence of the increases in [CO_2] and other gases that absorb infrared radiation, global warming probably already has begun. It has been predicted that temperature increases of about 4°C will take place by the end of the twenty-first century in crop production areas. In some circumstances, these increases in air temperature could result in substantial decreases in economic yield if cultivars, management methods, or crop species are not changed. In contrast, the many crop plants with the C_3 photosynthetic system could exhibit a tendency for increases in photosynthesis and productivity as [CO_2] continues to increase. Consequently, it is important to determine whether elevated [CO_2] can reduce the potentially detrimental effects on productivity of increases in air temperature.

Up to now there has been relatively little emphasis on breeding for heat tolerance in either the public or the private sector but significant progress has been made with a small number of crop species [24]. Breeding the first heat-resistant cultivars by incorporating heat tolerance has taken at least 10 years with annual crop species that are relatively easy to breed. The approach taken has involved incorporating heat tolerance during pollination and seed or fruit set [24]. Breeding heat-resistant cultivars of hard-to-breed perennial species, such as trees or vines, likely would take several decades and many resources. The effects of high temperature, elevated [CO_2], and their interaction on crop plants will be examined. Circumstances where heat-resistant cultivars could help to sustain productivity in the future will be discussed, including consideration of potential negative effects of genes that confer heat tolerance. In addition, circumstances will be described where attempting to incorporate heat tolerance may not be justified and alternative mitigation procedures will be needed.

Climate Change Effects on Crop Plants

Increased Temperature Effects on Crop Plants

From 1950 through 1979, daily minimum temperatures increased more than daily maximum temperatures. However, from 1979 through 2004, there were comparable increases in daily maximum and minimum temperatures [75]. Models predict that daytime and nighttime temperatures will both increase by about 4°C in the twenty-first century. This is important because in some cases high nighttime temperatures have been more damaging to crop productivity than high daytime temperatures [24]. There is some uncertainty with respect to how fast air temperatures may increase and how much they may increase in different parts of the world and seasons of the year.

Higher temperatures can have complex effects on plants. The approach taken in this analysis is to determine: what aspect of temperature is most damaging to productivity (Is it high day temperature or high night temperature or both and at what stage of development?); what developmental stage or physiological process is most damaged by heat stress; and what extent is economic yield (i.e., grain or fruit yield) decreased when the developmental or physiological process is damaged. Answers to these questions provide the information needed to design efficient programs for breeding heat-resistant plants.

The most reliable method to determine the effects of increases in temperature on productivity is to subject different plots of crop plants growing in the field to increases in temperature. Unfortunately, only a few studies of this type have been conducted. In one of these studies, plots of cowpea that were flowering were enclosed with plastic only during the nighttime hours and a system involving a fan, air-distribution system, heater, and differential thermostat were used to raise the air temperature in the enclosure a fixed number of degrees above ambient air temperature [53]. With this system, daytime conditions on the treated and control plots were not changed, so it is likely that the differences in productivity between the plots were caused by the differences in nighttime temperature. The cowpea plants exhibited a 4.4% decrease in grain yield per degree Celsius increase in nighttime temperature above a threshold daily minimum temperature of 15°C [54]. The lower yields were due to reductions in the proportions of flowers producing pods. In another field study, sorghum exhibited a 28% decrease in grain yield and a 30% decrease in seed number when night

temperatures were increased 5°C for 1 week during floret differentiation [17]. When rice plots were subjected to a 4°C increase in temperature both day and night using open-top chambers, there was a decrease in grain yield of 18% [47] due to reductions in spikelet and pollen fertility [45].

Another approach involves growing a crop cultivar with similar management methods in field environments with contrasting thermal regimes. A major problem with correlation studies of this type is that the different field environments also differ with respect to factors other than nighttime temperature, although an attempt was made to minimize these differences in the following examples. When cowpea was grown in four locations in a subtropical zone with contrasting thermal regimes over 2 years a 13.6% decrease in grain yield per degree Celsius increase in average minimum night temperature above a threshold of 16°C between emergence and first flowering was observed [32]. The reduction in productivity was mainly due to reductions in pod set and harvest index (HI is the ratio of grain yield to total shoot biomass). Correlation studies also have been conducted where the productivity of a rice cultivar was examined over 12 years during which time the temperature varied in a tropical zone [60]. In this case, grain yield decreased 10% per degree Celsius increase in minimum night temperature.

A third approach involves studies using glasshouses or growth chambers with artificial lighting systems. While these types of studies can produce information on the mechanisms of heat stress effects because many factors can be kept constant and the studies can be repeated, they tend not to produce reliable data concerning the yield losses due to heat that might occur in field conditions. Studies with cowpea will be discussed because much research on heat-stress effects has been conducted with this crop species and there are indications that some other crop species are affected in a similar manner. Growth chamber studies demonstrated that high night temperatures can reduce pod set of cowpea due to impaired pollination [79], whereas much higher temperatures during the day did not reduce pod set [78]. Artificial pollination studies demonstrated that the female part of the flower, the pistil, was not damaged by high night temperatures [77]. Reciprocal transfers of plants between growth chambers with high or optimal night temperatures demonstrated that the stage of floral development most sensitive to heat stress occurred 9–7 days before anthesis [1]. This stage is after meiosis, which occurs 11 days before anthesis. Damage occurred at the time that the tetrads are released from the microspore mother cell sac [1, 48, 79]. Premature degeneration of the tapetal tissue and lack of endothecium formation were observed which could have been responsible for the low pollen viability, low anther dehiscence, and low pod set under high night temperatures [1]. Tapetal tissue plays an important role in providing nutrients to developing pollen grains and its premature degeneration could thereby stunt pollen development. Based on studies with contrasting cowpea genotypes, Mutters et al. [49] proposed that heat injury during floral development of sensitive cowpea genotypes may be due to reduced translocation of proline from anther walls and tapetal tissue to developing pollen. Tapetal malfunction has been considered to be the causal mechanism of much of the cytoplasmic and genetic male sterility occurring in plant species [16, 51]. Growth chamber [50] and glasshouse studies [19] have shown that high night temperatures can be more damaging to reproductive development of cowpea under the long days typical of subtropical zones than under the short days that can occur in tropical zones.

Why is pod set in cowpea sensitive to high night temperature while it is not sensitive to much higher temperatures during the day? Growth chamber studies demonstrated that pod set of cowpea is sensitive to heat during a particular time in the night, the last 6 h and not the first 6 h of a 12-h night [48]. The greater sensitivity of pod set to heat under long days was shown to be a phytochrome-mediated effect [50]. Phytochrome-mediated events have a degree of circadian control occurring at a particular time in the 24-h cycle. Mutters and Hall [48] hypothesized that there is a heat-sensitive physiological or developmental process in pollen development that is under circadian control. Natural selection would have favored plants in which this process takes place during the coolest part of the diurnal period, which is the late night and early morning.

As with cowpea, day length also can influence the effect of high temperature on pollination of rice

[83]. In addition, glasshouse studies have shown that higher nighttime temperatures that reduced grain yield of rice also reduced percentage of pollen germination and spikelet fertility [46]. Studies in sunlit growth chambers demonstrated, however, that both high day and high night temperature can reduce spikelet fertility in rice [84]. If there is a heat-sensitive process in pollen development that occurs in the coolest period in the 24-h cycle, it could be affected by both late night and early morning temperatures.

From the field and controlled-environment studies, it is apparent that, depending on the crop species, increases in night or day temperature of 4°C during early flowering could cause substantial reductions in fruit or grain yield. The reductions in yield in cowpea and rice appear to have been mainly caused by reduction in pollination. Similar effects have been seen in some other annual crop species including common bean [23]; peanut [72, 73]; tomatoes [59]; pepper [81]; and cotton [70]. The species that appear to be particularly sensitive to high night temperature include: cowpea, common bean, pepper, cotton, sorghum, and possibly rice. Few controlled-environment studies have been conducted of high temperature effects on perennial crops. In one case, for peach, higher temperatures resulted in reduced pollen viability, lack of synchronization in fertilization, reduced fruit set, and reduced fruit yield [52].

Where heat stress is most damaging to economic yield by reducing fruit set and/or seed set, it is a case of the reproductive sink suffering greater damage than the photosynthetic source of carbohydrates. Alternative possibilities for the effects of heat stress include the photosynthetic source suffering significant damage that then reduces economic yield, and cases where both the reproductive sink and the photosynthetic source suffer significant damage.

For Irish potatoes, economic yield may be reduced because of heat-induced reductions in photosynthesis [67]; although initiation of tuberization may be even more sensitive to heat stress than photosynthesis [66]. Grain yield of wheat may be reduced in hot environments because of heat-induced reductions in photosynthesis and stomatal conductance [68]. However, high temperatures during the early floral development can result in infertile pollen and low grain set in wheat [14]. Consequently, reductions in grain yield of wheat in hot environments may be due to both reductions in photosynthesis and damage to reproductive development.

Elevated Atmospheric Carbon Dioxide Concentration [CO_2] Effects

Analyses of air trapped in polar ice indicates that, prior to the year 1800, the [CO_2] fluctuated between 180 and 290 ppm for at least 220,000 years [29]. Since 1800, ice core data indicate increases in [CO_2] from 280 to 315 ppm by 1958. Direct measurements of [CO_2] indicate accelerating increases since 1958 from 315 to 380 ppm by the early 2000s. Predictions indicate [CO_2] could exceed 600 ppm by the end of the twenty-first century.

Plants with the C_4 photosynthetic system evolved during an early period after the [CO_2] became low, and this system represents a specific adaptation to the low [CO_2] environments of the last 200,000 years. The yield responses of C_4 species to elevated [CO_2] are small, and will not be considered in this analysis. The extent and nature of the evolution of plants with the C_3 photosynthetic system, with respect to low [CO_2], are not known. It is likely that low [CO_2]s over 220,000 years resulted in evolutionary modifications to whole plant processes, such as increases in the ratio of photosynthetic source to carbohydrate sink tissues. Consequently, some C_3 plants may not be well adapted to either future or even present-day levels of [CO_2] due to inadequate investment in sink tissues [30]. Photosynthetic rates of these plants may increase when [CO_2] is increased from the current level of 380 ppm to say 600 ppm but the rates may not increase as much as they would if the whole plant system was adapted to function optimally at a [CO_2] of 600 ppm. Progress during the twenty-first century in increasing the productivity of several C_3 crops through plant breeding was estimated as mainly (77%) resulting from increases in HI with only 23% due to increases in total shoot biomass [22]. This indicates these crop species had an inadequate reproductive sink, for agricultural purposes, and with increasing [CO_2] the reproductive sink may become even more inadequate to support the full photosynthetic potential.

In early studies using controlled-environment enclosures, doubling [CO_2] increased grain yield of various small grain cereals by 32% and various grain legumes by 54% at intermediate temperatures [36]. More recent studies with free-air [CO_2] enrichment (FACE) experiments under field conditions, however, gave grain yield responses to elevated [CO_2] that were about 50% lower than those obtained using enclosures [39]. FACE experiments provide responses that farmers are more likely to get because the crops are grown under natural open-air field conditions. Yield increases in the FACE studies were less than the increases in photosynthesis that occurred with short-term doubling of [CO_2] at the same temperature [3, 61]. A possible explanation for the smaller yield responses to long-term [CO_2] enrichment is the downregulation of photosynthetic capacity that occurred in the FACE experiments [39]. This downregulation has been attributed to feedback mechanisms that operate when the supply of carbohydrates from photosynthesis exceeds sink demands for carbohydrates [3]. Limitations by sink demand were apparent in those FACE experiments where only a small proportion of the increase in photosynthate supply was partitioned to grain [39]. The results of the FACE experiments support the hypothesis that crop plants are not well adapted to the higher [CO_2]s likely to occur by the end of the twenty-first century.

Interactive Effects of Increases in Temperature and [CO_2]

The interactive effects of elevated [CO_2] and higher temperatures on plants are complex [13]. They can be simplified if one separates cases where heat stress limits the reproductive sink from cases in which heat stress limits the photosynthetic source. In many cases, reproductive development is more sensitive to heat stress than overall biomass production resulting in a decrease in HI. For soybean grown under controlled-environment field conditions, HI progressively decreased with increasing temperature under either 330 or 660 ppm [CO_2] and HI was lower with elevated [CO_2] indicating a more severe imbalance between the reproductive sink and the photosynthetic source [6]. Reproductive development of Pima cotton can be so sensitive to high temperatures that the plants do not produce either fruiting branches or bolls [63]. Studies in naturally sunlit, controlled-environment chambers demonstrated that elevated [CO_2] of 700 ppm did not ameliorate this problem [64, 65]. Controlled-environment field studies with rice demonstrated that grain yield decreased 10% per degree Celsius in average temperature above 26°C at [CO_2]s of either 330 or 660 ppm [5]. The decrease in grain yield was mainly due to fewer grains per panicle. High day and high night temperatures can cause decreases in viability of pollen grains at anthesis, increases in floret sterility, and decreases in seed set in rice [84]. Elevated [CO_2] aggravated the heat stress effect on pollen, causing a 1°C decrease in the threshold maximum canopy surface temperature after which the percentage of spikelets having ten or more germinated pollen grains exhibited a precipitous decline [45]. Heat-induced increases in floral sterility may have been responsible for the downregulation of photosynthesis observed in rice under high temperatures and elevated [CO_2] through indirect effects associated with reductions in reproductive sink strength [40].

A study with genotypes that are either heat-tolerant or heat-sensitive during reproductive development has provided unique insights into the interactive effects of high night temperature and elevated [CO_2]. With high night temperatures, many cowpea genotypes do not produce flowers, while others produce flowers but no pods and the few with total heat tolerance produce flowers and pods [18]. In growth chamber studies with contrasting genotypes in pots under high night temperature, a totally heat-sensitive genotype did not produce any flowers and a partially heat-sensitive genotype did not set any pods under either 350 or 700 ppm [CO_2] [2]. A heat-tolerant genotype had greater pod production under elevated [CO_2] at both high and more optimal night temperatures than a genetically similar cultivar that does not have the heat-tolerance genes [2]. These results indicate that for those many annual crops that are sensitive to heat during reproductive development, incorporating heat tolerance may also enhance their yield responses to elevated [CO_2] over a range of temperatures [29, 30]. This important hypothesis should be more completely tested using other cultivars of cowpea and other crop species.

For cases where the photosynthetic source is particularly sensitive to heat stress, the interactive effects with elevated [CO_2] are less clear. The review of [3] indicates that for C_3 plants, photosynthetic responses to elevated [CO_2] of individual leaves often increased with

increasing temperature, up to some maximum temperature, and in some cases biomass responded in the same way. In field studies with wheat using temperature-gradient plastic tunnels, the response of grain yield to elevated [CO_2] of 700 ppm increased from 8% at low seasonal mean temperatures of 10°C to 58% at the highest mean air temperature of 24°C [62]. Other studies with wheat have not exhibited this interactive effect on grain yield of elevated [CO_2] and high temperature [38]. Irish potato responses to elevated [CO_2] of 700 ppm were determined in field canopy chambers maintained at either moderate temperatures or heat-stress temperatures [58]. Tuber yield and total biomass were increased substantially by elevated [CO_2] in the moderate-temperature chambers. In contrast, tuber yields in the heat-stress chambers were very low and there was no effect of elevated [CO_2]. Total biomass production was not affected by the heat-stress treatment. The authors stated that "These results should be viewed as preliminary since they are based on a single growing season and one variety, but they suggest that elevated [CO_2] will not mitigate the negative effects of high-temperature stress on tuberization and yield."

Mitigating Effects of Global Warming on Crop Plants

Circumstances Where Heat-Resistant Cultivars May Help to Sustain Productivity

For some of those annual crops in which reproductive development is particularly sensitive to high temperatures, breeding to incorporate heat tolerance during reproductive development has produced cultivars that are heat-resistant in that they have greater grain or fruit yields than other cultivars in hot environments. Some examples are provided of where breeding heat-resistant cultivars may help to sustain productivity as [CO_2] and temperatures increase including some crop species where heat-resistant cultivars already have been bred, and rice and wheat because of their global importance.

Cowpea and Common Bean Some emphasis is given to cowpea because much has been published on breeding for heat tolerance in this crop. A heat-resistant cowpea cultivar was bred for use during warm seasons in subtropical zones by incorporating heat tolerance during reproductive development [20]. Developing this cultivar required 18 years of breeding. The approach was to take an overall process, heat resistance, that has complex inheritance and divide it into a developmental sequence of simpler heat-tolerant processes which individually were shown to be conferred by one or two major genes [25]. This sequence involved tolerance at the early floral bud stage that conferred the ability to produce flowers under hot long-day conditions; tolerance during pollen development that conferred the ability to set pods under high night temperature; and tolerance during embryo development that conferred the ability to produce large numbers of seeds per pod under high day or high night temperatures. A pedigree breeding program based on this approach and heat-tolerant germplasm are available that would enable additional heat-resistant cowpea cultivars to be bred for use in subtropical zones in about 6 years [28]. The University of California at Riverside has heat-tolerant cowpea lines that are available for use as parents. In breeding for heat resistance, one of these lines could be crossed with the best available cultivar for the target production zone. Heat tolerance could be incorporated by subjecting a large F_2 generation to a long-day field environment with very high night temperatures (or a glasshouse with very high night temperature and long days) and selecting plants with abundant flower production and pod set. The major gene responsible for heat tolerance during early floral development and the ability to produce flowers is recessive [25], and can be fixed by this selection. A major gene responsible for some of the heat tolerance during pod set is dominant [44]; consequently, selection during additional generations is required to fix this trait. During the fall and winter, one could either select for low leaf-electrolyte-leakage, as a measure of cellular membrane thermostability, to indirectly select for heat tolerance during pod set in the F_3 and F_4 generations in moderate-temperature glasshouses or simply advance two generations using single-seed decent. Many earlier studies have been conducted using leaf-electrolyte-leakage as a measure of cellular membrane thermostability with several crop species [10]. Definitive genetic selection studies with cowpea have demonstrated that low leaf-electrolyte-leakage can be associated with heat tolerance during pod set [71]. Individuals selected for low

leaf-electrolyte-leakage under heat stress also tended to have high pod set in hot environments. Individuals selected for high pod set in hot environments also tended to have low leaf-electrolyte-leakage under heat stress. During the next summer, replicate F_5 families could be grown in the extremely hot field nursery (or the glasshouse with very high night temperature) and in parallel nurseries to screen for agronomic traits. Families would be chosen that have abundant flower production, pod set, and number of seed per pod under high night temperature, and suitable agronomic traits in parallel nurseries. The best individual plants would be chosen from within these families. In the following fall and winter, two generations could be advanced in moderate-temperature glasshouses or in a suitable off-season field nursery. While making these generation advances, an adequate quantity of seed should be produced to enable the F_8 lines to be tested for yield and other agronomic traits in several hot, long-day, commercial production environments during the third summer. Potential new cultivars would be selected from these lines and then subjected to one more years yield testing on experiment stations followed by 2 years of yield testing on both experiment stations and farmers' fields.

Most cowpea is produced in tropical zones, such as in the Savanna and Sahelian zones of Africa and in Brazil. Progress has been made in breeding heat-tolerant cowpea cultivars for use in Africa [56, 57]. The approach used involved crossing heat-resistant cowpea parents from the University of California at Riverside with cultivars from Ghana. Selection for heat tolerance during reproductive development was conducted in subtropical long-day environments in California. Final selection for agronomic traits was conducted in northern Ghana. A modified breeding method, however, may be more effective for breeding heat-resistant cowpea cultivars for the tropics than was used for subtropical zones [28]. Heat-resistant African breeding lines could be used as parents and all selection, including that for heat tolerance, should be conducted in Africa. Selection would emphasize ability to set pods and maintenance of large numbers of seeds per pod. Many cowpea cultivars have the ability to produce about 15 ovules per pod, but they rarely produce this many seeds per pod. Under optimal field conditions, the average number of seeds per pod is about 10. In short-day conditions typical of the tropics, heat-sensitive cowpea genotypes produced 50% fewer seeds per pod in a glasshouse with high night temperatures than in a glasshouse with moderate night temperatures [19]. In these studies, several cowpea breeding lines from West Africa were shown to either have high pod set or maintain large numbers of seeds per pod under high night temperatures. These lines could be used as parents to combine heat-tolerance genes and thereby breed cowpea cultivars for Sub-Saharan Africa with greater heat resistance.

Future breeding of heat-resistant cowpea cultivars likely will be done by public plant breeders since as of 2009 there were no significant private breeding programs for cowpea in the world. Some collaborative support for breeding heat-resistant cowpea cultivars can be provided by the University of California at Riverside [31] and the Kano station of the International Institute of Tropical Agriculture in Nigeria. Breeding heat-resistant cowpea cultivars for Africa likely will proceed slowly because national programs have relatively few resources and other high priorities, such as the need to breed cowpea cultivars with resistance to various pests and diseases.

There are many similarities in the responses to heat of common bean and cowpea [27]. Progress has been made in breeding snap bean types of common beans with heat resistance through incorporating heat tolerance during reproductive development by selecting for high pod set in glasshouses with high night temperatures over two generations [15]. In addition, heat-resistant cultivars of dry bean types of common bean have been bred [7] by selecting for grain yield in hot commercial production environments [8].

Tomato Major contributions to the breeding of heat-resistant tomato cultivars for tropical zones have been made by the Asian Vegetable Research and Development Center in Taiwan [55]. This tomato improvement program gave high priority to incorporating genes for heat tolerance during fruit set. This was achieved by selecting for high fruit set in field nurseries with a sowing date that resulted in strong reductions in fruit set due to heat in sensitive materials. This program has produced open-pollinated and hybrid cultivars with heat resistance, and breeding lines that have

been released in at least 32 countries. For subtropical zones, initially, heat-resistant cultivars of tomato were bred by public programs that selected for fruit set in very hot summer field conditions, such as in the Lower Rio Grande Valley in Texas. Subsequently, since the late 1960s, several private breeding programs have been breeding heat-resistant hybrid tomato cultivars for use in the Central Valley of California and elsewhere. For understandable reasons, these private breeding programs have not divulged the methods they have used to incorporate heat tolerance.

Rice A large area of rice is grown in tropical zones at low elevation between the tropics of Cancer and Capricorn. As further warming occurs in this area the major practical solution is to develop heat-resistant rice cultivars. Breeding heat-resistant rice cultivars has received much less attention than breeding for other abiotic stresses [80] and biotic stresses or yield potential. Pollen development, pollination, and spikelet fertility are particularly sensitive to high temperatures. Rice accessions have been discovered that have heat tolerance during flowering, and potentially useful selection criteria have been determined including: flowering earlier in the morning to escape heat; substantial pollen shed (i.e., a large number of pollen grains on the stigma); and high spikelet fertility [80]. Significant research on breeding for heat tolerance in rice has been conducted by the International Rice Research Institute (IRRI) in the past (e.g., [43]). Public rice breeding programs, such as those in China and India, should be able to develop heat-resistant rice cultivars for use in tropical zones by using morphological selection criteria related to pollen and spikelet fertility. National programs in China already have bred heat-sensitive rice lines that are male sterile in hot long-day environments for use in achieving outcrossing in field environments to facilitate the production of hybrid cultivars of rice [83]. All that is needed now is for the national programs in China and elsewhere to select in the opposite direction and breed rice that is not male sterile in hot environments by incorporating greater heat tolerance.

Pima Cotton and Upland Cotton Under high temperatures, cotton plants may not produce fruiting branches or set bolls with Pima cotton being more sensitive to heat stress than upland cotton [37]. Since the early 1960s, selecting for high boll set on low nodes in very hot field conditions has been an important component of the public American Pima cotton breeding program [37]. This program has released a series of six heat-resistant cotton cultivars that have produced progressively higher lint yields under hot field conditions [42]. Surprisingly, these heat-resistant cultivars had progressively higher stomatal conductances as measured in the early afternoon on hot days during peak flowering and fruiting [42]. The authors argued that the adaptive advantage of the higher stomatal conductance appears to be associated with leaf cooling. A tendency to maintain higher stomatal conductances may be even more important in the future since elevated [CO_2] can cause stomata to partially close.

Heat-resistant upland cotton cultivars have been and are being bred for use in subtropical zones by private breeding programs. Several public programs are breeding heat-resistant cotton cultivars for use in tropical zones [70].

Wheat A large area of wheat is grown in both temperate and subtropical zones. As temperatures increase, some of this production could concentrate in temperate zones; however, heat-resistant wheat cultivars will be useful in many areas as temperatures increase. Due to current farmer interest in growing wheat in hot irrigated conditions, the International Maize and Wheat Improvement Center (CIMMT) has conducted a research program to breed cultivars for this environment. Comparisons of spring wheat cultivars with contrasting heat resistance growing in hot, irrigated environments demonstrated that grain yield was positively correlated with photosynthetic rate and leaf conductance of flag leaves, and canopy temperature depression [68]. Canopy temperature depression (CTD) is the number of degrees Celsius that the crop canopy is cooler than air temperature. Since CTD is dependent on transpiration rate, it provides an alternative to measuring leaf conductance which determines transpiration rate. The CTD is more effective for use in selection in that it can be measured more quickly and covers a larger foliage area than do measurements of leaf conductance. For environments with

sunny conditions and a large vapor pressure deficit, CTD was proposed to be a useful trait for selecting for heat tolerance in wheat grown under irrigated conditions that is correlated with higher stomatal conductance and greater photosynthesis [4]. Progress has been made in using measurements of CTD in breeding programs to enhance heat tolerance in spring wheat [69]. In a different approach, studies with genetic lines of wheat that varied in heat resistance demonstrated that low leaf-electrolyte-leakage, as a measure of cellular membrane thermostability, may provide a useful selection criterion for heat resistance in wheat [11]. The authors pointed out, however, that it may only be valuable as a supplemental selection criterion in final breeding stages or as a rough selection tool to reduce a large population into the most likely heat-tolerant core at the early stages of a breeding program. Similarly, selection for CTD may only be useful as a supplemental selection criterion [69]. For a while at least, breeding heat-resistant wheat cultivars likely will mainly depend on empirical selection for grain yield in hot commercial production environments [9].

Do Heat-Tolerance Genes Have Any Negative Effects on Crop Plants?

Genes can have negative as well as positive effects on crop performance due to either pleiotropy, where a single gene has multiple effects, or genetic linkage. One method for evaluating the negative as well as the positive effects of genes is to use backcross breeding to create pairs of near isogenic lines with and without the trait. Ideally this should be done by creating several pairs of lines with different genetic backgrounds to test for potential gene interactions. The performances of the pairs of lines are then compared in contrasting environments to document any positive and negative effects. Six pairs of cowpea lines with differences in heat tolerance during reproductive development have been compared in eight field environments with average night temperatures ranging from being cool to very hot in a subtropical zone [32]. Positive effects were apparent in that the heat-tolerant lines had greater pod set and grain yield than the heat-sensitive lines in the very hot environments. A potential negative effect was detected in that all of the heat-tolerant lines exhibited a progressive dwarfing with increases in night temperature due to shorter main-stem internodes. Performances of the semidwarf heat-tolerant lines and the standard-height heat-sensitive lines were compared at different row spacings with moderate temperatures in a subtropical zone [34]. The semidwarf heat-tolerant lines produced greater grain yields than the standard-height heat-sensitive lines when grown under narrow row spacing and similar grain yields under wide row spacing. In the good growing conditions of these experiments, the semidwarf habit of the heat-tolerant lines appears to be advantageous. In more stressful field conditions and hotter (i.e., tropical zone) environments, the dwarfing would be greater and could seriously reduce the competitiveness of the plants against weeds. The dwarfing associated with heat tolerance may have been due to genetic linkage with the recessive gene that confers heat tolerance during early floral development, and lines are available with heat tolerance during floral development that are not dwarfed [33]. Consequently, it should be possible to breed heat-tolerant cowpea lines that are not dwarfed. It should be noted, however, that the dwarfing is associated with greater HI [32] and may be responsible for the greater yield response of the heat-tolerant semidwarf line to elevated [CO_2] [2].

Potential negative effects of genes may be discovered by theoretical analysis. Heat tolerance during pod set has been associated with low membrane leakage in hot temperatures [71]. Membrane fluidity and function depend on their chemical composition and temperature [76]. Membranes with a chemical composition that is suited to function in hot conditions may not function as well in cool conditions. No evidence for this effect has been reported for cowpea. The heat-tolerant lines had similar grain yields as the heat-sensitive lines in cool field environments [32]. Lines have been bred at the University of California, Riverside that have both chilling tolerance during emergence, associated with low membrane leakage in chilling temperatures [35], and heat tolerance during pod set showing that it is possible to combine these two membrane-dependent traits. In germplasm screening studies, no association was seen between heat tolerance during emergence, chilling tolerance during emergence, and heat tolerance during flowering [21].

Circumstances Where Attempting to Incorporate Heat Tolerance into Cultivars May Not Be Justified and Alternative Mitigation Procedures Will Be Needed

There are many crop species where attempting to incorporate heat tolerance into cultivars may not be effective in sustaining productivity as temperatures increase. Tree and vine crops are difficult to breed using crossing and selection among progeny, and no models are available for incorporating heat tolerance using molecular transgenic methods that have been proven to be effective in enhancing heat resistance.

With crossing and selection, it is necessary to evaluate large numbers of progeny to find individuals that have many of the desired traits. In particular, fruit quality traits are critical for consumer acceptance and must be maintained. This phenotyping takes extensive land area and many years with tree or vine crops.

Much molecular work on heat stress has focused on heat-shock proteins. The classical studies of [82] demonstrated that leaves subjected to high temperatures (50°C) for short periods (15–30 s) tolerated high temperatures (55°C) longer than untreated leaves. Since then there have been many molecular studies of these heat-shock responses that associated them with specific proteins [74]. These proteins have been shown to play a role in enabling seedlings to survive extreme heat shock but they have not been associated with heat tolerance during reproductive development [74]. For example, cowpea genotypes with contrasting heat tolerance during reproductive development [18] have been examined by several laboratories but no differences in heat-shock proteins were detected among them. The heat-shock proteins have not been shown to be useful in enhancing heat resistance, the economic yield of crops in hot environments (e.g., reviews by [12, 76]).

Impacts of future climate changes on some perennial crops in California have been modeled [41]. The authors concluded that climate change in California is very likely to put downward pressure on yields of almonds, walnuts, avocados, and table grapes by 2050; although, they did not include effects of elevated [CO_2] in their analysis. FACE studies with tree species showed an average 28% increase in aboveground biomass production under elevated [CO_2] [39]. Given the long timescales for orchards and vineyards of about 30 years, temperature increases should be considered when selecting perennial cultivars for new plantings. Temperature effects on tree crops can be complex. For example, some deciduous tree crops have a chilling requirement that must be met during the winter to overcome bud dormancy. Insufficient chilling due to warming in winter could result in delayed opening of leaf buds and delayed bloom, and the flowers could be abnormal such that fruit set is reduced [26]. Cultivars of tree crops are available that have smaller chilling requirements but removing an orchard and then replacing it with another cultivar would incur considerable costs since the new orchard would not produce many fruit for several years [41].

Future Directions

For several important annual grain and fruit crops, incorporating heat tolerance during reproductive development by selecting for seed or fruit set under hot conditions can be used to breed heat-resistant cultivars that will help to sustain productivity as air temperatures increase. These cultivars also may be more responsive to the elevated [CO_2] that will occur in the future. For cases where private plant breeding companies are unlikely to do the necessary breeding, such as with rice and cowpea, more funding and resources should be provided to the public national and international programs that could do this breeding.

For annual crops that produce neither grain nor fruit or for which the main lesion due to heat stress is reduced photosynthetic function, more efficient selection methods could facilitate breeding for heat resistance. More efficient selection methods might be developed if more funds were provided for applied research on heat tolerance in plants. Over the last three decades, relatively little research funding has been devoted to the physiology and genetics of breeding for heat tolerance. In contrast, during this same period, substantial funding has been devoted to molecular studies of heat-shock proteins which, as of 2009, has not produced a documented practical benefit to crop breeding.

For perennial crops, breeding for heat tolerance is extremely difficult. In this case, alternative mitigation methods should be pursued. Predictive methods

should be developed and applied to assist farmers to determine when orchards should be removed and what crop and cultivar should be grown instead.

Bibliography

Primary Literature

1. Ahmed FE, Hall AE, DeMason DA (1992) Heat injury during floral development of cowpea (*Vigna unguiculata*, Fabaceae). Am J Bot 79:784–791
2. Ahmed FE, Hall AE, Madore MA (1993) Interactive effects of high temperature and elevated carbon dioxide concentration on cowpea (*Vigna unguiculata* (L.) Walp.). Plant Cell Environ 16:835–842
3. Allen LH (1994) Carbon dioxide increase: direct impacts on crops and indirect effects mediated through anticipated climate changes. In: Boote KJ, Bennett JM, Sinclair TR, Paulsen GM (eds) Physiology and determination of crop yield. Crop Science Society of America, Madison
4. Amani I, Fischer RA, Reynolds MP (1996) Canopy temperature depression associated with yield of irrigated spring wheat cultivars in a hot climate. J Agron Crop Sci 176:119–129
5. Baker JT, Allen LH (1993) Contrasting crop species response to CO_2 and temperature: rice, soybean and citrus. Vegetatio 104/105:239–260
6. Baker JT, Allen LH, Boote KJ, Jones P, Jones JW (1989) Responses of soybean to air temperature and carbon dioxide concentration. Crop Sci 29:98–105
7. Beaver JS, Miklas PN, Echavez-Badel R (1999) Registration of 'Rosada Nativa' pink bean. Crop Sci 39:1257
8. Beaver JS, Rosas JC, Myers J, Acosta J, Kelly JD, Nchimbi-Msolla S, Misangu R, Bokosi J, Temple S, Arnaud-Santana E, Coyne DP (2003) Contributions of the bean/cowpea CRSP to cultivar and germplasm development in common bean. Field Crops Res 82:87–102
9. Behl RK, Nainawatee HS, Singh KP (1993) High temperature tolerance in wheat. In: Buxton DR, Shibles R, Forsberg RA, Blad BL, Asay KH, Paulsen GM, Wilson RF (eds) International crop science I. Crop Science Society of America, Madison
10. Blum A (1988) Plant breeding for stress environments. CRC, Boca Raton
11. Blum A, Klueva N, Nguyen HT (2001) Wheat thermotolerance is related to yield under heat stress. Euphytica 117:117–123
12. Cheikh N, Miller PR, Kishore G (2000) Role of biotechnology in crop productivity in a changing environment. In: Reddy KR, Hodges HF (eds) Climate change and global crop productivity. CAB International, Wallingford
13. Conroy JP, Seneweera S, Basra AS, Rogers G, Nissen-Wooler B (1994) Influence of rising atmospheric CO_2 concentrations and temperature on growth, yield and grain quality of cereal crops. Aust J Plant Physiol 21:741–758
14. Dawson IA, Wardlaw IF (1989) The tolerance of wheat to high temperatures during reproductive growth. III. Booting to anthesis. Aust J Agric Res 40:965–980
15. Dickson MH (1993) Breeding for heat tolerance in green beans and broccoli. In: Kuo CG (ed) Adaptation of food crops to temperature and water stress. Asian Vegetable Research and Development Center, Shanhua, Publication No 93-410
16. Dundas I, Saxena KB, Byth DE (1981) Microsporogenesis and anther wall development in male-sterile and fertile lines of pigeon pea (*Cajanus cajan* [L.] Millsp.). Euphytica 30:431–435
17. Eastin JD, Castleberry RM, Gerik TJ, Hutquist JH, Mahalakshmi V, Ogunela VB, Rice JR (1983) Physiological aspects of high temperature and water stress. In: Raper CD, Kramer PJ (eds) Crop reactions to water and temperature stresses in humid, temperate climates. Westview Press, Boulder
18. Ehlers JD, Hall AE (1996) Genotypic classification of cowpea based on responses to heat and photoperiod. Crop Sci 36:673–679
19. Ehlers JD, Hall AE (1998) Heat tolerance of contrasting cowpea lines in short and long days. Field Crops Res 55:11–21
20. Ehlers JD, Hall AE, Patel PN, Roberts PA, Matthews WC (2000) Registration of 'California Blackeye 27' cowpea. Crop Sci 40:854–855
21. El-Kholy AS, Hall AE, Mohsen AA (1997) Heat and chilling tolerance during germination and heat tolerance during flowering are not associated in cowpea. Crop Sci 37:456–463
22. Gifford RM (1986) Partitioning of photoassimilate in the development of crop yield. In: Luca WJ, Cronshaw J (eds) Phloem transport. Alan R. Liss, New York
23. Gross Y, Kigel J (1994) Differential sensitivity to high temperatures of stages in the reproductive development of common bean (*Phaseolus vulgaris* L.). Field Crops Res 36:201–212
24. Hall AE (1992) Breeding for heat tolerance. Plant Breed Rev 10:129–168
25. Hall AE (1993) Physiology and breeding for heat tolerance in cowpea, and comparison with other crops. In: Kuo CG (ed) Adaptation of food crops to temperature and water stress. Asian Vegetable Research and Development Center, Shanhua, Publication No 93-410
26. Hall AE (2001) Crop responses to environment. CRC, Boca Raton
27. Hall AE (2004) Comparative ecophysiology of cowpea, common bean and peanut. In: Nguyen HT, Blum A (eds) Physiology and biotechnology integration for plant breeding. Marcel Decker, New York
28. Hall AE (2011) Breeding cowpea for future climates. In: Yadaf S, Redden B, Hatfield JL, Lotze-Campen H, Hall AE (eds) Crop adaptation to climate change. Wiley-Blackwell, Ames
29. Hall AE, Allen LH (1993) Designing cultivars for the climatic conditions of the next century. In: Buxton DR, Shibles R, Forsberg RA, Blad BL, Asay KH, Paulsen GM, Wilson RF (eds) International crop science I. Crop Science Society of America, Madison
30. Hall AE, Ziska LH (2000) Crop breeding strategies for the 21st century. In: Reddy KR, Hodges HF (eds) Climate change and global crop productivity. CAB International, Wallingford
31. Hall AE, Cisse N, Thiaw S, Elawad HOA, Ehlers JD, Ismail AM, Fery RL, Roberts PA, Kitch LW, Murdock LL, Boukar O, Phillips RD,

McWatters KH (2003) Development of cowpea cultivars and germplasm by the bean/cowpea CRSP. Field Crops Res 82:103–134
32. Ismail AM, Hall AE (1998) Positive and potential negative effects of heat-tolerance genes in cowpea lines. Crop Sci 38:381–390
33. Ismail AM, Hall AE (1999) Reproductive-stage heat tolerance, leaf membrane thermostability and plant morphology in cowpea. Crop Sci 39:1762–1768
34. Ismail AM, Hall AE (2000) Semidwarf and standard-height cowpea responses to row spacing in different environments. Crop Sci 40:1618–1623
35. Ismail AM, Hall AE, Close TJ (1999) Allelic variation of a dehydrin gene co-segregates with chilling tolerance during seedling emergence. Proc Natl Acad Sci 96:13566–13570
36. Kimball BA (1983) Carbon dioxide and agricultural yield: an assembly and analysis of 430 prior observations. Agron J 75:779–788
37. Kittock DL, Turcotte EL, Hofman WC (1988) Estimation of heat tolerance improvement in recent American Pima cotton cultivars. J Agron Crop Sci 161:305–309
38. Lawlor DW, Mitchell RAC (2000) Crop ecosystems responses to climate change: wheat. In: Reddy KR, Hodges HF (eds) Climate change and global crop productivity. CAB International, Wallingford
39. Leakey ADB, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR (2009) Elevated CO_2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. J Exp Bot 60:2859–2876
40. Lin W, Ziska LH, Namuco OS, Bai K (1997) The interaction of high temperature and elevated CO_2 on photosynthetic acclimation of single leaves of rice in situ. Physiol Plant 99:178–184
41. Lobell DB, Field CB, Cahill KN, Bonfils C (2006) Impacts of future climate change on California perennial crop yields: model predictions with climate and crop uncertainties. Agric For Meteor 141:208–218
42. Lu Z, Percy RG, Qualset CO, Zeiger E (1998) Stomatal conductance predicts yields in irrigated Pima cotton and bread wheat grown at high temperatures. J Exp Bot 49:453–460
43. Mackill DJ, Coffman WR (1983) Inheritance of high temperature tolerance and pollen shedding in a rice cross. Z Pflanzenzüchtg 91:61–69
44. Marfo KO, Hall AE (1992) Inheritance of heat tolerance during pod set in cowpea. Crop Sci 32:912–918
45. Matsui T, Namuco OS, Ziska LH, Horie T (1997) Effects of high temperature and CO_2 concentration on spikelet sterility in indica rice. Field Crops Res 51:213–219
46. Mohammed AR, Tarpley L (2009) High nighttime temperatures affect rice productivity through altered pollen germination and spikelet fertility. Agric For Meteor 149:999–1008
47. Moya TB, Ziska LH, Namuco OS, Olszyk D (1998) Growth dynamics and genotypic variation in tropical, field-grown paddy rice (*Oryza sativa* L.) in response to increasing carbon dioxide and temperature. Glob Change Biol 4: 645–656
48. Mutters RG, Hall AE (1992) Reproductive responses of cowpea to high temperatures during different night periods. Crop Sci 32:202–206
49. Mutters RG, Ferreira GR, Hall AE (1989) Proline content of the anthers and pollen of heat-tolerant and heat-sensitive cowpea subjected to different temperatures. Crop Sci 29: 1497–1500
50. Mutters RG, Hall AE, Patel PN (1989) Photoperiod and light quality effects on cowpea floral development at high temperatures. Crop Sci 29:1501–1505
51. Nakishima H, Horner HT, Palmer RG (1984) Histological features of anthers from normal and ms3 mutant soybean. Crop Sci 24:735–739
52. Nava GA, Damalgo GA, Bergamaschi H, Paniz R, Pires dos Santos R, Marodin GAB (2009) Effect of high temperatures in the pre-blooming and blooming periods on ovule formation, pollen grains and yield of 'Granada' peach. Sci Hortic 122:37–44
53. Nielsen CL, Hall AE (1985) Responses of cowpea (*Vigna unguiculata* (L.) Walp.) in the field to high night air temperatures during flowering. I. Thermal regimes of production regions and field experimental system. Field Crops Res 10:167–179
54. Nielsen CL, Hall AE (1985) Responses of cowpea (*Vigna unguiculata* (L.) Walp.) in the field to high night air temperatures during flowering. II. Plant responses. Field Crops Res 10:181–196
55. Opeña RT, Chen JT, Kuo CG, Chen HM (1993) Genetic and physiological aspects of tropical adaptation in tomato. In: Kuo CG (ed) Adaptation of food crops to temperature and water stress. Asian Vegetable Research and Development Center, Shanhua, Publication No 93-410
56. Padi FK, Denwar NN, Kaleem FZ, Salifu AB, Clottey VA, Kombiok J, Haruna M, Hall AE, Marfo KO (2004) Registration of 'Apagbaala' cowpea. Crop Sci 44:1486
57. Padi FK, Denwar NN, Kaleem FZ, Salifu AB, Clottey VA, Kombiok J, Haruna M, Hall AE, Marfo KO (2004) Registration of 'Marfo-Tuya' cowpea. Crop Sci 44:1486–1487
58. Peet MM, Wolfe DW (2000) Crop ecosystem responses to climate change: vegetable crops. In: Reddy KR, Hodges HF (eds) Climate change and global crop productivity. CAB International, Wallingford
59. Peet MM, Sato S, Gardner R (1998) Comparing heat stress on male-fertile and male-sterile tomatoes. Plant Cell Environ 21:225–231
60. Peng S, Huang J, Sheehy JE, Laza RC, Visperas RM, Zhong X, Centeno GS, Kush GS, Cassman KG (2004) Rice yields decline with higher night temperature from global warming. Proc Natl Acad Sci 101:9971–9975
61. Poorter H (1993) Interspecific variation in the growth responses of plants to an elevated ambient CO_2 concentration. Vegetatio 104/105:77–97
62. Rawson HM (1995) Yield responses of two wheat genotypes to carbon dioxide and temperature in field studies using temperature gradient tunnels. Aust J Plant Physiol 22:23–32

63. Reddy KR, Hodges HF, McKinion JM, Wall GW (1992) Temperature effects on Pima cotton growth and development. Agron J 84:237–243
64. Reddy KR, Hodges HF, McKinion JM (1995) Carbon dioxide and temperature effects on Pima cotton development. Agron J 87:820–826
65. Reddy KR, Hodges HF, McKinion JM (1997) A comparison of scenarios for the effect of global climate change on cotton growth and yield. Aust J Plant Physiol 24:707–713
66. Reynolds MP, Ewing EE (1989) Effects of high air and soil temperature stress on growth and tuberization in *Solanum tuberosum*. Ann Bot 64:241–247
67. Reynolds MP, Ewing EE, Owens TG (1990) Photosynthesis at high temperature in tuber-bearing *Solanum* species. Plant Physiol 93:791–797
68. Reynolds MP, Balota M, Delgado MIB, Amani I, Fischer RA (1994) Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. Aust J Plant Physiol 21:717–730
69. Reynolds MP, Singh RP, Ibrahim A, Ageeb OAA, Larqué-Saavedra A, Quick JS (1998) Evaluating physiological traits to complement empirical selection for wheat in warm environments. Euphytica 100:85–94
70. Singh RP, Vara Prasad PV, Sunita K, Giri SN, Reddy KR (2007) Influence of high temperature and breeding for heat tolerance in cotton: a review. Adv Agron 93:313–385
71. Thiaw S, Hall AE (2004) Comparison of selection for either leaf-electrolyte-leakage or pod set in enhancing heat tolerance and grain yield of cowpea. Field Crops Res 86: 239–253
72. Vara Prasad PV, Craufurd PQ, Summerfield RJ (1999) Sensitivity of peanut to timing of heat stress during reproductive development. Crop Sci 39:1352–1357
73. Vara Prasad PV, Craufurd PQ, Summerfield RJ (1999) Fruit number in relation to pollen production and viability in groundnut exposed to short periods of heat stress. Ann Bot 84:381–386
74. Vierling E (1991) The roles of heat-shock proteins in plants. Annu Rev Plant Physiol 42:579–620
75. Vose RS, Easterling DR, Gleason B (2005) Maximum and minimum temperature trends for the globe: an update through 2004. Geophys Res Lett 32:L23822
76. Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61:199–223
77. Warrag MOA, Hall AE (1983) Reproductive responses of cowpea to heat stress: genotypic differences in tolerance to heat at flowering. Crop Sci 23:1088–1092
78. Warrag MOA, Hall AE (1984) Reproductive responses of cowpea (*Vigna unguiculata* (L.) Walp.) to heat stress. I. Responses to soil and day air temperatures. Field Crops Res 8:3–16
79. Warrag MOA, Hall AE (1984) Reproductive responses of cowpea (*Vigna unguiculata* (L.) Walp.) to heat stress. II. Responses to night air temperature. Field Crops Res 8:17–33
80. Wasserman R, Jagadish SVK, Heuer S, Ismail A, Redona E, Serraj R, Singh RK, Howell G, Pathak H, Sumfleth K (2009) Climate change affecting rice production: the physiological and agronomic basis for possible adaptation strategies. Adv Agron 101:59–67
81. Wien HC (1997) Pepper. In: Wien HC (ed) The physiology of vegetable crops. CAB International, Wallingford
82. Yarwood CE (1961) Acquired heat tolerance of leaves to heat. Science 134:941–942
83. Ziguo Z, Hanlai Z (1992) Fertility alteration in photoperiod-sensitive genic male sterile (PGMS) rice in response to photoperiod and temperature. Int Rice Res Newsl 17:7–8
84. Ziska LH, Manalo PA (1996) Increasing night temperature can reduce seed set and potential yield of tropical rice. Aust J Plant Physiol 23:791–794

Websites

Hall AE (2000) Heat stress section in www.plantstress.com

Transgene Expression in Plants, Control of

Sylvie De Buck[1], Annelies De Paepe[2], Ann Depicker[3]
[1]Department of Plant Biotechnology and Genetics, Ghent University, Ghent, Belgium
[2]Department of Plant Systems Biology, Flanders Institute for Biotechnology VIB, Ghent, Belgium
[3]UGent Vakgroep Plantenbiotechnologie en Genetica, VIB Plant Systems Biology Department, Ghent University, Ghent, Belgium

Article Outline

Glossary

CRE/*loxP* A site-specific recombination system in which the CRE recombinase catalyzes recombination between *loxP* sequences. A *loxP* sequence is 34 bp long and consists of two inverted repeats of 13 bp and one spacer region of 8 bp. The orientation of the *loxP* sequence is determined by the spacer region.

Inverted T-DNA repeat Repeat obtained from the integration of two T-DNA copies at one genetic locus, but with one T-DNA integrated in inverted orientation compared to the other.

Position effect Influence of the position of a gene in the genome upon its expression.

Posttranscriptional gene silencing (PTGS) Silencing mechanism leading to the sequence-specific degradation of target mRNAs and sequence-specific suppression of translation.

Single-copy transformant Transgenic plant harboring only one copy of the introduced DNA segment.

Transcriptional gene silencing (TGS) Silencing mechanism that targets homologous DNA sequences in the promoter, suppressing transcription and correlating with promoter sequence methylation.

Transfer DNA (T-DNA) The DNA fragment that is delineated by the right and left border repeats and is transferred from *Agrobacterium* to the plant cell by the type-IV secretion system.

Transgene expression variability Variability that is higher in a population of transformants than expected based on gene dosage effects.

Transgenic plant Plant harboring one or more external DNA segments that had been introduced and stabilized by integration into the plant genome. The foreign DNA is transferred to the plant cell via *Agrobacterium*-mediated transformation or direct gene transfer.

Definition of the Subject

To define the subject in relation to the title, we would like to emphasize that most of the results and conclusions described below were obtained in the model plants *Arabidopsis thaliana* and *Nicotiana tabacum* after *Agrobacterium*-mediated transformation, and in a limited number of crops, such as wheat, rice, and soybean, after direct gene transfer. Additionally, most of the transgenes described below are transcriptional fusions with the 35S promoter from the cauliflower mosaic virus. Indeed, to obtain transgenic plants with high and constitutive expression of a transgene, the 35S promoter is commonly used. This 35S promoter is very powerful, because it generally leads to constitutively high transcript and protein levels of the transgene in most dicot plants and is not greatly influenced by

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

environmental conditions or tissue types. However, over the years, it became clear that 35S-driven transgene expression is very variable in the different transformants of a transgenic plant population with the same construct. For most analyzed overexpression constructs containing this 35S promoter, more than a 100-fold difference in protein accumulation levels has been observed. Approximately 20% of the transformants have high recombinant protein accumulation levels, but approximately 80% display an intermediate or low and unstable transgene expression. Additionally, high-expression transformants often segregate progeny plants with low recombinant protein accumulation levels. Thus, it is imperative that for scientific analysis and applications, the variability of 35S-driven transgenes is known in different transformants and generations. In this contribution, the possible causes of variation in transgene expression in a population of transgenic plants are discussed and several approaches to diminish the variability are reviewed.

Introduction

From the mid-1980s on, transgenic plants were generated by adding one or more genes to a plant's genome. Transformation was usually achieved with gold-particle bombardment or through the gene transfer process via *Agrobacterium tumefaciens*, a soil bacterium carrying an engineered T-DNA vector. The inserted gene sequence, known as the transgene, can be derived from any living prokaryotic or eukaryotic organism. Transgenic plants were originally engineered to address specific scientific questions and to investigate the plant biology in general. However, the ability to create transgenic plants also opened the way to manipulate crop plants for better characteristics, such as increased yield, enhanced quality, pest or disease resistance, superior tolerance to heat, cold, and drought, and nutritional improvement. In addition, transformation of plants allows molecular farming of many limiting components, such as proteins, metabolites, fatty acids, and bioenergy precursors.

To generate transgenic plants, transformation methods had to be explored and ameliorated. Furthermore, the vectors and expression cassettes for controlled integration and expression of the introduced transgenes had to be worked out. Once the *Agrobacterium*-based system for T-DNA transfer had been unraveled, the simple view was that a particular T-DNA sequence could be constructed to be integrated as a single copy into the plant genome. However, it turned out that transformants quite often integrate two to ten T-DNA copies at one locus or dispersed over several loci [1]. Moreover, the transferred DNA is integrated randomly and, thus, every transformant containing the same transgene differs regarding the integration site and the transgene copy number [2]. Concerning transgene expression, in principle, it is very straightforward to combine particular gene-controlling elements with a coding sequence of interest to obtain a predictable level of transgene expression. The most frequently used promoter elements are the promoter of the nopaline synthase gene (pNOS), driving weak and constitutive expression of the downstream transcribed sequence, and the promoter of the cauliflower virus 35S transcript (p35S), driving in general at least 20-fold higher expression levels. Whereas the majority of transformants with transgenes controlled by the pNOS promoter display the same mRNA level, independent transgenic lines generated with a p35S-controlled transgene construct show variable transgene expression with more than 100-fold difference in recombinant protein levels. In the latter case, frequently the recombinant protein accumulation levels in the different transformants are distributed bimodally [3]. Low p35S transgene expression occurs regularly in transgenic lines with multiple T-DNA copies, particularly when they are integrated into inverted orientation. As a consequence, the transgenes are transcribed convergently [4]. However, also single T-DNA copies can result in low or even no transgene expression [5]. Originally, the mean of recombinant protein accumulation levels in different transformants was considered to represent the expression strength of a particular transgene construct. Afterward, it was realized that the class of transformants with high transgene expression correlated better with the transgene expression capacity [6], implying that all transformants with intermediate and low transgene expression have controlling mechanisms that result in transgene suppression or transgene silencing.

A first and visually dramatic example of transgene silencing was found in plants that overexpressed gene constructs coding for pigment production in

wild-type petunia (*Petunia hybrida*) with purple flowers [7, 8]. Contrary to their expectations, the flowers of the transgenic petunia exhibited reduced and variable instead of increased pigmentation. As both the expression of endogenes and transgenes were suppressed, this phenomenon was called cosuppression [7, 8]. Later on, the transgene silencing mechanisms were divided into three groups [9]. One group harbors the transgene silencing events based on position effects and heterochromatization. As transgenes integrate randomly, different transformants contain the transgene at a different chromosomal position and nearby located chromatin-controlling elements, enhancers and silencers, are expected to influence the activity of the integrated gene. The other two groups of silencing mechanisms operate via homology between the silencer sequences and the target silenced genes, do not depend on position, and exert their effects *in trans* [9, 10]. Transcriptional silencing (TGS) refers to the silencing process that suppresses transcription of the silenced genes based on homology in the silencer locus-containing promoter region. TGS functions at the level of prevention of transcription initiation of the silenced genes; is often accompanied with concomitant DNA methylation, altered histone modifications, and heterochromatin formation; and is stably inherited through meiosis and mitosis [11]. In contrast, post-transcriptional gene silencing (PTGS) needs homology in the transcribed region, results in specific transcript degradation and/or specific repression of translational initiation, and resets through sexual propagation [10, 12].

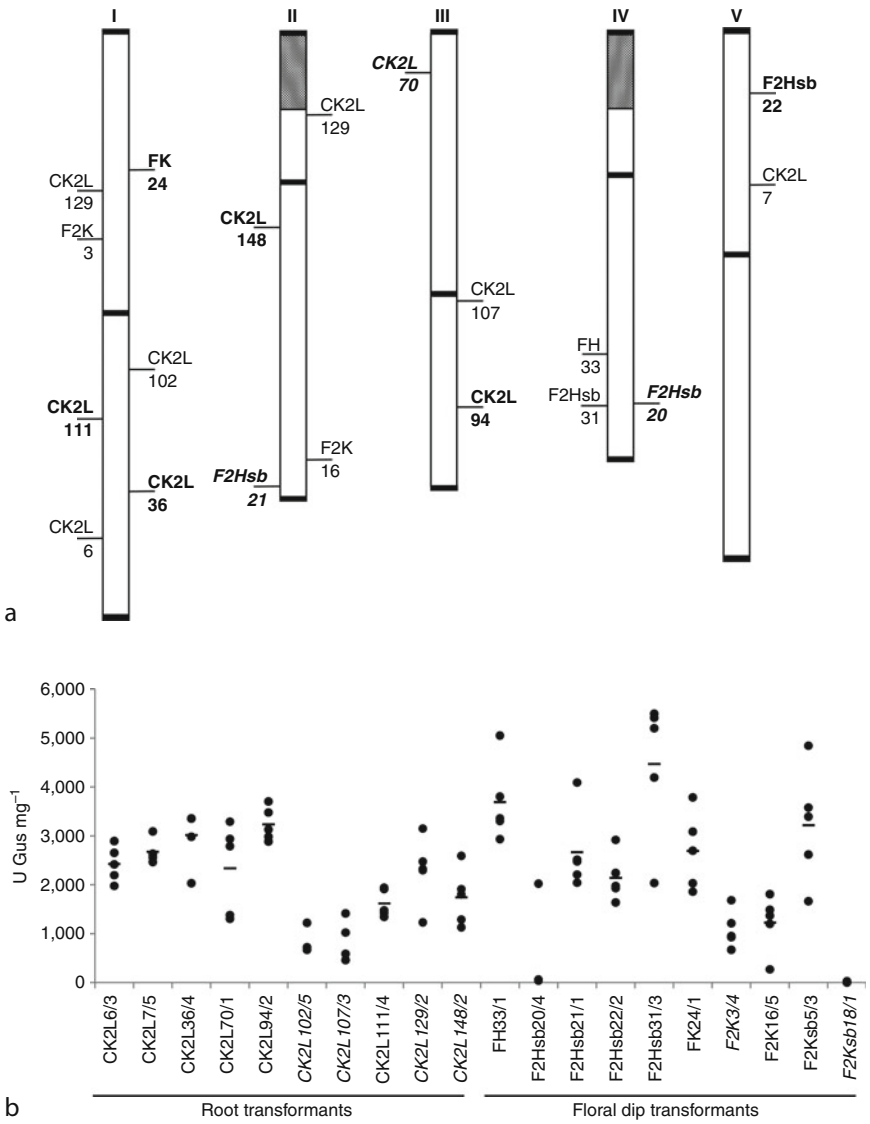
Here, different parameters that influence transgene expression in plants are described: (1) the transformation process, which results in plants with transgene loci that can be either simple or complex; (2) the different regulatory sequences used in the transgene expression construct; (3) the position of the integrated transgene into the plant genome; and (4) the correlation between repeat structures and transgene silencing. Several approaches to minimize the transgene expression variability are discussed: (1) the resolution of complex integration loci by the CRE/*loxP* recombination process, (2) site-specific or site-directed integration, and (3) the use of gene silencing mutants to create a population of transgenic plants with high and stable transgene expression.

Generation of Transgenic Plants by *Agrobacterium*-Mediated Transformation or Direct Gene Transfer

Agrobacterium-mediated transformation is the most widely used method to generate transgenic plants [13, 14], although direct transformation methods are still very important for certain crop plants (see below). During *Agrobacterium* transformation, the transferred DNA (T-DNA), delineated by the right border (RB) and left border (LB) repeats, is integrated into the plant genome by illegitimate recombination. Overall, approximately one half of the transformants contains only one T-DNA copy and the other half multiple T-DNAs (two to ten), integrated at one or several independent loci [2, 15].

The T-DNA integrates at random in any chromosome [2] (Fig. 1) and is as efficient in all five chromosomes of *Arabidopsis thaliana* as in intergenic and intragenic sequences and in exons and introns (Fig. 1) [16, 17]. With promoterless selectable markers and consecutive selection on those markers, many transformants could be recovered, indicating that T-DNAs frequently integrate in transcriptionally active regions [18, 19], but in the absence of transcriptional activation selection, many transformants integrated the T-DNA between genes and in repetitive DNA. Under nonselective conditions, silent T-DNA insertions were found in heterochromatic regions, centromeres, telomeres, and rDNA repeats [20, 21]. T-DNA integration is accompanied with small deletions of the plant target DNA and/or T-DNA ends. Very often, microhomology of 2–10 bp is observed between the plant pre-insertion site and the T-DNA ends, suggesting that T-DNA integration occurs via the nonhomology-dependent double-stranded break repair [2, 15, 22].

In theory, only the T-DNA, delineated by the LB and RB, is transferred to the plant cell, but in many plants, vector backbone sequences are transferred and integrated into the genome as well [23–26]. Two mechanisms might account for this vector backbone transfer. First, the LB repeat could mistakenly be recognized as initiation site for T-strand production and, as such, result in the transfer of vector backbone sequences. Second, the consequence of inefficient recognition of the LB repeat might be read-through from the T-DNA into the vector sequences [23, 27]. Both mechanisms



Transgene Expression in Plants, Control of. Figure 1

Random integration of single-copy T-DNAs into the *Arabidopsis* genome and the similar GUS activity levels yielded by single-copy 35S-*GUS* transgenes in the different transformants (Adapted from [17]). (**a**) Distribution of 18 single-copy T-DNA transformants on the five chromosomes of the *Arabidopsis* genome. In nine transformants, the T-DNA is integrated into an intergenic region and nine in a transcribed annotated gene (indicated in *bold*). Of these nine intragenic inserted T-DNAs, six were integrated into an exon and three into an intron (indicated in *italics*). Centromeres and telomeres are indicated in *vertical black boxes* and rDNA repeats in *gray boxes*. For transformant CK2L129, the LB and RB regions were fused to sequences of chromosomes 1 and 2. (**b**) GUS activity analysis in the T2 generation of 20 different single-copy T-DNA transformants. GUS activity was measured in the leaves of five 6-week-old seedlings per transformant (represented as a *dot*). All transformants were homozygous for the T-DNA insertion, except for the transformants indicated in *italics*. The average GUS activity level in the five seedlings of the homozygous transformants is marked by a *line*. The intra-transformant variability in GUS activity was as high as the inter-transformant variability, except for two transformants without detectable GUS activity. GUS activity levels are given as units GUS mg^{-1} of total soluble protein. For transformants F2Ksb5 and F2Ksb18, the integration position into the genome could not be determined (see **a**)

can be distinguished by analyzing whether or not the LB T-DNA sequences are directly linked to the vector sequences. The vector backbone transfer and integration is seemingly not influenced by the plant species, the explant type used for transformation, the T-DNA vector replicon type, or the selection [25]. Molecular DNA blot and polymerase chain reaction analyses revealed that the vector backbone sequences were mostly linked to both the LB and RB T-DNAs. In fact, the complete vector backbone sequence was integrated between two in tandem oriented T-DNAs, emphasizing the importance of the second mechanism in which the integration of complete vector backbone sequences results from a conjugative transfer initiated at the RB, followed by copying of the T-DNA, the vector, and again a T-DNA from a circular template due to read-through of consecutively the LB and RB [25]. Furthermore, the results demonstrated that neither the RB nor the LB was efficiently recognized as termination and initiation sites, respectively [25]. Additional evidence that the surrounding regions of the border repeats are important for the recognition of the repeat as initiation or termination site was obtained from hybrid border regions. In the absence of the surrounding border regions, the LB repeat was not recognized as T-DNA termination site; addition of the natural LB inner region and occurrence of both the octopine and nopaline LB regions with their repeats, improved the correct recognition of the LB repeat when compared to an LB with only the LB outer region [26].

A number of agronomically important plant species are still recalcitrant for *Agrobacterium*-mediated transformation and are, therefore, transformed with direct gene transfer methods, such as particle bombardment or biolistics, electroporation, and polyethylene glycol transformation [28]. A disadvantage of these techniques is that transformants tend to have multiple transgene copies integrated as a concatemeric array at one locus, and contain inverted repeats [29–33]. However, several approaches have been described to circumvent this problem (see below).

Transgene Loci Can Be Either Simple or Complex

Upon *Agrobacterium*-mediated transformation, a significant number of transformants contain a single-copy T-DNA insert, but, dependent on the transformation method used, a high percentage of transformants contain multiple T-DNAs integrated into the plant host genome. These multiple T-DNA copies are mostly clustered in one genetic locus, but they can also be present in two or more loci. Multiple T-DNAs integrated at one genetic locus are organized as direct and/or inverted repeats of the T-DNA segment. Complex T-DNA loci were found in many plant species, such as *Arabidopsis*, tobacco (*Nicotiana tabacum*), petunia, and potato (*Solanum tuberosum*) [34–38]. As direct and inverted T-DNA repeats were not found in the bacteria, they are believed to be formed in the plant cell prior to or during the T-DNA integration [34, 35, 39, 40].

Two models have been proposed for the formation of multicopy T-DNA loci upon *Agrobacterium*-mediated transformation: the replication and the ligation models [38]. In the replication model, the repeats originate from a single T-DNA copy that is replicated after introduction in the plant cell and before or during integration into the genome. This hypothesis is favored because all T-DNAs involved in repeat structures had analogous breakpoints in a restriction analysis and because three identical T-DNA copies were observed after transformation with a library of T-DNAs containing different promoters [38, 41, 42]. However, cotransformation experiments with different T-DNAs originating from different agrobacteria gave, with a similar frequency, rise to T-DNA loci with different T-DNAs in direct or inverted orientation [1, 34, 35, 43–46]. As these T-DNA structures cannot be formed by replication, but only by ligation of the cotransformed T-DNA copies before or during integration [34, 35], the ligation model postulates that repeats originate from extrachromosomal ligation of two or more individual T-DNAs prior to or during integration into the genome [34] and even that repeat structures are formed by the cointegration of several T-strand intermediates in one target site rather than by ligation of T-strands [39]. In any case, T-DNA inverted repeats about the RB, an integration structure that is frequently observed upon *Agrobacterium*-mediated transformation, can only be formed after duplication of the transferred single-stranded T-DNAs. Sequence analysis of T-DNA junctions revealed that, during the formation of T-DNA repeat structures, end-to-end ligation of double-stranded T-DNAs occurs especially between right T-DNA ends, whereas recombination based on microhomology regions and insertions of filler

DNA was more frequent at LB junctions [22, 35]. None of the sequenced T-DNA/T-DNA junctions contained plant DNA, suggesting that T-DNA recombination and ligation occurred in the majority of cases before integration [35].

Analysis of which parameter determined the structure and complexity of the transgene locus indicated a possible correlation with either the used *Agrobacterium* strain, the titer, or the physiology of the bacterial inocula, bacterial vectors, plant species, or ecotype, but overall no clear picture was obtained [1, 34, 38, 44, 47, 48]. Single-copy T-DNA insertions were found predominantly upon *Arabidopsis* root transformation, whereas multiple insertions, especially organized in T-DNA repeat structures over the RB occurred in transformants generated by leaf disc transformation [48]. However, this was not found in root and leaf disc transformants in an independent experiment (M. De Neve and A. Depicker, unpublished results), indicating that other parameters had to be involved. Nevertheless, a detailed study revealed that the structure of the T-DNA integration locus is especially determined by the transformed target cell [1, 48]. Whereas 20 years ago, *Agrobacterium*-mediated transformation of *Arabidopsis* was achieved mainly by in vitro tissue explant methods, such as root transformation [49], nowadays floral dip is commonly used [50], but the obtained T-DNA copy number in transformants obtained after both transformation methods varies much. To analyze whether this difference was due to cotransformation frequencies or to replication in the plant cell, several floral dip and root transformations were done with mixtures of *Agrobacterium* strains, each carrying one or two different T-DNA vectors, allowing to trace back the origin of complex T-DNA loci [1]. Although the cotransformation frequencies of T-DNAs originating from different agrobacteria after floral dip and root transformation were comparable, and the frequencies of T-DNA originating from one bacterium only slightly higher than those after floral dip transformation, the T-DNA copy numbers in the transformants differed completely. Upon floral dip transformation, on average T-DNA copies were integrated at one genetic locus versus one to three after root transformation [1]. The cotransformation frequencies might explain the T-DNA copy number in most root transformants, but not in floral dip transformants. Therefore, it was postulated that T-DNA replication of a single T-strand occurs in the plant cell before or during T-DNA integration and that its frequency is much higher upon floral dip than upon root transformation [1]. Because the same *Agrobacterium* strains had been used in both transformation methods, not the bacterium but the type of target cell determined the complexity of the T-DNA pattern [1].

In general, transformants obtained after direct gene transfer contain more transgene copies than after *Agrobacterium*-mediated transformation [30–32]. In maize (*Zea mays*), comparison of transgene copy numbers and RNA expression levels upon transformation via direct gene transfer and *Agrobacterium*-mediated transformation revealed that more than 90% of the transformants obtained after *Agrobacterium*-mediated transformation harbored fewer than three T-DNA copies, while most of the transformants obtained after particle bombardment contained more than three copies [32], of which some of the transformants contained as many as 100 copies of the transgene [32]. Another observation was that the transformants obtained after *Agrobacterium*-mediated transformation displayed a higher transgene expression than after particle bombardment. Besides the expected positive correlation between transgene copy number and transgene expression, frequently a negative correlation between gene dosage and transgene expression was observed (see below) [51].

Recent research revealed that more single-copy integrants could be obtained by particle bombardment when a limited quantity of DNA was used for bombardment [52, 53]. A decrease in DNA from 1.5 to 2.5 ng per shot resulted in an increase of single-copy transformants from 30% to 70% [52, 53]. The 10% reduction in transformation efficiency was more than compensated by the more efficient screening for single-copy transformants [53]. Additionally, complex integration patterns could also be avoided when cassette DNA, harboring a promoter, a coding region and a 3′ end region, instead of whole plasmids were used for bombardment [52, 54–56].

Regulatory Sequences Have a Major Impact on Transgene Expression and Expression Variability

To obtain transformants with high and stable transgene expression, the construct should be designed carefully

because several sequences might have a great impact on the transgene expression and expression variability. Especially, the selected promoter that drives the transgene expression will determine the accumulation levels of the transgene transcript and, indirectly, of the transgene-encoded protein. Since the beginning of chimeric gene constructions, plant scientists predominantly chose the 35S promoter of the cauliflower mosaic virus as a strong and constitutive promoter to overexpress a coding sequence of interest [57] and for the promoters of the nopaline (pNOS) and octopine (pOCS) synthase-encoding genes, as weak promoters, for instance to drive the selectable marker genes in dicots.

The strength of the 35S promoter increases when upstream activator sequences are added [58], but as a consequence, single-copy transgenes driven by a 35S promoter with two upstream activator regions are more prone to gene silencing [59–61]. In general, silencing frequency correlates positively with the promoter strength [61–64]. Indeed, silencing did not occur frequently when a weak pNOS promoter was used [62], whereas the 35S promoter is very prone to transgene silencing, most probably because the high expression leads to transgene RNA accumulation levels above a certain threshold that triggers RNA degradation [65]. Strikingly, promoters do not only influence the levels, but also the variability of expression in a population of independently transformed plants with the same transgene [66]. Transformation of *Arabidopsis* plants with a p35S-*β-glucuronidase* (*GUS*) construct resulted in a bimodal expression pattern, with more than 80% of the primary transformants showing a very low and less than 20% a high GUS accumulation [3, 66]. The mannopine synthase promoter (pMAS) yielded gene expression levels that were only slightly lower than those of the 35S promoter, but the variation in transgene expression was at least eightfold lower [66]. The bimodal character of the p35S promoter versus the normal distribution of the pMAS-driven gene expression suggests that silencing phenomena occur less frequently in plants transformed with pMAS than with p35S.

Not only the promoter, but also the codon usage might play a role in determining the transcript stability and/or translation efficiency. For *Arabidopsis*, highly expressed endogenous genes seem to contain a C at the third codon position, whereas genes with low expression levels have predominantly a T in that position [67]. By adapting codon usage in the green fluorescent protein (GFP)-encoding gene toward a higher proportion of codons with a C or a G in the third position, more highly *GFP*-expressing plants were generated [68]. Additionally, the nature of the coding sequence and the sequence composition seem to be important parameters in determining the transcript accumulation and/or threshold above which silencing is initiated [62, 69]. The transcript levels of the three reporter genes *GUS*, *GFP*, and streptomycin phosphotransferase (*SPT*) controlled by the same p35S, varied, reflecting the expected dissimilar transcript stability and turnover for different sequences. Furthermore, other effects also determined the transcript accumulation because the steady-state transgene transcript levels in transformants containing two copies of the *GUS* transgene were lower than in those carrying only one copy, while in transformants with two copies of the *SPT* or the *GFP* genes, the levels were higher than those with only one of the respective genes. The transcript length and GC content of the coding sequences were unlikely to be primary determinants for the gene-specific threshold for the silencing trigger [69]. Indeed, although the *GFP* and *SPT* transgenes have almost identical transcript lengths and similar GC content, silencing was activated at a different copy number. Silencing of *GUS* expression was already observed in plants with three P35S-*GUS* copies, while *GFP* and *SPT* expression were only reduced in plants carrying five or more 35S-*GFP* and nine or more 35S-*SPT* copies.

In tobacco plants, different 5′ leader sequences were modulated by P35S-driven expression [6]. A 31-nucleotide random leader stimulated translation 20- and 100-fold compared to the nine- and four-nucleotide synthetic leaders, respectively. However, the 30-nucleotide satellite tobacco necrosis virus leader and both the 79-nucleotide tobacco mosaic virus (TMV) and the plant chlorophyll *a/b*-binding protein (66-Cab22L) leaders were approximately two- to threefold and fivefold stronger than this 31-nucleotide random leader, respectively [6]. On the contrary, the 5′-untranslated regions (5′ UTRs) of MAS and TMV did not influence the transgene expression variability [66]. Similarly, also four terminators (tMAS, tNOS,

tG7, and tOCS) were analyzed for their effect on transgene expression, but none affected levels and variability [66]. That especially certain combinations of different elements can lead to high and stable transgene expression was clearly demonstrated [70]. The combination of the strong *ß-phaseolin* promoter, with the 5′ and 3′ regulatory sequences of the *arceline 5-I* gene and efficient endoplasmic reticulum-targeting signals boosted the expression of an antibody fragment to 36% of the total soluble protein in *Arabidopsis* seeds. Furthermore, the variation in transgene expression was very low [70].

A significantly increased level of transgene expression can be observed for particular intron-containing transgenes when compared to their respective intronless constructs. This phenomenon, designated "intron-mediated enhancement," has been demonstrated both in monocots and dicots [71–74]. The ability of an intron to enhance transgene expression depends on the sequence and position of the intron within the transgene [72, 75]. Generally, stimulation of mRNA accumulation decreases with increasing distance of the intron from the promoter and its effect is greatly diminished or entirely lost when the intron is placed in the 3′ UTR [71, 75]. The mechanism of intron-mediated enhancement is largely unknown, but has been postulated to be due to an increased accumulation of mRNA, enhanced transcript stability, and/or an enhanced translation [71, 72, 74, 76].

The effect of matrix attachment regions (MARs) on transgene expression levels and stability is still unclear and contradictory results have been obtained [66, 77–79]. In some studies, MARs significantly increased the average protein accumulation levels in transgenic plants and reduced the transgene expression variability [80–83]. For instance, the chicken lysozyme MAR (chiMAR) reduced the expression variability by seven- to eightfold in transgenic potato [83], whereas this chiMAR had no influence on the transgene expression levels and variability in *Arabidopsis* transformants, unless the gene silencing mutants were used as genetic background for transformation [3, 66, 78, 84]. The lack of positive effect of the MARs was caused neither by the transformation method, nor by the plant species used for transformation, because this chiMAR did not influence the *Arabidopsis* transformants obtained after root or floral dip transformation and the tobacco transformants [78]. Moreover, a tobacco-derived MAR sequence had no positive effect on transgene expression in the *Arabidopsis* wild-type Columbia-0 transformants [78].

The Influence of the Integration Position on Transgene Expression

Although the presence of multiple transgene copies, especially in inverted orientation, is the major trigger to induce transgene silencing (see below), also single-copy plants can show variation in transgene expression and undergo transgene silencing [5, 17, 60, 85–88]. Two general explanations for the inactivation of single-copy transgenes have been proposed that are not mutually exclusive: recognition of a transgene as foreign DNA and subsequent TGS and neighborhood of spreading heterochromatin domains. In the first explanation for position effects, transgenes might be recognized as nonself sequences, and especially the difference in GC content of the transgene versus the flanking sequences might induce chromatin changes and epigenetic variation [12, 89]. Indeed, a single copy of the GC-rich *A1* gene from maize became specifically methylated in transgenic petunia (*Petunia hybrida*), while the homolog of gerbera (*Gerbera hybrida*), with a GC content similar to that of petunia, remained unmethylated [89, 90]. In the second explanation, position effects result from heterochromatization of the transgene integrated close to heterochromatin domains, which is similar to the position effect variegation, extensively studied in fruit fly (*Drosophila melanogaster*). Thus, also in plants, the chromosomal position of a transgene is expected to cause variability in transgene expression [9, 91]. In petunia, single-copy transgenes were strongly silenced when they were integrated into a highly repetitive region [5]. Likewise, two single-copy transformants were identified with unstable transgene expression that were integrated into intercalary and paracentromeric locations [85], while the two stably expressing loci were integrated into the vicinity of telomeres and flanked at least on one side by plant DNA with AT-rich regions. However, the number of examined cases is limited and T-DNA insertion into heterochromatic regions has been demonstrated not to be necessarily associated with transgene silencing [16].

To determine whether the integration position had a major effect on transgene expression variability, a large transformant population had been screened for single-copy T-DNA transformants and the transgene accumulation levels were compared [17]. Twenty single-copy transformants were selected based on antibiotic resistance marker expression and in all of them the T-DNA integration was characterized at the sequence level in the *Arabidopsis* genome (Fig. 1a). In 18 of the 20 transformants, the p35S-driven *GUS* expression was high and stable in two subsequent generations. Furthermore, the GUS activity levels in these 18 different transformants could be considered as similar, because the intra-transformant variability was as high as the inter-transformant variability (Fig. 1b). Integration into an intergenic or genic region and into an exon or intron did not result in differential transgene expression (Fig. 1) and, additionally, integration into a gene in sense or antisense orientation had no influence on the transgene expression, indicating that overlapping transcription from the endogene and transgene did not induce transgene silencing in *Arabidopsis* [17].

Another hypothesis was that transgene expression might be reduced by the absence of MAR sequences near the T-DNA integration site or by the presence of neighboring highly methylated sequences [92, 93]. However, no general correlation with these factors was observed [17]. There has also been some debate on the influence of vector backbone sequences, cointegrated into the plant genome on transgene expression. In a first report based on the observation that two unstably expressed loci harbored binary vector sequences directly contiguous to the right T-DNA border, whereas two stably expressed loci contained no vector backbone sequences, vector sequences were postulated to trigger TGS [85]. Later, several studies showed that the integration of vector sequences, even with a GC content strongly diverged from that of the plant genome, had no negative influence on transgene expression [17, 94, 95].

In general, one can conclude that in *Arabidopsis*, the integration position of single-copy T-DNAs does not strongly influence transgene expression [17, 62, 94], but one should take care not to extrapolate this finding too far. The *Arabidopsis* genome is small (125 Mb) and has a low amount of heterochromatic DNA, while the genome of *Drosophila*, in which position effect variegation is often observed, is comparable in size, but consists for one third of centric heterochromatin [96]. Also, one should be aware that all the above-described observations were based on transformants that were selected on the expression of one selectable marker on the same T-DNA, implying that silenced heterochromatic T-DNA insertions were not recovered. Indeed, an increased number of T-DNA integrations into heterochromatic regions were found without selection in *Arabidopsis* [20, 21]. Apparently, the kanamycin selection had failed to identify approximately 30% of all integration events, because the expression of the selectable marker was absent or very low [21]. Analysis revealed that not PTGS, but TGS, caused this discrepancy in transformation efficiency. Furthermore, the integration sites of the lines with silenced transgenes mapped to heterochromatic regions, including telomeres, centromeres, and rDNA repeats [20, 21], which are regions that are significantly underrepresented in T-DNA integration studies with transformants identified by selection [21].

Nevertheless, the holy grail of plant biotechnology is to integrate the transgenes into the plant genome via homologous recombination. Indeed, in contrast to yeast and the moss *Physcomitrella patens*, the frequency of transgene integration via homologous integration is extremely low. When compared to random integration, targeted integration of a transgene into the homologous sequence of the plant genome occurs in the range from 0.01% to 0.1% [97, 98]. Several approaches, with variable success, have already been applied to detect gene targeting events among the random integration events: (1) application of gene-specific selection or screening for the target genes; (2) use of positive–negative selection and, hence, reduction of the transformants with randomly integrated transgenes; and (3) overexpression of genes involved in homologous recombination in yeast, such as the *RAD54* gene [99–101]. Additionally, induction of double-stranded breaks in the genome has been reported to result in an increased targeted integration frequency [102, 103]. Nowadays, these double-stranded breaks are produced by zinc finger nucleases (ZFNs) [104–106]. These ZFNs are synthetic restriction enzymes that can be specifically designed to cleave virtually any long stretch of double-stranded DNA sequence [107]. A defective *GUS:NPTII* reporter gene, carrying a recognition site for ZFN and integrated at

various chromosomal sites in ten different tobacco lines, was restored via homologous recombination in 10% of the transformed protoplasts, regardless of the chromosomal position of the reporter gene [105]. Approximately 20% of the *GUS:NPTII* reporter gene was repaired solely with homologous recombination, but the other events still contained additional DNA insertions and deletions [105]. Moreover, ZFNs are probably a promising tool to efficiently achieve targeted integration into plants. Once this procedure is optimized, plants with high, stable, and predictable accumulation levels of important heterologous proteins might be obtained with an increased frequency.

Correlation Between T-DNA Locus Structure and Homology-Based Silencing

Based on the gene dosage rationale, screening for transgenic plants with multiple transgene copies might be predicted to result in plants with high transgene expression. A direct correlation between transgene copy number and expression level has indeed been reported [51, 62]. However, also the inverse correlation is observed with p35S-driven transgenes inserted as convergently transcribed inverted repeats: the reproduction of the expression of the inverted repeat transgene copies is much lower than that of a single-copy transgene [51, 108].

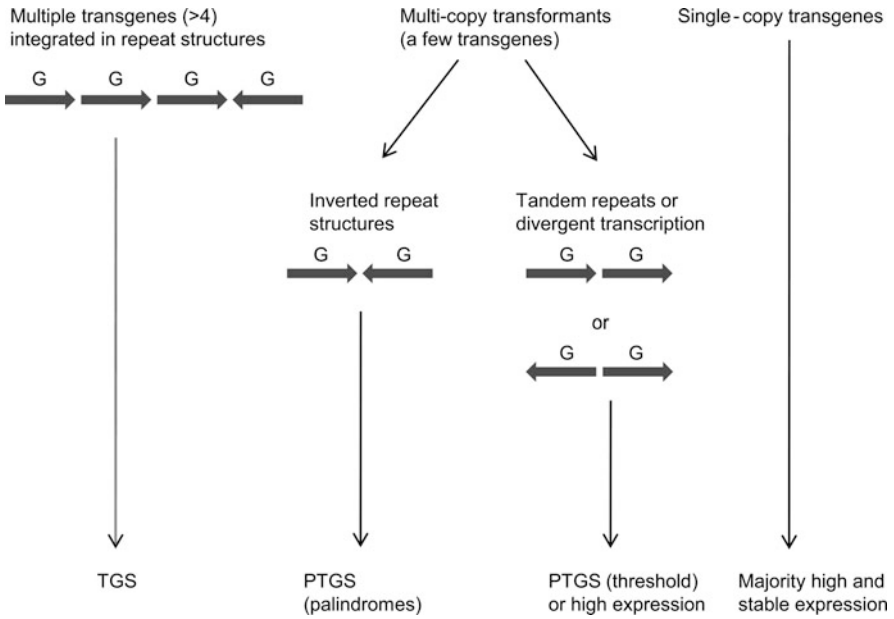
Increased Transgene Dosage Can Result in PTGS

Below a certain number of identical p35S-driven transgenes, gene copy number and expression correlated positively [62]. Transgene expression seemed stable and high over all generations analyzed and a comparable expression level was obtained for all independent lines harboring the same copy number of a certain transgene. However, once above a certain copy number, gene silencing occurred, implying that silencing was triggered by threshold concentrations of either transgene transcript or another product of transgene expression (Fig. 2) [62]. In many reports, the correlation between high transgene copy number and transgene expression was negative: the higher the copy number, the lower the expression level per gene copy [59, 61, 109]. Not the copy number per se, but especially the arrangement of the transgene copies in one genetic locus, seemed responsible for the low transgene expression. Indeed, as described above, multiple T-DNAs at one genetic locus are frequently integrated as a direct or an inverted repeat (Fig. 2) [1, 34, 35].

Several other examples show a correlation between transgene silencing and the presence of tandem repeats, which might be linked to exceeding the mRNA threshold level above which PTGS is initiated (Fig. 2) [59, 110, 111]. The same is true for transgene loci that are not silenced under hemizygous, but become silenced under homozygous conditions, as, for instance, in tobacco and *Arabidopsis* [60, 87, 112, 113].

Convergent Transcription from Inverted T-DNA Repeat Structures Is a Strong Trigger for PTGS

Invariably, convergently transcribed 35S-driven transgenes from invertedly repeated T-DNAs are posttranscriptionally silenced to a level that is only 1% or less of the single-copy transgene (Fig. 2) [4, 51, 59, 61, 108, 113–118]. Additionally, invertedly repeated transgenes can very efficiently silence *in trans* homologous sequences located elsewhere in the genome [51, 108, 117, 119]. This direct correlation between invertedly repeated trangenes and transgene silencing has been demonstrated experimentally by the deletion of one of the transgenes from the inverted repeat that released transgene silencing and a 100-fold increase in expression [108]. Indeed, in two parental lines, harboring two invertedly repeated 35S-driven *GUS* genes, the GUS activity was low and both the coding sequences and the center of the inverted repeat were densely methylated. Homologous transgenes at other chromosomal positions were silenced *in trans* by the inverted repeat silencer locus [108, 118]. Removal of one of the *GUS* copies from this silencer locus by the CRE recombinase resulted in all cases in constitutively high *GUS* expression, a significant decrease in methylation and lack of *in trans* silencing capacity [108, 118]. Strikingly, the spacer region in-between the two invertedly repeated transgenes seemed to determine the efficiency of transgene silencing. The presence of an 826-bp non-repetitive spacer region in-between the invertedly repeated transgenes strongly decreased the degree and the stability of silencing [108], but in another system, inverted repeats interrupted by a non-palindromic spacer region varying from 500 bp to 1,022 bp still gave efficient silencing [120].



Transgene Expression in Plants, Control of. Figure 2
Relation between locus structure and the expression of a p35S-driven transgene. Upon transformation, both single-copy and multicopy transformants are generated. Both single-copy plants and plants with multiple T-DNAs arranged in tandem repeat or in repeats giving rise to transgene divergent transcription generally display high and stable transgene expression. Plants carrying convergently transcribed transgenes show low transgene expression as a result of PTGS, whereas plants harboring multiple transgenes integrated into a concatemeric array frequently result in TGS. Abbreviations: *G* gene, *TGS* transcriptional gene silencing, *PTGS* posttranscriptional gene silencing

Multiple T-DNA Copies at One Locus Tend to Become Transcriptionally Silenced After Several Generations

Multiple T-DNA repeats not only induce PTGS, but also TGS. Induction of TGS occurs especially when a concatemeric array or inverted repeats of transgenes are formed and when different genes are regulated by the same promoter [119, 121–124]. The 271 locus, consisting of multiple T-DNAs with an antisense nitrite reductase driven by the 35S promoter, is transcriptionally silenced and is also able to very efficiently *in trans* silence another single-copy p35S-driven transgene [119]. Transgene expression is seemingly less stable over sexually than over vegetatively propagated generations [125]. Indeed, expression of the sulfur-rich sunflower (*Helianthus annuus*) seed albumin (*SSA*) and the phosphinothricin (*BAR*) genes, all driven by p35S, progressively decreased in transgenic clover (*Trifolium subterraneum*) [122]. The expression of both genes was stable until the T3 generation, but in the T7 generation, all plants were completely susceptible to the herbicide and the mean level of the SSA protein was much lower than those observed in the T3 generation. This progressive decrease in expression correlated with the reduced transcription level of both genes and strong CpG methylation in the promoter [122]. Not only the strong 35S promoter, but also the weak pNOS can trigger TGS. The H2 locus, a locus harboring six copies of pNOS, all transcriptionally silenced loci, strongly methylated the promoter regions [121]. Also, transcription of an inverted repeat of pNOS could trigger TGS and methylation of pNOS *in trans*, and this progressively decreased through generations [124].

How to Prevent Transgene Silencing?

To prevent transgene silencing, single-copy transgene inserts should be screened (see below) and inclusion of identical sequences in different transgene constructs

should be avoided [126]. Even a homology of 90 bp in the promoter region can be sufficient to trigger TGS [119]. Additionally, the use of multiple identical 3′ end regions can result in low transgene expression levels. A homology of 239 nucleotides in the 3′ UTR is sufficient to be recognized efficiently by the homology-based RNA degradation machinery [117] and also a homology of approximately 204 nucleotides in the 3′ UTR is enough for a single-copy transgene to activate the *in trans* silencing of another transgene with the same 3′ UTR [113]. Also designing transgenes without any inverted repeat is important to guarantee high and stable expression. Indeed, upon transcription of these inverted repeats, hairpin structures and double-stranded RNA are formed initiating transgene silencing [127]. Almost complete cosuppression of an endogenous gene in tomato (*Solanum esculentum*) was obtained after transformation with a homologous transgene construct that harbored two upstream inverted repeats in the 5′ UTR.

Control of Gene Expression by the Generation of Single-Copy Transformants

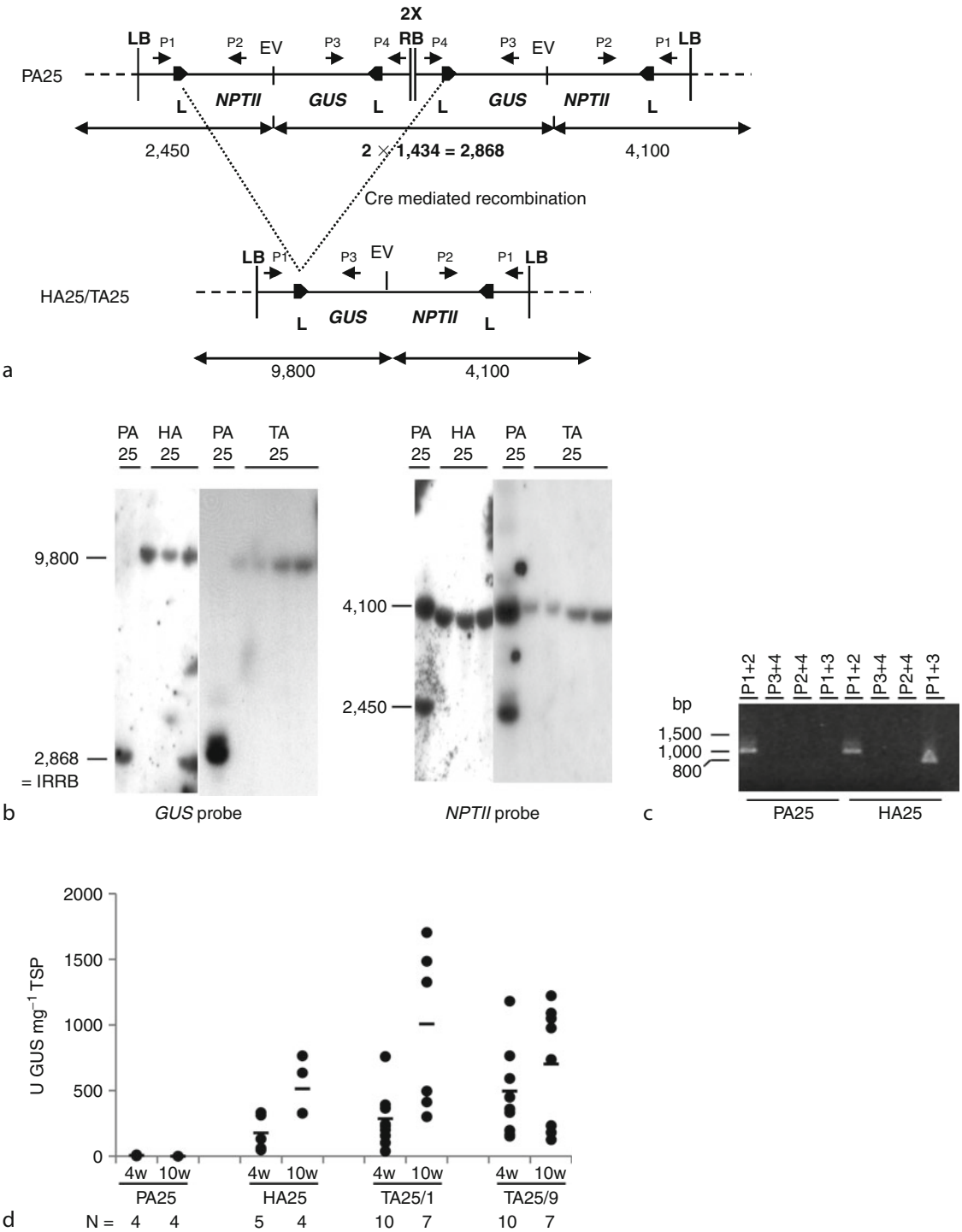
As mentioned above, transformants obtained after *Agrobacterium*-mediated gene transfer often contain multiple T-DNA copies in direct or inverted orientations, and the frequency of single-copy transformants is rather low [1, 17]. Because screening for single-copy T-DNA transformants strongly enriches for transformants with stable and high transgene expression [17], single-copy transformants need to be identified in a large pool of plants. Currently, conventional, but time-consuming and labor-intensive methods, such as DNA gel blot analysis and T-DNA fingerprinting are used [128]. Recently, transformation technologies and vectors have been developed to increase the frequency of single-copy transformants during or after transformation with site-specific recombination.

Generation of Single-Copy Transformants by Resolution of Complex Integration Loci by Site-Specific Recombination

A T-DNA construct, harboring site-specific *loxP* recombination sites, was designed to reduce complex T-DNA loci into a single T-DNA insert (Fig. 3a) [129]. This T-DNA harbored two invertedly oriented *loxP* recombination sites inside and immediately adjacent to the left and right T-DNA border ends [129]. Recombination between the outermost *loxP* sequences in direct orientation should resolve multiple copies into a single T-DNA copy, regardless of the number and orientation of the *loxP*-derived T-DNA copies inserted at one locus.

In a first approach, seven *Arabidopsis* transformants with multiple T-DNA inserts on a single locus were crossed with a homozygous *CRE*-expressing line [129]. In three hybrids, the complex T-DNA locus was reduced efficiently to a single-copy locus and in two of them, the resolution of the inverted repeat locus was accompanied by an at least tenfold-enhanced and stable transgene expression (Fig. 3a–d). In the progeny plants, only the simplified T-DNA locus was detected upon segregation of the *CRE* recombinase gene, proving that excision took place in the progenitor cells of the gametes (Fig. 3b and c) [129]. Unfortunately, in four of the seven transformants, the complex T-DNA locus could not be resolved by CRE-mediated transformation to a single T-DNA copy, although some rearrangements occurred as demonstrated on DNA gel blots. Strikingly, these complex T-DNA loci that were not resolved, had variable expression levels in different progeny plants with the same complex locus, implying some epigenetic imprints imposed by the interaction with the CRE recombinase. Possible reasons for the lack of resolution in these transformants might be deletion of the most extreme *loxP* sequences, too low CRE activity, or inaccessibility of the *loxP* sequences due to heterochromatinization of the complex locus [129]. Indeed, a correlation between the efficiency of CRE-mediated recombination and the CRE mRNA levels had been demonstrated [130].

In a second approach, the T-DNA vector with oppositely oriented *loxP* sequences was transformed into *CRE*-expressing *Arabidopsis* plants [88] and 55% of the primary transformants were single-copy T-DNA plants versus only 15% in control plants. Most of the single-copy transformants in *CRE*-expressing background (70%) displayed a continuous somatic inversion of the DNA fragment between the two inverted *loxP* sequences. To avoid this phenomenon, a new T-DNA vector, harboring only one *loxP* sequence adjacent to the LB or RB, was transformed in a *CRE*-expressing



Transgene Expression in Plants, Control of. Figure 3

Single-copy T-DNA transformants with high and stable transgene expression as a result of CRE/*loxP* recombination-mediated resolution (Adapted from [129]). (**a**) Schematic representation of the CRE/*loxP*-mediated resolution of a complex locus to a single T-DNA (not on scale). Parental (P) plant PA25 contained two T-DNAs in inverted orientation. After the plant

plant and up to 70% of the transformants were single copy. As resolution to single-copy plants is only efficient when the outermost *loxP* sites are in direct orientation, inverted T-DNA repeats might be mostly internal to multiple T-DNA copy arrays and they rarely occur at the ends after floral dip transformation in *Arabidopsis* [88]. Most single-copy transformants, obtained with both T-DNA vectors, displayed high and stable transgene expression. Strikingly however, the transgene expression in the majority of multicopy CRE transformants was stable and uniform, suggesting that the CRE recombinase prevented also the generation of inverted T-DNA repeats or modified the chromatin structure of the locus, so that it became less sensitive for gene silencing [88]. In both strategies, the *CRE* expression cassette is still present after resolution of the complex T-DNA locus, which can be a disadvantage, because, except for *Arabidopsis*, high *CRE* expression can result in severe growth phenotypes and reduced fertility in some plant species [131]. To avoid this drawback, *loxP*-containing T-DNAs could be transformed into hemizygous *CRE*-expressing plants, with 25% of the transformed progenies without *CRE* expression cassette as a consequence [88]. Also backcrossing of a homozygous *CRE*-expressing line with a wild-type plant would result in transformants without the *CRE*-containing T-DNA. Alternatively, the CRE recombinase protein could also be transiently introduced into the plant cell nucleus. Vergunst et al. [132] developed a VirB/D4-dependent translocation system in which the CRE recombinase was fused with the VIRE2 or VIRF proteins of *A. tumefaciens*. Together with the VIR proteins, the CRE recombinase was transferred to the plant cell and site-specific recombination took place. This recombination did not require any transferred DNA [132], but the question remains whether this CRE recombinase activity would be high enough to mediate complex T-DNA loci resolution and to transfer the CRE recombinase very efficiciently.

Simplification of complex T-DNA loci was also observed by Verweire et al. [133]. To ultimately develop a T-DNA vector that generates homozygous marker-free transgenic *Arabidopsis* plants without the need of additional transformation rounds or crosses, a germline-specific auto-excision vector was designed that harbored both the selectable marker and the *CRE* expression cassette between two *lox*P sequences in direct repeat. Additionally, the *CRE* recombinase was driven by the germline-specific 2.2-kb promoter fragment of the *SOLO DANCERS* gene, which is active in both the male and female meiocytes of *Arabidopsis*. In this manner, the transgenic plants become genetically programmed so that the marker

DNA was digested with the *Eco*RV (EV) enzyme, a fragment of 2,868 bp after hybridization with the *GUS* probe is indicative for the inverted T-DNA repeat about the RB, while two T-DNA/plant junctions of 2,450 bp and 4,100 bp were observed after hybridization with the *NPTII* probe, revealing the left T-DNA/plant DNA junctions. The hybrid (H) HA25 was obtained after crossing the parental plant with a *CRE*-expressing plant. CRE/*loxP*-mediated resolution between the two outermost *loxP* sites resulted in a new T-DNA harboring two LB regions in HA25 and the F2 progeny plants TA25. The primers P1, P2, P3, and P4 are indicated, showing homology with the LB region, the *GUS*-coding sequence, the *NPTII* coding sequence, and the RB region, respectively. (**b**) DNA gel blot analysis on *Eco*RV-digested DNA of PA25, HA25, and TA25. After CRE/*loxP*-mediated resolution, only one original LB-T-DNA/plant junction fragment was detected after hybridization with the *NPTII* probe fragment. Additionally, as expected, one new T-DNA/plant junction was observed after hybridization with the *GUS* probe. (**c**) Polymerase chain reaction (PCR) analysis on PA25 and HA25 transformants to identify the newly formed T-DNA configurations. Primer combination P1+2 amplified an LB/NPTII junction of 1,142 bp; primer combination P1+3 amplified an LB/GUS junction of 914 bp (*arrow 2*). With primer P4, no PCR fragment was obtained in PA25, because no inverted repeats could be amplified by PCR. Upon CRE/*loxP*-mediated resolution, the RB inverted palindrome was deleted, and only PCR products with the LB region of primer P1 were obtained. (**d**) GUS activity analysis in leaves of 4- and 10-week-old PA25, HA25, and TA25 seedlings. The measurements and the mean are indicated by *dots* and *line*, respectively. GUS activity levels are given as units GUS mg^{-1} of total soluble protein. The number (*N*) of the analyzed seedlings is indicated

gene is lost after the initial selection of the primary transformants. Surprisingly, not only marker-free homozygous progenies were obtained very efficiently, but also the locus structure in these progeny plants was simplified [133].

A similar approach to resolve complex transgene loci had been developed previously for the generation of single-copy transgenic wheat plants after direct gene transfer. In this case, the selectable marker was removed simultaneously with the resolution of the complex locus [134]. The transformation vector consisted of a transgene flanked by two *lox511* sites, a mutant variant of the wild-type *loxP* sequence, in inverted orientation. Additionally, the selectable marker gene was flanked by two wild-type *loxP* recombination sites in the same orientation. Two correct and independent recombination reactions could take place, because *lox511* and *loxP* did not recombine, with transformants with a marker-free, single-copy insertion of the transgene in the plant genome as a consequence. Crossing of four wheat transformants, harboring a multicopy locus, with a *CRE*-expressing wheat transformant, resulted in 62/72 F2 plants with a single-copy, marker-free, transgene locus [134]. Because the resolved locus still contained two *lox511* sites in inverted orientation, the CRE recombinase could still mediate inversion of the transgene between these two sites. Therefore, all F1 plants were chimeric for the inversion and harbored both transgene orientations.

To avoid crossing with a *CRE*-expressing plant, the transgene, flanked by the oppositely oriented *loxP* sites in maize cells, was cobombarded with a *CRE*-expressing construct [135]. This cotransformation resulted in 85% primary transformants harboring one or two copies of the introduced gene, of which 38% harbored a single copy. Of these single-copy plants, 60% lacked also the recombinase gene, because during cobombardment a molar ratio of 3:1 transgene to CRE construct was used. In this manner, an overall efficiency of 23% of plants with only one copy of the transgene construct was obtained [135]. This cotransformation strategy requires that the selectable marker is retained in the primary transformants. However, a strategy with two different recombination systems and two different selectable markers could result in the integration of one transgene without the incorporation of additional unneeded DNA in the transgenic plant [135].

Besides the use of the CRE/*loxP* recombination system to obtain efficiently single-copy transformants, other strategies have been proposed and evaluated. A site-specific recombination strategy was described in which two sets of directly repeated FLP recognition target (FRT) sites [136] flanked a "to-be-removed" (TBR) region. The FRT sites flanking one TBR are inverted in relation to the sites flanking the other TBR. Each TBR can be excised independently, because only recombination between directly repeated FRT sites can cause excision. Furthermore, one site-specific recombinase reaction could simultaneously induce double excisions, resulting in the resolution of complex T-DNA loci. Indeed, the progenies of 70% of the *Arabidopsis* transformants harbored only one or two copies of the transgene, while 40% contained only one T-DNA copy. This frequency of transformants with a simple T-DNA integration pattern is significantly higher than the 5–20% single-copy transformants normally obtained after floral dip transformation [1, 17]. Most interestingly, the reduced copy number was also strongly associated with increased expression [137].

Generation of Single-Copy Transformants by Site-Specific or Site-Directed Integration

Besides the use of site-specific recombination to resolve complex transgene loci into simple loci before, during or after integration, this site-specific recombination system can also be applied to produce plants with a simple integration pattern through site-specific integration [86, 138–141]. In addition, with this technology, transgenes can be targeted to a specific preselected chromosomal site to eliminate variation in gene expression. The strategy to obtain single-copy transformants via site-specific integration consists of two transformation rounds. In the first round, a target line is generated, harboring a target *lox* site between the promoter and the *CRE* gene. In the second transformation, the vector carrying the gene of interest and two *lox* sites is introduced into the target line. When the gene of interest is integrated into the *lox* site of the target line, the expression of

the recombinase gene is stopped. Site-specific gene integration in plants has been described with various DNA delivery methods, such as polyethylene glycol-mediated tobacco protoplast transformation [86], *Agrobacterium*-mediated transformation of *Arabidopsis* [138], and biolistic-mediated transformation of rice [139, 141]. After *Agrobacterium*-mediated transformation, the frequency of *CRE*-mediated gene integration was very low (approximately 2%) compared to the DNA delivery via direct gene transfer, probably because the transferred DNA during *Agrobacterium* transformation is single stranded, which is not a substrate for the CRE recombinase [139, 140].

By combining site-specific gene integration with gene expression analysis, the absolute level of transgene expression could be shown to vary up to tenfold, depending on the target site in the tobacco genome, indicating that the chromosome position can affect the level of transgene expression [86]. Additionally, despite the identical integration pattern, half of the transgenic tobacco lines showed the expected expression of the transgene and the other half very low *GUS* expression. This low *GUS* expression was due to transgene silencing, because it was correlated with DNA methylation and low transcript levels. Furthermore, this DNA methylation was specific for the newly introduced DNA sequences. Also in independent experiments, regenerants could be divided also upon site-directed integration in two categories: the single-copy lines that contained one site-specific insert without additional sequence integration and multicopy lines in which, besides the site-specific integration, additional transgene copies were integrated into the plant genome [140, 141]. The transgene expression of the single-copy rice transformants was high and stable and the variation was lower than that of the multicopy lines. In approximately half of the multicopy lines, expression of the site-specific integrated transgene could be reactivated and stabilized when the illegitimate integrated inserts were segregated away. In the remaining half, the expression could not be restored, because the random integrations were genetically linked to the site-specific integration event [141].

A site-directed integration system for *Agrobacterium*-mediated transformation of tobacco was developed in which a single transgene copy could be integrated precisely into a predefined target locus by recombinase-mediated cassette exchange (RMCE) [142]. In the RMCE-based site-directed integration strategy that uses the R-RS system from *Zygosaccharomyces rouxii*, a single-copy of the target cassette, surrounded by two oppositely oriented RS sites, is randomly integrated into the plant genome. The exchange cassette contains the selectable marker gene and the gene of interest between two opposite RS sites, and the recombinase gene, the selectable marker gene, and the gene of interest between directly oriented RS sites. This third RS site excluded random integration events. The recombinase will catalyze a double crossover between the two RS sites, replacing the target cassette by the exchange cassette and removing the recombinase gene. Expression analysis revealed that the obtained site-specific recombined plants from the same target line had approximately the same transgene expression level and less transgene expression variability than the random-integration transgenic plants and no transgene silencing [142]. Strikingly, transgenes in the same direction at the same target locus had the same level of activity, in contrast to transgenes in different directions, indicating that the surrounding genome DNA sequence outside the target locus might affect the activity of the gene. Also after direct gene transfer of soybean (*Glycine max*), site-specific integration via RMCE occurred efficiently and stable transgene expression was stable [143].

Another approach to obtain single-copy transgenic lines is the "Agrolistic" method, in which the advantages of *Agrobacterium*-mediated transformation and biolistics were combined [144, 145]. The *VIRD* genes were cobombarded with the T-DNA borders, flanking the introduced transgene. Approximately, 20% of the transformed tobacco calli contained only the T-DNA with correctly processed border sequences and no integrated vector backbone sequences [144], whereas 10–35% of transgenic maize calli contained one to two transgene copies. The addition of *VIRE2* genes even doubled this transformation efficiency [145]. Finally, the application of niacimide, a product that reduces recombination of extrachromosomal molecules, resulted in the generation of single-copy transformants in wheat with an efficiency of 8% [146].

Use of Gene Silencing Mutants to Overcome Transgene Expression Variability

Variation in transgene expression in a population of plants is frequently due to PTGS of the transgenes. Several genes are involved in PTGS and mutant screens revealed that the RNA-dependent RNA polymerase (RDRP6, SGS2) is essential for PTGS to occur [147, 148]. Therefore, a logical approach to overcome PTGS in a population of transgenic plants was to generate transformants in an *rdr6* mutant background, impaired in PTGS [3, 84]. Introduction of p35S-*GUS* or p35S-*GFP* transgenes into the *Arabidopsis sgs2* and *sgs3* mutants (two different alleles of *RDR6*) resulted in stable and high expression of these transgenes in 100% of the transformants analyzed [3], while, similarly, the outcome of introduction of a cyclin-dependent kinase inhibitor 6 gene under the control of p35S was a clear overexpression phenotype, typical for high expression of the kinase inhibitor, in all transformants. Also the transgene expression in transformants with convergent transcription of invertedly repeated T-DNAs was high. Interestingly, immediate transgene silencing in the F1 progeny plants occurred by backcrossing of these high-expressing transformants containing an inverted repeat T-DNA locus with a wild-type nontransformed plant. This observation clearly demonstrates that RDRP6 is required for the generation of double-stranded RNA, also from inverted repeat loci, and that not the read-through transcripts from the convergently transcribed genes generate the double-stranded RNA, as is widely believed. Similar results were obtained when the transgenes were driven by the pCAS promoter, a strong constitutive promoter derived from the cassava vein mosaic virus [149]. On the contrary, expression variability was not significantly reduced in the plants transformed with *sgs2* and *sgs3* when the transgenes were under control of the hybrid pOMA1 promoter, a hybrid promoter consisting of parts of the pMAS and pOCS, which might be too weak to trigger PTGS [3], in contrast to the strong p35S and pCAS. When the T-DNA constructs were flanked by MARs of the chicken lysozyme gene, the GUS activity was boosted fivefold and 12-fold in *sgs2* and *sgs3* background, respectively, in contrast to the wild-type background, making this PTGS-MAR expression system very attractive for the production of heterologous proteins in plants [3, 84].

Future Directions

The ultimate aim is to generate transgenic plants with a predictable, well-controlled, and stable transgene expression over many generations. As described above, to achieve this goal, many parameters should be taken into account. Most importantly, the transgene construct should be optimally designed: a suitable promoter should be selected that drives transgene expression in the desired way, multiple homologous sequences should be avoided in the transgene construct, and foreign unnecessary DNA should be as much as possible prevented to be integrated into the plant genome. Until now, the transgene construct was integrated via illegitimate recombination, but recent reports on site-specific integration let one assume that targeted integration via ZFNs will soon become feasible routinely [105]. At the same time, this method will enrich for single-copy transformants ensuring stable transgene expression. Importantly, the majority of the above-described observations and conclusions are all based on genes driven by the strong constitutive p35S. Therefore, it is perfectly conceivable that all these observations are typical for this promoter, but cannot be extrapolated to other promoters. In the future, the use of strong plant promoters instead of the 35S promoter in transgene constructs might be a big improvement to obtain controllable and stable high transgene expression. Indeed, in general, plant promoter–gene fusions show limited quantitative and qualitative variations in transgene expression in different transformants. Analysis of the expression profile of the *ARCELIN-5* regulatory sequences in *Arabidopsis* seeds revealed that, surprisingly, the *Arabidopsis* seeds accumulated ARCELIN-5, an abundant protein found in common bean (*Phaseolus vulgaris*), to 15% of the total soluble protein content [150]. All transformants had low plant-to-plant variation and even in plants harboring a complex T-DNA integration pattern, with invertedly and directly repeated T-DNAs, the transgene expression was high and stable [150]. Similar results were found for genes driven by the *ß-PHASEOLIN* promoter of common bean [70].

However, to really control transgene expression in plants, the underlying triggers that initiate PTGS and TGS have to be understood in much more detail. Do all plant promoters give less variation in transgene expression compared to viral and bacterial promoters? Do plant promoters also induce silencing and with which frequency? Intriguingly, why do certain loci with multiple p35S-driven transgene copies provoke silencing and why others not? Silencing is often triggered when gene expression rises above a certain threshold, but what is the nature of this threshold and how is the threshold measured? Can transcript levels be increased by the insertion of particular stabilizing elements? To answer all these questions, an integrated approach will have to be followed, including the use of well-designed transgene constructs differing only in a single transgene element in combination with different mutants. Molecular characterization of the transgene locus, the transcript steady state and turnover levels, and the translation efficiency should allow to unravel the network of gene expression control and identify the most important master elements. The ultimate goal is to be able to construct transgenes with a predefined expression pattern in a reliable way.

Acknowledgments

The authors thank Martine De Cock for help with the manuscript. This work was supported in part by grants from the European Union BIOTECH program (QLRT-2000-00078) with additional cofinancing from the Flemish Community, the "Bijzonder Onderzoeksfonds" of Ghent University (BOF 01111400), and the Research Foundation-Flanders (no. G.021106).

Bibliography

1. De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in *Arabidopsis* transformants is highly determined by the transformed target cell. Plant J 60:134–145
2. Windels P, De Buck S, Depicker A (2008) *Agrobacterium tumefaciens*-mediated transformation: patterns of T-DNA integration into the host genome. In: Tzfira T, Citovsky V (eds) *Agrobacterium*: from biology to biotechnology. Springer, New York, pp 441–481
3. Butaye KMJ, Goderis IJWM, Wouters PFJ, Pues JM-TG, Delauré SL, Broekaert WF, Depicker A, Cammue BPA, De Bolle MFC (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. Plant J 39:440–449
4. Muskens MWM, Vissers APA, Mol JNM, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol Biol 43:243–260
5. Pröls F, Meyer P (1992) The methylation pattern of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrida*. Plant J 2:465–475
6. De Loose M, Danthinne X, Van Bockstaele E, Van Montagu M, Depicker A (1995) Different 5′ leader sequences modulate β-glucuronidase accumulation levels in transgenic *Nicotiana tabacum* plants. Euphytica 85:209–216
7. Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. Plant Cell 2:279–289
8. van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291–299
9. Depicker A, Van Montagu M (1997) Post-transcriptional gene silencing in plants. Curr Opin Cell Biol 9:373–382
10. Frizzi A, Huang S (2010) Tapping RNA silencing pathways for plant biotechnology. Plant Biotechnol J 8:655–677
11. Vaucheret H, Fagard M (2001) Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet 17:29–35
12. Matzke AJM, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1:142–148
13. Tzfira T, Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol 17:147–154
14. Banta LM, Montenegro M (2008) *Agrobacterium* and plant biotechnology. In: Tzfira T, Citovsky V (eds) *Agrobacterium*: from biology to biotechnology. Springer, New York, pp 73–148
15. Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet 20:375–383
16. Forsbach A, Schubert D, Lechtenberg B, Gils M, Schmidt R (2003) A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. Plant Mol Biol 52:161–176
17. De Buck S, Windels P, De Loose M, Depicker A (2004) Single-copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable β-glucuronidase accumulation levels. Cell Mol Life Sci 61:2632–2645
18. Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Körber H, Redei GP, Schell J (1989) High-frequency T-DNA-mediated gene tagging in plants. Proc Natl Acad Sci USA 86:8467–8471

19. Herman L, Jacobs A, Van Montagu M, Depicker A (1990) Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. Mol Gen Genet 224:248–256
20. Kim S-I, Veena GSB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. Plant J 51:779–791
21. Francis KE, Spiker S (2005) Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integrations. Plant J 41:464–477
22. Windels P, De Buck S, Van Bockstaele E, De Loose M, Depicker A (2003) T-DNA integration in *Arabidopsis* chromosomes: presence and origin of filler DNA sequences. Plant Physiol 133:2061–2068
23. Wenck A, Czakó M, Kanevski I, Márton L (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. Plant Mol Biol 34:913–922
24. Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J 11:945–957
25. De Buck S, De Wilde C, Van Montagu M, Depicker A (2000) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Mol Breed 6:459–468
26. Podevin N, De Buck S, De Wilde C, Depicker A (2006) Insights into recognition of the T-DNA border repeats as termination sites for T-strand synthesis by *Agrobacterium tumefaciens*. Transgenic Res 15:557–571
27. van der Graaff E, den Dulk-Ras A, Hooykaas PJJ (1996) Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. Plant Mol Biol 31:677–681
28. Barcelo P, Lazzeri PA (1998) Direct gene transfer: chemical, electrical and physical methds. In: Lindsey K (ed) Transgenic plant research. Harwood Academic Publishers, Amsterdam, pp 35–55
29. Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci USA 95:7203–7208
30. Zhao ZY, Gu W, Cai T, Tagliani LA, Hondred D, Bond D, Krell S, Rudert ML, Bruce WB, Pierce DA (1998) Molecular analysis of T0 plants transformed by *Agrobacterium* and comparison of *Agrobacterium*-mediated transformation with bombardment transformation in maize. Maize Genet Coop Newsletter 72:34–37
31. Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardement. Mol Breed 7:25–33
32. Shou H, Frame BR, Whitham SA, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Mol Breed 13:201–208
33. Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TH, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15:305–327
34. De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J 11:15–29
35. De Buck S, Jacobs A, Van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. Plant J 20:295–304
36. Cluster PD, O'Dell M, Metzlaff M, Flavell RB (1996) Details of T-DNA structural organization from a transgenic Petunia population exhibiting co-suppression. Plant Mol Biol 32:1197–1203
37. Wolters A-MA, Trindade LM, Jacobsen E, Visser RGF (1998) Fluorescence *in situ* hybridization on extended DNA fibres as a tool to analyse complex T-DNA loci in potato. Plant J 13:837–847
38. Jorgensen R, Snyder C, Jones JDG (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet 207:471–477
39. Krizkova L, Hrouda M (1998) Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. Plant J 16:673–680
40. Kumar S, Fladung M (2000) Transgene repeats in aspen: molecular characterisation suggests simultaneous integration of independent T-DNAs into receptive hotspots in the host genome. Mol Gen Genet 264:20–28
41. Van Lijsebettens M, Inzé D, Schell J, Van Montagu M (1986) Transformed cell clones as a tool to study T-DNA integration mediated by *Agrobacterium tumefaciens*. J Mol Biol 188:129–145
42. Herman LMF, Van Montagu M, Depicker AG (1986) Isolation of tobacco DNA segments with plant promoter activity. Mol Cell Biol 6:4486–4492
43. Depicker A, Herman L, Jacobs A, Schell J, Van Montagu M (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol Gen Genet 201:477–484
44. De Block M, Debrouwer D (1991) Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor Appl Genet 82:257–263
45. Poirier Y, Ventre G, Nawrath C (2000) High-frequency linkage of co-expressing T-DNA in transgenic *Arabidopsis*

thaliana transformed by vacuum-infiltration of *Agrobacterium tumefaciens*. Theor Appl Genet 100:487–493
46. Radchuk VV, Van DT, Klocke E (2005) Multiple gene co-integration in *Arabidopsis thaliana* predominantly occurs in the same genetic locus after simultaneous in planta transformation with distinct *Agrobacterium tumefaciens* strains. Plant Sci 168:1515–1523
47. Nam J, Matthysse AG, Gelvin SB (1997) Differences in susceptibility of Arabidopsis ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell 9:317–333
48. Grevelding C, Fantes V, Kemper E, Schell J, Masterson R (1993) Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. Plant Mol Biol 23:847–860
49. Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA 85:5536–5540
50. Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743
51. Hobbs SLA, Warkentin TD, DeLong CMO (1993) Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol Biol 21:17–26
52. Shiva Prakash N, Bhojaraja R, Shivbachan SK, Hari Priya GG, Nagraj TK, Prasad V, Srikanth Babu V, Jayaprakash TL, Dasgupta S, Spencer TM, Boddupalli RS (2009) Marker-free transgenic corn plant production through co-bombardement. Plant Cell Rep 28:1655–1668
53. Lowe BA, Shiva Prakash N, Way M, Mann MT, Spencer TM, Boddupalli RS (2009) Enhanced single copy integration events in corn via particle bombardment using low quantities of DNA. Transgenic Res 18:831–840
54. Loc TN, Tinjuangjun P, Gatehouse AMR, Christou P, Gatehouse AJ (2002) Linear transgene constructs lacking vector backbone sequences generate transgenic rice plants which accumulate higher levels of proteins conferring insect resistance. Mol Breed 9:231–244
55. Yao Q, Cong L, Chang JL, Li KX, Yang GX, He GY (2006) Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment. J Exp Bot 57:3737–3746
56. Fu X, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, Twyman RM, Christou P, Kohli A (2000) Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res 9:11–19
57. Odell JT, Nagy F, Chua N-H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812
58. Fang R-X, Nagy F, Sivasubramaniam S, Chua N-H (1989) Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1:141–150
59. Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol Biol 31:957–973
60. Elmayan T, Vaucheret H (1996) Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally. Plant J 9:787–797
61. Que Q, Wang H-Y, English JJ, Jorgensen RA (1997) The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. Plant Cell 9: 1357–1368
62. Lechtenberg B, Schubert D, Forsbach A, Gils M, Schmidt R (2003) Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing. Plant J 34:507–517
63. Marjanac G, Karimi M, Naudts M, Beeckman T, Depicker A, De Buck S (2009) Gene silencing induced by hairpin or inverted repeated sense transgenes varies among promoters and cell types. New Phytol 184:851–864
64. Chuang C-F, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 97:4985–4990
65. Vaucheret H, Elmayan T, Thierry D, van der Geest A, Hall T, Conner AJ, Mlynarova L, Nap J-P (1998) Flank matrix attachment regions (MARs) from chicken, bean, yeast or tobacco do not prevent homology-dependent *trans*-silencing in transgenic tobacco plants. Mol Gen Genet 259:388–392
66. De Bolle MFC, Butaye KMJ, Coucke WJW, Goderis IJWM, Wouters PFJ, van Boxel N, Broekaert WF, Cammue BPA (2003) Analysis of the influence of promoter elements and a matrix attachment region on the inter-individual variation of transgene expression in populations of *Arabidopsis thaliana*. Plant Sci 165:169–179
67. Mathé C, Peresetsky A, Déhais P, Van Montagu M, Rouzé P (1999) Classification of *Arabidopsis thaliana* gene sequences: clustering of coding sequences into two groups according to codon usage improves gene prediction. J Mol Biol 285:1977–1991
68. Rouwendal GJA, Mendes O, Wolbert EJH, de Boer AD (1997) Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. Plant Mol Biol 33:989–999
69. Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in Arabidopsis T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. Plant Cell 16:2561–2572

70. De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, Depicker A, Angenon G (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. Nat Biotechnol 20:1265–1268
71. Mascarenhas D, Mettler IJ, Pierce DA, Lowe HW (1990) Intron-mediated enhancement of heterologous gene expression in maize. Plant Mol Biol 15:913–920
72. Bourdon V, Harvey A, Lonsdale DM (2001) Introns and their positions affect the translational activity of mRNA in plant cells. EMBO Rep 2:394–398
73. Rose AB, Elfersi T, Parra G, Korf I (2008) Promoter-proximal introns in *Arabidopsis thaliana* are enriched in dispersed signals that elevate gene expression. Plant Cell 20: 543–551
74. Barlett JG, Snape JW, Harwood WA (2009) Intron-mediated enhancement as a method for increasing transgene expression levels in barley. Plant Biotechnol J 7:856–866
75. Rose AB (2004) The effect of intron location on intron-mediated enhancement of gene expression in *Arabidopsis*. Plant J 40:744–751
76. Tanaka A, Mita S, Ohta S, Kyozuka J, Shimamoto K, Nakamura K (1990) Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. Nucleic Acids Res 18:6767–6770
77. Allen GC, Spiker S, Thompson WF (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Mol Biol 43:361–376
78. De Bolle MFC, Butaye KMJ, Goderis IJWM, Wouters PFJ, Jacobs A, Delauré SL, Depicker A, Cammue BPA (2007) The influence of matrix attachment regions on transgene expression in *Arabidopsis thaliana* wild type and gene silencing mutants. Plant Mol Biol 63:533–543
79. Butaye KMJ, Cammue BPA, Delauré SL, De Bolle MFC (2005) Approaches to minimize variation of transgene expression in plants. Mol Breed 16:79–91
80. Petersen K, Leah R, Knudsen S, Cameron-Mills V (2002) Matrix attachment regions (MARs) enhance transformation frequencies and reduce variance of transgene expression of barley. Plant Mol Biol 49:45–58
81. Ülker B, Allen GC, Thompson WF, Spiker S, Weissinger AK (1999) A tobacco matrix attachment region reduces the loss of transgene expression in the progeny of transgenic tobacco plants. Plant J 18:253–263
82. Vain P, Worland B, Kohli A, Snape JW, Christou P, Allen GC, Thompson WF (1999) Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. Plant J 18:233–242
83. Mlynárová L, Jansen RC, Conner AJ, Stiekema WJ, Nap J-P (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. Plant Cell 7:599–609
84. Sels J, Delauré SL, Aerts AM, Proost P, Cammue BPA, De Bolle MFC (2007) Use of a PTGS-MAR expression system for efficient in planta production of bioactive *Arabidopsis thaliana* plant defensins. Transgenic Res 16:531–538
85. Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T, Spiker S, Matzke M, Matzke AJM (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9:1251–1264
86. Day CD, Lee E, Kobayashi J, Holappa LD, Albert H, Ow DW (2000) Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. Genes Dev 14:2869–2880
87. De Wilde C, Podevin N, Windels P, Depicker A (2001) Silencing of antibody genes in plants with single-copy transgene inserts as a result of gene dosage effects. Mol Genet Genomics 265:647–653
88. De Paepe A, De Buck S, Hoorelbeke K, Nolf J, Peck I, Depicker A (2009) High frequency of single-copy T-DNA transformants by floral dip in *CRE*-expressing *Arabidopsis* plants. Plant J 59: 517–527
89. Meyer P, Heidmann I (1994) Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. Mol Gen Genet 243:390–399
90. Elomaa P, Helariutta Y, Griesbach RJ, Kotilainen M, Seppänen P, Teeri TH (1995) Transgene inactivation in *Petunia hybrida* is influenced by the properties of the foreign gene. Mol Gen Genet 248:649–656
91. Peach C, Velten J (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. Plant Mol Biol 17:49–60
92. Meyer P, Heidmann I, Niedenhof I (1993) Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. Plant J 4:89–100
93. Holmes-Davis R, Comai L (1998) Nuclear matrix attachment regions and plant gene expression. Trends Plant Sci 3: 91–97
94. Meza TJ, Stangeland B, Mercy IS, Skårn M, Nymoen DA, Berg A, Butenko MA, Håkelien A-M, Haslekås C, Meza-Zepeda LA, Aalen RB (2002) Analyses of single-copy *Arabidopsis* T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. Nucleic Acids Res 30:4556–4566
95. Kunz C, Narangajavana J, Jakowitsch J, Park Y-D, Delon TR, Kovarik A, Koukaková B, van der Winden J, Moscone E, Aufsatz W, Mette MF, Matzke M, Matzke AJM (2003) Studies on the effects of a flanking repetitive sequence on the expression of single-copy transgenes in *Nicotiana sylvestris* and in *N. sylvestris-N. tomentosiformis* hybrids. Plant Mol Biol 52: 203–215

96. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers Y-HC, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor Miklos GL, Abril JF, Agbayani A, An H-J, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Deslattes May A, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferriera S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei M-H, Ibegwam X, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacleb JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RDC, Scheeler F, Shen H, Shue BC, Sidén-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang Z-Y, Wassarman DA, Weinstock GM, Weissenbach J, Williams SM, Woodage T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh R-F, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC (2000) The genome sequence of *Drosophila melanogaster*. Science 287:2185–2195
97. Puchta H (2002) Gene replacement by homologous recombination in plants. Plant Mol Biol 48:173–182
98. Hanin M, Paszkowski J (2003) Plant genome modification by homologous recombination. Curr Opin Plant Biol 6:157–162
99. Hanin M, Volrath S, Bogucki A, Briker M, Ward E, Paszkowski J (2001) Gene targeting in *Arabidopsis*. Plant J 28:671–677
100. Shaked H, Melamed-Bessudo C, Levy AA (2005) High-frequency gene targeting in *Arabidopsis* plants expressing the yeast *RAD54* gene. Proc Natl Acad Sci USA 102:12265–12269
101. Terada R, Johzuka-Hisatomi Y, Saitoh M, Asao H, Iida S (2007) Gene targeting by homologous recombination as a biotechnological tool for rice functional genomics. Plant Physiol 144:846–856
102. Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J 17:6086–6095
103. Tzfira T, Frankman LR, Vaidya M, Citovsky V (2003) Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. Plant Physiol 133: 1011–1023
104. Lloyd A, Plaisier CL, Carroll D, Drews GN (2005) Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis*. Proc Natl Acad Sci USA 102:2232–2237
105. Wright DA, Townsend JA, Winfrey RJ Jr, Irwin PA, Rajagopal J, Lonosky PM, Hall BD, Jondle MD, Voytas DF (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J 44:693–705
106. Cai CQ, Doyon Y, Ainley WM, Miller JC, DeKelver RC, Moehle EA, Rock JM, Lee Y-L, Garrison R, Schulenberg L, Blue R, Worden A, Baker L, Faraji F, Zhang L, Holmes MC, Rebar EJ, Collingwood TN, Rubin-Wilson B, Gregory PD, Urnov FD, Petolino JF (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol 69:699–709
107. Carroll D, Morton JJ, Beumer KJ, Segal DJ (2006) Design, construction and *in vitro* testing of zinc finger nucleases. Nat Protoc 1:1329–1341
108. De Buck S, Van Montagu M, Depicker A (2001) Transgene silencing of invertedly repeated transgenes is released upon deletion of one of the transgenes involved. Plant Mol Biol 46:433–445
109. Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol Biol 15:851–864
110. Assaad FF, Tucker KL, Signer ER (1993) Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*. Plant Mol Biol 22:1067–1085
111. Sijen T, Wellink J, Hiriart J-B, van Kammen A (1996) RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. Plant Cell 8:2277–2294
112. de Carvalho F, Gheysen G, Kushnir S, Van Montagu M, Inzé D, Castresana C (1992) Suppression of β-1, 3-glucanase transgene expression in homozygous plants. EMBO J 11:2595–2602
113. De Neve M, De Buck S, De Wilde C, Van Houdt H, Strobbe I, Jacobs A, Van Montagu M, Depicker A (1999) Gene silencing results in instability of antibody production in transgenic plants. Mol Gen Genet 260:582–592
114. Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc Natl Acad Sci USA 91:10502–10506
115. Stam M, de Bruin R, Kenter S, van der Hoorn RAL, van Blokland R, Mol JNM, Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. Plant J 12:63–82
116. Jones JDG, Gilbert DE, Grady KL, Jorgensen RA (1987) T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet 207:478–485

117. Van Houdt H, Kova_ík A, Van Montagu M, Depicker A (2000) Cross-talk between posttranscriptionally silenced neomycin phosphotransferase II transgenes. FEBS Lett 467:41–46
118. De Buck S, Depicker A (2001) Disruption of their palindromic arrangement leads to selective loss of DNA methylation in inversely repeated *gus* transgenes in Arabidopsis. Mol Genet Genomics 265:1060–1068
119. Vaucheret H (1993) Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of homology in the promoter sequence are sufficient for trans-inactivation. CR Acad Sci Paris, Life Sci 316:1471–1483
120. Hutvágner G, Mlynárová L, Nap J-P (2000) Detailed characterization of the posttranscriptional gene-silencing-related small RNA in a GUS gene-silenced tobacco. RNA 6:1445–1454
121. Matzke AJM, Neuhuber F, Park Y-D, Ambros PF, Matzke MA (1994) Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. Mol Gen Genet 244:219–229
122. Hagan ND, Spencer D, Moore AE, Higgins TJV (2003) Changes in methylation during progressive transcriptional silencing in transgenic subterranean clover. Plant Biotechnol J 1:479–490
123. Meza TJ, Enerly E, Børud B, Larsen F, Mandal A, Aalen RB, Jakobsen KS (2002) A human CpG island randomly inserted into a plant genome is protected from methylation. Transgenic Res 11:133–142
124. Fischer U, Kuhlmann M, Pecinka A, Schmidt R, Mette MF (2008) Local DNA features affect RNA-directed transcriptional gene silencing and DNA methylation. Plant J 53:1–10
125. Brunner AM, Li J, DiFazio SP, Shevchenko O, Montgomery BE, Mohamed R, Wei H, Ma C, Elias AA, VanWormer K, Strauss SH (2007) Genetic containment of forest plantations. Tree Genet Genomes 3:75–100
126. De Wilde C, Van Houdt H, De Buck S, Angenon G, De Jaeger G, Depicker A (2000) Plants as bioreactors for protein production: avoiding the problem of transgene silencing. Plant Mol Biol 43:347–359
127. Hamilton AJ, Brown S, Yuanhai H, Ishizuka M, Lowe A, Alpuche Solis A-G, Grierson D (1998) A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. Plant J 15:737–746
128. De Buck S, Depicker A (2004) Gene expression and level of expression. In: Christou P, Klee H (eds) Handbook of plant biotechnology, vol 1. Wiley, Chichester, pp 331–345
129. De Buck S, Peck I, De Wilde C, Marjanac G, Nolf J, De Paepe A, Depicker A (2007) Generation of single-copy T-DNA transformants in *Arabidopsis* by the CRE/*loxP* recombination-mediated resolution system. Plant Physiol 145:1171–1182
130. Marjanac G, De Paepe A, Peck I, Jacobs A, De Buck S, Depicker A (2008) Evaluation of CRE-mediated excision approaches in *Arabidopsis thaliana*. Transgenic Res 17:239–250
131. Coppoolse ER, de Vroomen MJ, Roelofs D, Smit J, van Gennip F, Hersmus BJM, Nijkamp HJJ, van Haaren MJJ (2003) Cre recombinase expression can result in phenotypic aberrations in plants. Plant Mol Biol 51:263–279
132. Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CMT, Regensburg-Tuïnk TJG, Hooykaas PJJ (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science 290:979–982
133. Verweire D, Verleyen K, De Buck S, Claeys M, Angenon G (2007) Marker-free transgenic plants through genetically programmed auto-excision. Plant Physiol 145:1220–1231
134. Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA 96:11117–11121
135. Srivastava V, Ow DW (2001) Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct. Plant Mol Biol 46:561–566
136. Kilby NJ, Davies GJ, Snaith MR, Murray JAH (1995) FLP recombinase in transgenic plants: constitutive activity in stably transformed tobacco and generation of marked cell clones in *Arabidopsis*. Plant J 8:637–652
137. Kumar S, Thompson WF (2009) Simultaneous excision of two transgene flanking sequences and resolution of complex integration loci. Plant Mol Biol 69:23–32
138. Vergunst AC, Jansen LET, Hooykaas PJJ (1998) Site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase. Nucleic Acids Res 26: 2729–2734
139. Srivastava V, Ow DW (2001) Biolistic mediated site-specific integration in rice. Mol Breed 8:345–349
140. Srivastava V, Ow DW (2004) Marker-free site-specific gene integration in plants. Trends Biotechnol 22:627–629
141. Chawla R, Ariza-Nieto M, Wilson AJ, Moore SK, Srivastava V (2006) Transgene expression produced by biolistic-mediated, site-specific gene integration is consistently inherited by the subsequent generations. Plant Biotechnol J 4:209–218
142. Nanto K, Sato K, Katayama Y, Ebinuma H (2009) Expression of a transgene exchanged by the recombinase-mediated cassette exchange (RMCE) method in plants. Plant Cell Rep 28:777–785
143. Li Z, Xing A, Moon BP, McCardell RP, Mills K, Falco SC (2009) Site-specific integration of transgenes in soybean via recombinase-mediated DNA cassette exchange. Plant Physiol 151:1087–1095
144. Hansen G, Chilton M-D (1996) "Agrolistic" transformation of plant cells: integration of T-strands generated *in planta*. Proc Natl Acad Sci USA 93:14978–14983
145. Hansen G, Shillito RD, Chilton M-D (1997) T-strand integration in maize protoplasts after codelivery of a T-DNA substrate and virulence genes. Proc Natl Acad Sci USA 94:11726–11730
146. De Block M, Debrouwer D, Moens T (1997) The development of a nuclear male sterility system in wheat. Expression of the

barnase gene under the control of tapetum specific promoters. Theor Appl Genet 95:125–131

147. Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell 101:543–553
148. Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, Jouette D, Lacombe A-M, Nikic S, Picault N, Rémoué K, Sanial M, Vo T-A, Vaucheret H (2000) *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 101:533–542
149. Verdaguer B, de Kochko A, Beachy RN, Fauquet C (1996) Isolation and expression in transgenic tobacco and rice plants of the cassava vein mosaic virus (CVMV) promoter. Plant Mol Biol 31:1129–1139
150. Goossens A, Dillen W, De Clercq J, Van Montagu M, Angenon G (1999) The *arcelin-5* gene of *Phaseolus vulgaris* directs high seed-specific expression in transgenic *Phaseolus acutifolius* and *Arabidopsis* plants. Plant Physiol 120: 1095–1104

Transgenic Crops Resistant to Fungal, Bacterial and Viral Pathogens

Tennant Paula[1], Fuchs Marc[2]
[1]Department of Life Sciences, University of the West Indies, Mona, Kingston, WI, Jamaica
[2]Department of Plant Pathology and Plant-Microbe Biology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY, USA

Article Outline

Glossary

Bacterium A unicellular, prokaryotic microorganism with a wide range of shapes that is surrounded by a lipid membrane and a cell wall made of peptidoglycan, and has few intracellular structures, no nucleus but a single circular chromosome located in the cytoplasm and reproduces by binary fission.

Fungus A eukaryotic organism such as a yeast or a mold, with cell walls that contain glucans and chitin, membrane-bound nuclei with chromosomes and cytoplasmic organelles, soluble carbohydrates, and storage compounds, and exhibits enormous diversity in life cycle strategies, morphologies, and ecologies.

Pathogen An infectious agent that causes disease to its host.

Pathogenicity The capacity of a pathogen to cause disease.

Pathogenicity factors Products encoded by a pathogen that are crucial for the establishment of disease, e.g., proteins involved in the attachment of the pathogen to the plant surface or penetration and colonization of the host.

Resistance The reaction of a host to a pathogen that derives from preformed defenses and/or defense responses induced following infection.

Systemic acquired resistance (SAR) A mechanism of induced defense that acts nonspecifically throughout the plant, is associated with the accumulation of pathogenesis-related proteins and requires the signal molecule, salicylic acid.

Transgenic plant A plant that results from the application of recombinant DNA and plant tissue culture technologies.

Virus A small obligate parasite that utilizes the cellular metabolic pathways of its host organism to replicate its genes which are made up of deoxyribonucleic or ribonucleic acid and are encapsidated in a protein coat.

Definition of the Subject

Loss of crops due to fungal, bacterial, and viral diseases can have a large impact on human food supplies and local economies, as well as on the social stability of rural communities. It is conservatively estimated that diseases, insects, and weeds together cause 30–40% loss of all crops worldwide [1]. Annual losses worldwide due to plant diseases are estimated to ~14% of total losses and about $220 billion. In addition, the need for measures to control diseases limits the acreage of land available for cultivation, restricts the crops that can be grown in fields already contaminated with certain pathogens and necessitates the use of agrochemicals for treating seeds, fumigating soils, spraying plants, and applying fruit postharvest treatments. Such control measures add to the cost of food production and toxic chemicals can be harmful to human health and the environment [1].

Since the beginning of agriculture, crop plants have been domesticated and improved for various traits such as increased yield, improved nutritious composition, enhanced disease resistance, and tolerance to abiotic factors, among others. The selection and use of resistant crop cultivars is the most efficient and most environmentally friendly strategy to mitigate the impact of

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

fungal, bacterial, and viral diseases. Resistance to pathogens can be achieved by application of disease-suppressing cultural practices, use of plant defense-promoting substances, deployment of biological agents antagonistic to the pathogens that cause disease, agrochemicals, conventional breeding strategies, and genetic engineering [1]. The need for controlling plant diseases effectively is not only a major challenge, but also a necessity to reduce food losses while improving food quality and safeguarding the environment.

The advent of molecular genetics and plant transformation, in combination with an increased understanding of pathogen–host interactions, has opened new avenues for the development of disease-resistant crops through genetic engineering. Research groups from academia and industry created the first transgenic plants in the early 1980s [2–5]. These early transgenic plants were laboratory specimens (tobacco, petunia, and sunflower). Subsequent research developed transgenic plants, including horticultural crops, with useful traits such as disease resistance. Transgenic crops resistant to fungal, bacterial, and viral diseases were created following extensive research. The first disease-resistant transgenic crop, i.e., a virus-resistant transgenic summer squash, was deregulated in the USA in 1994. Several cultivars derived from the deregulated transgenic summer squash lines were commercialized in the USA in 1996 [6]. The adoption rate of virus-resistant transgenic summer squash has been increasing steadily since the initial releases. Other commercially available virus-resistant transgenic crops include papaya in the USA, and sweet pepper and tomato in the People's Republic of China. Transgenic crops resistant to fungal and bacterial diseases have been developed and evaluated under field conditions. However, in spite of remarkable progress, their development and release lags behind virus-resistant transgenic crops.

Introduction

Plants provide microorganisms, including viruses, with a diversity of habitats [7]. Ensuing interactions are either detrimental or beneficial and are classified as neutralism, commensalism, synergism, mutualism, amensalism, or parasitism. It is the latter association that has been a major challenge to crop production for centuries.

In the parasitic interaction, an organism lives within the plant host or on its outer surfaces and obtains its nutrients from the latter [1]. The removal of nutrients often affects normal growth and development of the plant and may be associated with pathogenicity. Virus infections commonly result in the inhibition of plant growth as well as the development of undesirable morphological changes and decreased yield [8]. Unlike fungal and bacterial pathogens, viruses induce disease in plant hosts primarily by utilizing cellular components and disrupting cellular processes. Fungi are responsible for diseases referred to as blight, gray mold, bunts, powdery, and downy mildews. These microbes have evolved at least three strategies that lead to less-than-healthy plants; enzymes that breakdown the plant cell wall and cuticle, toxins that either reduce the activity of the host cells or inhibit it completely, and plant-specific hormones that interfere with the hormonal equilibrium of the plant cell [9]. Bacterial pathogens cause pre- and post-harvest diseases that are characterized by cankers, galls, wilts, spotting of leaves, and stem or fruit rots of various vegetable and fruit crops. The invasion of healthy plant tissue through a wound or an area of dead or dying tissue is generally followed by the initiation of infection, which is similar to that of fungi, and is associated with the breakdown of cell walls, the production of toxins, exo-polysaccharides or proteins that mimic plant hormones.

Plant diseases, whether initiated by viruses, bacteria, or fungi, are of paramount importance because of the destruction they cause to plants and plant products. The effects not only threaten crop productivity and reduce farmers' net income, but also the food supply, and by extension, the economies of rural areas and even countries [10]. Zadoks and Schein [11] differentiate between primary and secondary losses and direct and indirect losses. Primary losses refer to those sustained because of plant disease (before or after harvest) in yield or wages, while secondary losses are losses of future production capacity. On the other hand, losses in quality, quantity, and production capacity, and socioeconomic losses are classified as direct and indirect losses, respectively.

Disease control efforts to date have focused on various crop husbandry techniques including crop rotation, and the avoidance of spread of infested soil

and pathogen-carrying plant materials. The most widely used management strategy is the application of agrochemicals, particularly against fungal pathogens. Chemical protection against bacterial pathogens is not as evolved as that against fungi, and is not applicable to viruses. The most widely used methods against bacterial diseases primarily center on phytosanitary practices, harvesting techniques, and storage conditions, and eventually on the use of antibiotics. However, the intensive use of agrochemicals has led to the development of resistance in various microbial populations and in some cases the chemicals are no longer used because of their high toxicity or problems with persistence in the soil. Nowadays, the focus is on strategies that allow for crop production with minimal use of agrochemicals. The most effective strategy to mitigate the impact of diseases on agriculture involves breeding-resistant crop cultivars. The majority of the cultivars available today have been generated through the classical method of hybridization [12] and the introgression of genes responsible for resistance. Resistant plants ward off pathogen attack by an innate immunity of each cell, systemic signals emanating from infection sites and a suite of cellular responses that follow the activation of the gene for resistance. However, there are many pathogens for which no effective sources of disease resistance have been identified. Technologies such as genetic engineering and gene transfer methods offer an alternative means for the development of disease resistant cultivars. In contrast to conventional breeding which involves the random mixing of the tens of thousands of genes present in both the resistant and susceptible plant, the latter technology allows the transfer of only the resistance gene to the susceptible plant and the preservation of its valuable economic traits. Moreover, the genetic sources for disease resistance are not limited to closely related plant species. During the last decade, considerable progress has been made in the development of virus resistant transgenic plants, while the development of fungal- and bacterial-resistant transgenic plants has lagged behind.

This chapter examines the different strategies devised for the development of resistance to fungal, bacterial, and viral diseases through genetic engineering. A description of the genes employed to confer resistance against given pathogens and the mechanisms underlying engineered resistance are provided. Emphasis will be on horticultural crops and the recent applications of transgenic resistance to agriculture. Factors affecting the development, release, and adoption of transgenic crops with resistance to fungal, bacterial, and viral diseases will be identified and discussed. The final sections offer overall conclusions, cover the impending direction of the development of transgenic disease resistance and some reflection on the impact of transgenic crops resistant to fungi, bacteria, and viruses in terms of effective disease management, mitigation of disease impact on agriculture, improved production of quality food, and better preservation of the environment.

Transgenic Resistance to Fungal Pathogens

Plants have evolved a complex pathogen defense system that is driven by tight balances between the induction of specific defense pathways and cell death. Much progress has recently been made in identifying and understanding a number of the genes and gene products involved in these defenses as well as the signaling pathways. The findings have prompted the development of a variety of strategies for producing fungus-resistant transgenic plants that are based on the manipulation of the plant's innate defense system, the production of antifungal proteins or the neutralization of fungal pathogenicity factors.

The earliest strategy employed in the development of resistance against fungal pathogens involved the enhancement of the plant's natural defenses through the overexpression of pathogenesis-related proteins (PR- proteins). PR- proteins form part of the general resistance mechanism in plants, that is, systemic acquired resistance (SAR), which is induced by necrotizing pathogens or by treatment with chemicals such as salicylic acid. Initial studies on the development of fungus-resistant transgenic plants examined the effects of expressing a single PR-protein gene in transgenic plants, while later studies looked at the co-expression of combinations thereof, in an attempt to improve on the levels of resistance, to target a wider spectrum of fungal pathogens and to provide longer-term protection.

In 1991, Broglie and coworkers [13] reported on the first fungus-resistant transgenic plants. Transgenic tobacco and rapeseed plants, transformed with a chitinase gene isolated from bean, exhibited enhanced

resistance against the soil-borne fungus, *Rhizoctonia solani*. Presumably, expression of the chitinase transgene played a dual role in protecting against infection; that is, the enzyme inhibited growth of the pathogen by cell wall digestion and the released pathogen-borne elicitors induced additional defense reactions in the plants. Following the first pioneering work of Broglie et al. [13], Lorito et al. [14] showed that an endochitinase gene (*ThEn-42*) from the mycoparasitic fungus, *Trichoderma harzianum*, conferred similar resistance to the fungal pathogens, *Alternaria alternata, A. solani, Botrytis cinerea*, and *Rhizoctonia solani* in transgenic tobacco and potato (cv. 'Desiree'). The effectiveness of glucanase, another PR-protein, in transgenic plants was demonstrated in banana transformed with β-1, 3 glucanase from soybean. Increased protection was observed when transgenic banana was infected with *Fusarium oxysporum* f. sp. *cubense* [15]. Further, the combinatorial expression of two chitinase genes [16] or chitinase and glucanase genes was found to give increased levels of protection than the expression of the PR-proteins in single transformants [17]. Subsequent strategies employed the co-expression of transgenes encoding differently acting antifungal proteins with similar results. For example, ÓBrian et al. [18] confirmed protection against *Rhizoctonia solani* in transgenic tobacco carrying three barley proteins; chitinase, β-1, 3 glucanase, and plant ribosome-inactivating protein (RIP). While the first two enzymes targeted the cell wall of the pathogen, RIP targeted and inactivated fungal ribsomes. RIPs possess N-glycosidase activity and catalyze the removal of an adenine residue from the 28S rRNA, thus inhibiting protein elongation.

Another emerging strategy against fungal pathogens involves the use of genes encoding small cysteine-rich antifungal proteins. These proteins play a role in the plant's innate defense mechanism and include thionins, defensins, and lipid transfer proteins.

The antimicrobial activities of thionins are well known, particularly those found in barley. Antimicrobial action, most likely based on the induction of membrane permeabilization resulting in cell disruption and death, is not limited to fungi and these proteins are effective against bacteria and oomycetes. Given this growth inhibition activity, transgenic plants overexpressing thionins have been generated to provide fungal resistance. Epple et al. [19] overexpressed an endogenous endothionin *Thi2.1* gene in *Arabidopsis thaliana* and observed enhanced resistance against *Fusarium oxysporum* f. sp. *matthiolae*. Similar levels of resistance were obtained with *Thi2.1* transgenic tomato against bacterial and Fusarium wilt [20].

Other small cysteine- rich proteins referred to as defensins are increasingly becoming recognized as candidates of defense transgenes. Defensins were originally called γ-thionins due to similarities to *a*- and *b*-thionins, but it was later shown that the former proteins shared appreciable structural and functional commonalities to insect and mammalian defensins, hence the name change. As with thionins, the mechanism of action has yet to be confirmed. However there is strong evidence of the induction of ion fluxes across the plasma membranes of living fungal hyphae followed by interactions with glycosylceramides at the fungal cell surface. Expression of single-protein defensin gene constructs has been deployed in transgenic plants as well as cleavable, chimeric polyprotein constructs in an attempt to increase expression levels in planta and to introduce broad-spectrum resistance. The constitutive expression of a mustard defensin conferred resistance to *Fusarium moniliforme* and *Phytophthora parasitica* pv. *nicotianae* in tobacco and *Pheaoisariopsis personata* and *Cercospora arachidicola*, which jointly cause late leaf spot disease in peanuts [21]. François et al. [22] engineered a polyprotein construct using defensin genes *DmAMP1* from *Dahlia merckii* seeds and *Rs-AFP2* from *Raphanus sativus* seeds. The genes were joined by a linker peptide obtained from *Impatiens balsamina* seeds [22] and used for the transformation of *Arabidopsis*. Antifungal activity was obtained against *Fusarium culmorum* in in vitro assays using purified fractions from extracellular fluids of the transgenic plants.

Lipid transfer proteins (LTPs) are the third group of small cysteine-rich antifungal proteins (ca. 10 KDa) found in higher plants. As the name implies, LTPs are involved in shuttling phospholipids and other fatty acid groups between cell membranes and bind acyl chains; culminating in membrane permeabilization and a direct cytotoxic effect on fungal cells. Constitutive expression of pepper (cv. Habanero) LTPs, *CALTPI*, and *CALTPII*, in tobacco conferred enhanced resistance to *Phytophthora nicotianae* and the bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* [23].

When a wheat LTP (*Ltp3F1*) was linked with a chitinase gene from barley (*chi2*) and transformed into carrots, reduced disease symptoms against necrotrophic foliar fungal pathogens, *Alternaria radicicola* and *Botrytis cinerea*, were obtained; 95% for *Botrytis* and 90% for *Alternaria* infection compared to 40–50% for single-gene transformants [24].

Phytoalexins are another group of plant secondary metabolites with primarily antifungal activity that have been exploited in the development of fungus-resistant transgenic plants. These metabolites are of low molecular weight (ca. <1,000 Da) and are synthesized or accumulated in response to infection or stress related to infection. Resveratrol is the best studied phytoalexin of grapevine that is synthesized from the precursors, malonyl-CoA and *p*-coumaroyl-CoA, by the action of stilbene synthase. Apparently, the accumulation of resveratrol upon infection triggers the synthesis of a fungal phenol oxidase that results in self-intoxication through the conversion of resveratrol to a more toxic dimmer, viniferin. Grapevine stilbene synthase transformation of tomato and papaya [25] introduced increased protection against *Botrytis cinerea* and *Phytophthora palmivora*, respectively. However, in other cases, e.g., strawberry, increased resistance was not obtained [26].

Increased evidence in recent years indicate the possibility of achieving improved resistance in transgenic plants expressing transcription factors that participate in the regulation of plant defense responses. Five major families of plant transcription factors have been described; basic region/leucine zipper motif (bZIP), zinc finger motif WRKY, MYB, ethylene-responsive element binding proteins (EREBP), and homeodomain proteins. Transcription factors play a crucial role in the transmission of pathogen-derived defense signals. They are involved either in the activation or suppression of downstream defense gene expression or the regulation of cross-talk between different signaling pathways. Presumably the mode of action is by binding promoter elements (W-box) of SAR gene promoters. Thus, the strategy is particularly attractive as the constitutive expression of a transcription factor may also modify the expression of all genes under its regulation. Solano et al. [27] engineered transgenic *Arabidopsis* plants with the ethylene-response-factor 1 (*ERF1*), an early ethylene-response gene that is regulated by the ethylene-insensitive gene EIN3 and, which in turn, regulates the expression of several pathogen responsive genes. The transgenic plants showed a clear reduction in *Botrytis cinerea* infection, with 40–70% of plants remaining symptomless after challenge and never developing macroscopic or microscopic necrosis. *ERF1* also mediated *Arabidopsis* resistance against the soil borne fungi, *Fusarium oxysporum* sp. *conglutinans* and *F. oxysporum* f. sp. *lycopersici* [28]. An ERF transcription factor (*GbERF2*) gene derived from cotton enhanced the resistance of transgenic tobacco to fungal infection by *Alternaria longipes* [29]. The accumulation of *GbERF2* transcripts along with transcripts of PR-proteins, such as *PR-1b*, *PR2*, and *PR4*, were detected.

Rather than using strategies based on the overexpression of PR-proteins or transcription factors that are induced during SAR for enhanced fungal resistance, other researchers have examined the effects of the constitutive expression of signaling molecules that play an important role in the induction of defense proteins. Components of the elicitor-receptor system were used to generate these transgenic plants. The system, explained by the gene-of gene-model, is the major line of plant defense against pathogens. Essentially, a pathogen protein encoded by an avirulence gene (*Avr*) is recognized by a plant protein encoded by a resistance gene (*R*), resulting in the activation of a number of defense mechanisms including the hypersensitive reaction and the restriction of the pathogen at the site of infection. Thus resistance against fungal pathogens carrying a particular *Avr* gene is introduced via the transfer of the corresponding *R* gene from a resistant plant to a susceptible plant. The success and durability of the strategy correlates strongly on the ability of the *R* gene product to recognize *Avr* proteins secreted by most if not all of the races of the pathogen. During the last decade, several sequences of *R* genes have been characterized into five classes according to their functional domains. Three classes contain leucine-rich repeats. The most common class encodes proteins with an amino-terminal nucleotide-binding site (NBS) and a carboxy-terminal leucine-rich repeat (LRR) and confers resistance against nematodes, sucking insects, viruses, bacteria, oomycetes, and fungi. The proof of concept has been conducted with the model system, tobacco, as well as various economic

crops. Rice plants transgenic for the *R* gene alleles, *Pi9*, *Pi2*, and *Piz*, against the rice blast fungus, *Magnaporthe grisea,* were reported highly resistant to 43 isolates collected from 13 countries [30]. In flax, three alleles of the flax rust resistance gene, *L* (*L2*, *L6,* and *L10*) when transformed into a highly susceptible rust cultivar, unequivocally demonstrated resistance against strains of pathogen (*Melampsora lini*) with the corresponding *Avr* gene [31]. Increased resistance was also obtained when transgenic tobacco transformed with the same three flax genes was challenged with two pathogens of tobacco. Increased resistance was conferred against *Cercospora nicotianae* and the oomycete, *Phytophthora parasitica* pv. *nicotianae* in *L6* transgenics and one *L10* transgenic plant [32]. Resistance was attributed to the constitutive expression of defense genes rather than recognition of the pathogens by the *L6* gene product. Of note, the *L6* transgenic plants exhibited a stunted phenotype.

Another arm of the plants' innate defense that is being investigated is that of RNA silencing. Accumulating evidence suggests the involvement of small interfering RNAs (siRNAs) and microRNAs (miRNAs) in the response to fungal pathogens. The involvement of plant siRNAs, short, noncoding RNAs between 20 and 24 nucleotides (nt) in length, have been implicated in the defense against viruses, and more recently bacterial pathogens, and is discussed in a later section of the chapter. miRNAs comprise the 21-nt class of siRNAs and short-interfering RNAs comprise the 24- nt class of siRNAs. Work with pine and the canker- forming rust fungus, *Cronartium quercuum* f. sp. fusiforme, identified 82 putative miRNA targets, including NBS-LRR proteins, receptor-like kinases, laccases, and MYB transcription factors, which are most likely associated with disease responses that restrict fungal growth [33]. Ellendorff et al. [34] showed that RNA silencing plays a role in the defense against *Verticillium* in *Arabidopsis*, but the siRNA involved were not identified. Further research into the targets and processes will invariably have broad implications in potentiating basal defenses against fungal phytopathogens.

Other strategies have explored the development of enhanced resistance through the expression of gene products that target components of the arsenal of the fungus. One approach employed the transformation a susceptible cultivar with sequences encoding polygalacturonase inhibiting proteins (PGIPs). PGIPs, proteins structurally related to several resistance gene products, are expressed in the cell wall of a number of plants and belong to a superfamily of LRR proteins, suggesting involvement in pathogen recognition. These proteins inhibit the activity of fungal endo-polygalacturonases presumably by binding at either the substrate binding site or the underside of the endo-polygalacturonase. The resulting oligogalacturonides induce a range of defense responses. *Arabidopsis* plants overexpressing *PGIP* isolated from pear showed decreases in disease symptoms produced by *Botrytis cinerea* [35]. Similarly, transgenic wheat accumulating bean PGIP developed smaller lesions when challenged with the necrotropic fungus, *Bipolaris sorokiniana* [36]. In earlier studies with transgenic tomato carrying a PGIP transgene isolated from bean, enhanced resistance against *Fusarium oxysporum* f. sp. *lycopersici, Botrytis cinerea,* and *Alternaria solani* was not obtained [37].

Another component of the arsenal that some fungi (e.g., *Sclerotinia sclerotiorum*) use to infect plants that is regarded as a pathogenicity factor is oxalate. Oxalic acid apparently assists in initiating infection through acidification, which facilitates cell wall–degrading enzyme activity, through pH-mediated tissue damage, or via the sequestration of calcium ions. Evidence for the utility of hydrogen peroxide–generating enzymes in protective plant responses was provided by the expression of oxalate oxidase, which catalyzes the degradation of oxalic acid to produce carbon dioxide and hydrogen peroxide (H_2O_2). Essentially increased H_2O_2 production limits the growth of the invading pathogen in planta and triggers the activation of a range of defense responses. Sunflower and tomato transformed with a wheat oxalate oxidase gene exhibited increased resistance to *Sclerotinia sclerotiorum* [38, 39]. Potato (cv. Bintje) and poplar expressing the same gene showed increased resistance to *S. musiva* [40] and the oomycete, *Phytophthora infestans* [41], respectively.

Transgenic Resistance to Bacterial Pathogens

Knowledge of bacteria–host interactions, in particular of the mechanisms of pathogenesis, and efforts to bolster plant defense mechanisms, has provided a strong basis for implementing transgenic approaches for bacterial disease management. Several strategies aiming at

the lysis or prevention of bacterial growth have been investigated based on the use of bacterial-resistance genes, avirulence genes, expression of antibacterial peptides that act as bactericidal or bacteriolytic agents, and the application of the concept of pathogen-derived resistance. The latter describes the use of genetic elements from a pathogen's own genome to confer resistance in an otherwise susceptible host via genetic engineering [42].

Members of the NBS-LRR class of disease *R* genes have been employed to provide effective protection against bacteria when transferred into new species. Transformation of a susceptible rice cultivar with the resistance gene *Xa21* that was isolated from a resistant rice line and characterized by positional sequencing and functional genetics conferred resistance to *Xanthomonas oryzae* [43]. Field tests of *Xa21* transgenic rice have shown satisfactory results in the Philippines, India, and the People's Republic of China [44], but deregulation of transgenic *Xa21* rice is still pending. It should be noted that hybrid rice containing *Xa21* was developed by conventional breeding [45]. Similarly, *Xa1* provided resistance to *Xanthomonas oryzae* pv. *oryzae* in transgenic rice [46]. Rice expressing an *R*-gene from maize were resistant against *Xanthomonas oryzae* pv. *oryzicola* [47]. Also, *Bs2* of pepper confers resistance to *Xanthommonas campestris* pv. *vesicatoria* in transgenic tomato [48]. Tomato plants expressing *Pto* showed resistance to *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *versicatoria* [49]. The transgenic approach based on *R*-genes circumvents tedious crosses and backcrosses of conventional breeding as well as linkages to undesired traits.

Another approach to confer resistance to bacterial diseases is based on the use of lytic peptides from insects that form pores in bacterial membranes. For example, cecropins are bactericidal peptides from the giant silk moth, *Hyalophora cecropia*, which interact with bacterial membranes and form transient ion channels. The peptides are active against a wide range of plant pathogenic bacteria including *Erwinia carotovora*, *E. amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas campestris*. Native, mutant (SB37 and MB39) or synthetic polypeptides (Shiva-1 and D4E1) that are designed for increased activity have been used for engineered resistance against bacterial diseases. Infection caused by *Erwinia carotovora* sp. *atroseptica* is reduced in transgenic potato expressing the Shiva-1 and SB-37 lytic peptide analogs [50]. Transgenic apple trees expressing SB-37 showed increased resistance to *E. amylovora* in field tests [51]. Poplar expressing D4E1 had more resistance to *Agrobacterium tumefaciens* and *Xanthomonas populi* than control nontransformed trees [52, 53]. Attacin is another small protein from the giant silk moth with antibacterial activity that inhibits synthesis of the outer bacterial membrane. Transgenic potato attacin expression has enhanced resistance to *Erwinia carotovora* sp. *atrospetica* [50]. Attacin [54] as well as lactoferrin [55] provide enhanced resistance against *Erwinia amylovora* in pear. Similarly, transgenic apple expression of the attacin E gene exhibit enhanced resistance to *Erwinia amylovora* [56] even under field conditions [57].

Lysozymes from various sources have been exploited in the development of transgenic resistance against bacterial diseases. Lysozymes are ubiquitous bacteriolytic enzymes that cleave the murein layer of bacterial petidoglycan, resulting in a weakening of the bacterial cell wall. A chimeric bacteriophage T4 lysozyme fused to a plant signal peptide reduces the susceptibility of transgenic potato plants to *Erwinia carotovora* spp. *atroseptica* [58]. Transgenic apple trees with the bacteriophage T4 lysozyme exhibited significant resistance to fire blight infection [59] and the bovine lysozyme isozyme was shown to confer resistance to *Xanthomonas campestris* in tomato, rice, and potato [60].

Tachyplesin, an antibacterial peptide isolated from the horseshoe crab (*Tachypleus tridentatus*), reduces the incidence of rubber rot caused by *E. carotovora* in transgenic potato [61].

Reactive oxygen species play important roles in various defense responses of plants, including pathogen infection. A prolonged local oxidative burst is one of the earliest events correlated with plant resistance at the site of pathogen invasion; see the previous section for detailed information on the role of oxalate oxidase in plant defense responses. Transgenic potato tubers expressing a glucose oxidase from *Aspergillus niger* for the production of large amounts of hydrogen peroxidase exhibit strong resistance to *Erwinia carotovora* [62].

Many bacteria control virulence factor expression by a cell-to-cell communication system referred to as quorum sensing. Disruption of bacterial quorum sensing has been proposed as a disease management strategy and several techniques with the potential to disrupt quorum sensing have been investigated. The enzyme *N*-acyl homoserine lactone (AHL)-lactonase (AiiA) isolated from the soil-borne *Bacillus* sp. 240B1, which catalyzes the degradation of AHL and attenuates symptoms caused by plant pathogens by degrading the quorum-sensing autoinducer AHL of the soft rot pathogen, *Erwinia carotovora*, induces enhanced resistance in transgenic potato [63].

Several pathovars of *Pseudomonas syringae* produce extracellular toxins that often increase their virulence toward plants. Phaseolotoxin is one such example. Phaseolotoxin is a modified tripeptide that inhibits the plant enzyme, ornithine carbamoyl transferase (OCTase), and is produced by the *P. syringae* pvs. *phaseolicola* and *actinidiae* as well as by a single strain of *P. syringae* pv. syringae, CFBP3388. Transgenic bean expressing *argK* that codes for a phaseolotoxin-insensitive OCTase show resistance to *Pseudomonas syringae* pv. *phaseolicola* [64].

An example of pathogen-derived resistance against a bacterial disease, crown gall, is reported by Krastanova et al. [65]. Based on an earlier report on the use of a truncated virE2 gene construct lacking the 215 C-terminal amino acids to confer resistance to grown gall disease in tobacco [66], these authors used a truncated form of *virD2* from *Agrobacterium tumefaciens* strains C58 and A6, and *Agrobacerium vitis* strain CG450 to engineer resistance to crown gall disease in transgenic grape rootstocks. Resistant lines show a substantial reduction in tumoreginicity and develop very small sized galls [65].

Other researchers used a different approach against the soil-borne bacterium, *Agrobacterium tumefaciens,* that utilized oncogenes of the phytopathogen. *Agrobacterium tumefaciens* possesses several oncogenes (e.g., *iaaM* and *ipt*) that trigger de novo synthesis of auxins and cytokinins to generate tumors following horizontal gene transfer into the plant genome. By expressing two self-complementary RNA constructs designed to initiate RNA interference (RNAi) of the *iaaM* and *ipt* oncogenes of *A. tumefaciens*, resistance to crown gall disease development was achieved in transgenic tomato plants. Transformed tomato lines display between 0.0% and 24.2% tumorigenesis, whereas controls averaged 100% tumorigenesis following stem inoculation with various strains of *A. tumefaciens* [67]. This mechanism of resistance is based on RNA silencing rather than the highly specific receptor–ligand-binding interactions characteristic of traditional plant resistance genes.

Manipulation of the plant's innate defense signaling pathways offers an alternative approach to conferring resistance against bacterial diseases by controlling a large number of induced genes either directly or indirectly. The nonexpressor of PR genes, *NPR1*, is a key regulatory gene of the salicylic acid-mediated systemic acquired resistance in *Arabidodpsis thaliana.* Moreover, *NPR1* plays a key role in ethylene and jasmonic acid signaling pathways that are involved in induced systemic resistance. Plants expressing *AtNPR1* are resistant to bacterial, fungal, and viral pathogens [68–70]. Similarly, transgenic carrots expressing *AtNPR1* are resistant to *Xanthomonas hortorum* as well as to fungal pathogens [71] and transgenic tomato plants expressing *AtNPR1* show enhanced resistance to *Ralstonia solanacearum* and to a lesser extent *to Xanthomonas campestris* [68]. When introduced into rice, *AtNPR1* confers resistance to the bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* [72]. Overexpression of the rice *AtNPR1* homolog, *OsNPR1*, also enhances resistance to *X. oryzae* pv. *oryzae* in rice [73] and overexpression of an apple *NPRI* confers increased resistance to *Erwinia amylovora* in transgenic apple cultivar 'Galaxy' and transgenic apple rootstock M26 [74].

The involvement of endogenous siRNA and miRNAs in mediating the triggering of defense mechanisms against bacteria is an area of considerable interest. In spite of tremendous progress at identifying and sequencing siRNAs and miRNAs, information available on the involvement of endogenous small RNAs at mediating defense regulation is scarce. One miRNA (miR393) was recently shown to contribute to basal defense against bacteria by targeting auxin-signaling components [75]. Agorio and Vera [76] characterized an *Arabidopsis* ocp (overexpressor of cationic peroxidase) mutant that overexpresses the H_2O_2-responsive Ep5C promoter. The ocp11 mutant exhibits enhanced disease susceptibility to several virulent and avirulent

Pseudomonas syringae strains. OCP11 was cloned and found to encode ARGONAUTE4 (AGO4), a component of the pathway that mediates the transcriptional gene silencing associated with siRNA. The mutant allele, ago4–1, was examined and found to be compromised in resistance to *P. syringae* [76]. This work provided insight into the involvement of small RNA gene silencing pathways in resistance to bacterial pathogens. Silencing the expression of key genes involved in pathogen–host interactions and disease susceptibility is opening new avenues for bacterial disease management.

Transgenic Resistance to Viral Pathogens

Transgenic resistance generally complements conventional breeding methodologies for the development of virus-resistant crops; however, the approach devised to engineer resistance against viral diseases differs from strategies employed in engineering resistance against fungal and bacterial diseases. This is due to the fact that viruses utilize cellular organelles and metabolic pathways for their replication. As such, they interfere with the basic functions in a living cell, unlike fungi and bacteria, which produce specialized reproductive structures or reproduce by binary fission. The approach to engineering resistance to viral diseases in crops essentially consists of activating anti-viral pathways of a natural, innate, and potent defense mechanism against viruses called RNA silencing [77–82].

Prior to the discovery of RNA silencing in the late 1990s and early 2000s, the concept of PDR [42] was commonly applied to engineering resistance against viral diseases. Initially, the viral coat protein gene was the preferred construct used to confer resistance to viruses in plants. However, it soon became apparent that almost any sequence derived from a viral genome, i.e., coat protein gene, movement protein gene, proteinase gene, RNA-dependent RNA polymerase gene, 5′ and 3′ noncoding regions, satellite RNA, defective interfering RNA, could provide resistance. Validated first in tobacco plants expressing the coat protein gene of *Tobacco mosaic virus* (TMV) [83], PDR was subsequently applied to horticultural crops for engineered resistance against viral diseases. In the first field trial of transgenic plants engineered for virus resistance, tomato plants expressing the coat protein gene of TMV were evaluated for resistance to mechanical inoculation by TMV [84]. Only 5% of the transgenic plants were symptomatic at the end of the trial compared with 99% of the nontransformed control plants. This study indicated that overexpression of the coat protein gene provided practical control of TMV under field conditions. Also, inoculated transgenic and uninoculated nontransformed plants had identical fruit yield, indicating that the transformation process and expression of the TMV coat protein gene did not alter the horticultural performance of the transgenic tomato plants.

The efficiency of viral genes at conferring resistance against vector-mediated virus transmission was first shown with cucumber plants engineered for resistance to *Cucumber mosaic virus* (CMV). Plants expressing the coat protein gene of CMV showed a significantly reduced incidence of CMV and a lower percentage of symptomatic plants than nontransformed control plants following CMV inoculation via aphid vectors [85]. In these studies, mechanically inoculated cucumber plants dispersed throughout the field provided reliable sources of inoculum for natural aphid populations to vector CMV. This approach coupled with the fact that field trials were established at a time of abundant endemic aphid flights caused sufficient disease pressure to make inferences about disease progress, resistance, and yield [85]. Subsequently, many other studies have confirmed the usefulness of engineered resistance at providing practical control of aphid-transmitted virus diseases [6].

PDR offers unique solutions to infection by multiple viruses, for example, by co-engineering and co-transferring genes from several viruses into a single host plant. The usefulness of multiple viral genes to control mixed virus infections was demonstrated early on with potato plants expressing the coat protein genes of *Potato virus X* (PVX) and *Potato virus Y* (PVY) [86, 87]. Potato line 303 was highly resistant to infections by PVX and PVY in the field [87]. Later, summer squash plants expressing coat protein gene constructs of CMV, *Zucchini yellow mosaic virus* (ZYMV) and/or *Watermelon mosaic virus* (WMV) were engineered for resistance to single viruses or combinations of these three viruses [88]. Among summer squash engineered for multiple virus resistance, line ZW-20 expressing the coat protein genes of ZYMV and WMV was highly resistant, regardless of whether the infections were

initiated by mechanical inoculations or mediated by aphid vectors [88, 89]. Similarly, transgenic line CZW-3 expressing the coat protein genes of CMV, ZYMV, and WMV was highly resistant to mixed infections by these three viruses following aphid transmission [88, 90].

Following the initial discovery by Powell Abel et al. [83], the viral coat protein gene from various viruses was introduced into numerous economically important crop species with the aim of achieving resistance. It was initially believed that resistance was provided by the viral protein itself via a mechanism involving excess plant-expressed coat protein that interfered with the uncoating step in viral replication [91]. However, it soon became apparent that resistance could be achieved in transgenic plants producing low or undetectable levels of coat protein [92]. The mechanism underlying the engineered resistance involved degradation of the transgene-derived mRNA into small fragments in a sequence-specific manner. This phenomenon was subsequently referred to as RNA silencing [78–82]. This antiviral plant defense mechanism is initiated by double stranded RNA (dsRNA) structures that are identical to the RNA to be degraded [93]. Silencing is associated with the production of 21–24 nt duplexes called small interfering RNAs (siRNAs) [94, 95]. The siRNAs are produced from dsRNA precursors by an endonuclease known as Dicer and become incorporated and converted to single stranded RNAs (ssRNAs) in an Argonaute-containing-RNA-induced silencing complex (RISC) that targets RNA for cleavage [80, 82, 96, 97].

RNA silencing is an innate and potent plant response to virus infection and a natural example of the concept of PDR that has provided new and unprecedented insights into virus–host interactions. Several approaches have been used to express dsRNA cognate to viral RNA for activation of RNA silencing. Expressing sense and antisense viral genes or inverted repeat viral genes to express hairpin RNAs (hpRNA) for the formation of duplex RNA are some of the most recent strategies used to engineer resistance against viruses [81]. For example, intron-spliced hairpin RNA (ihpRNA), ihpRNA overhang, and ihpRNA spacer were evaluated for resistance to PVY [98, 99]. The ihpRNA was found to be the most efficient construct to conferring resistance to PVY with 90% of the plants exhibiting RNA silencing [99]. The same strategy based on the use of highly conserved genetic segments of several viruses into a single transgene construct achieved multiple virus resistance [100].

Artificial plant micro RNAs (amiRNAs) have been used also to engineer virus resistance in plants. The *Arabidopsis thaliana* pre-miR159a precursor was used to generate two amiRNAs^{159} (amiR-P69^{159} and amiR-HC-Pro^{159}) with sequences complementary to *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV), respectively [101]. The amiR-P69159 was designed to target the viral suppressor P69 of TYMV while amiR-HC-Pro^{159} targeted the viral suppressor HC-Pro of TuMV. Transgenic plants carrying both transgenes expressed the corresponding amiRNAs and showed specific resistance to TYMV and TuMV. Low temperatures had no substantial effect on miRNA accumulation [101]. Similarly, the miR171 of *Nicotiana benthamiana* was used to target the 2b gene of CMV and confer resistance to CMV [102]. Although very promising, amiRNAs have not been used yet in crops for virus resistance.

Strategies other than PDR and RNA silencing have also been applied to confer resistance to viral diseases in plants. Peptides or protein microdomains designed to block protein-binding sites have been used. For example, peptide aptamers designed to microdomains of the nucleoprotein of *Tomato spotted wilt virus* (TSWV) that interact with one of the N-protein homomultimerization domains confer resistance to TSWV and other tospoviruses in *Nicotiana* species [103]. Similarly, antibody-based resistance has been investigated as an innovative strategy to confer virus resistance in plants. Hybridoma-derived single-chain variable antibody fragments (scFv) were engineered for resistance to various viruses in model plants [104–108]. A number of plant proteins with antiviral activity have been identified and exploited for engineered resistance to viruses in plants. A class of proteins termed ribosome-inactivating proteins [109], protease inhibitors (cystatins) [110], and the interferon-regulated 2–5A system [111, 112] were used successfully to confer virus resistance.

Status of Commercialized Disease Resistant Transgenic Crops

Since the early studies of Powell Abel and coworkers [83], numerous cereal, vegetable, legume, flower,

forage, turf, and fruit crops expressing virus-derived gene constructs have been created [6]. Many have been tested under field conditions and shown to be highly resistant to virus infections. Among the transgenic crops produced and evaluated in the field, two lines of transgenic summer squash, i.e., line ZW-20 resistant to ZYMV and WMV, and line CZW-3 resistant to ZYMV, WMV, and CMV [88], and papaya resistant to PRSV [113] have been deregulated and released for commercial use in the USA. Summer squash line ZW-20 received exemption status in 1994 and was the first disease-resistant transgenic crop to be commercialized. Summer squash line CZW-3 was deregulated and commercialized in 1996. Five transgenic zucchini cultivars and six transgenic straightneck or crookneck yellow squash cultivars derived from lines ZW-20 and CZW-3 were developed by conventional breeding. Their adoption is increasing steadily since their initial release in 1996. In 2006, the adoption rate was estimated to 22% (3,250 ha) across the country with an average rate of 70% in New Jersey and 20% in Florida, Georgia, and South Carolina [114]. Papaya expressing the coat protein gene of PRSV was deregulated and commercialized in Hawaii in 1998. PRSV is a major limiting factor to papaya production in Hawaii and around the world. After extensive testing, PRSV-resistant papaya was released in 1998 as devastation caused by the virus reached record proportions in the archipelago's main production region [113]. The impact of PRSV-resistant papaya on the papaya industry in Hawaii is evidenced by its rapid adoption rate. In 2000, the first wave of transgenic papaya bore fruit on more than 42% of the total acreage, and by 2009, transgenic papaya cultivars were planted on more than 90% of the total papaya land in Hawaii (780 of 866 total hectares) ([114], D. Gonsalves, personal communication October 2009).

Tomato and sweet pepper resistant to CMV and papaya resistant to PRSV are also released in the People's Republic of China [115, 116]. Limited, if any, information is available on their adoption rate. Two virus-resistant potato lines were deregulated also in 1998 and 2000 in the USA. After failed attempts to create a potato line resistant to *Potato leafroll virus* (PLRV) by coat protein gene expression, lines expressing a PLRV replicase gene were created, field tested, deregulated, and commercialized [117]. Resistance to PLRV was stacked with the coat protein gene of PVY. Many growers in the Pacific Northwest, Midwest USA and Canada are growing virus-resistant transgenic potato, and a breakdown in resistance has not been reported, neither any detrimental impact on the environmental or human health. Nonetheless, virus-resistant potato was withdrawn from the market after the 2001 growing season due to the reluctance of large processors and exporters to adopt these products [117].

Although not released yet, the transgenic plum cultivar 'Honeysweet' resistant to *Plum pox virus* (PPV) and another PRSV-resistant papaya are under consideration for deregulation in the USA. Plum trees expressing a coat protein gene of PPV are highly resistant to PPV infection [118–121]. The US Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) granted this plum cultivar deregulated status [122] and the Food and Drug Administration (FDA) has deemed a premarket review of the 'Honeysweet' unnecessary. Presently, the Environmental Protection Agency (EPA) is examining deregulation petitions for 'Honeysweet'. Papaya line X17-2 is also being considered for deregulation in the United States. Line X17-2 differs from the previously deregulated Hawaiian papaya in that it expresses the coat protein gene of a Florida isolate of PRSV and is suitable for cultivation in Florida [123]. APHIS and FDA granted X17-2-deregulated status and EPA is presently reviewing a deregulation petition [124].

Despite considerable progress in identifying genes that confer resistance against fungal and bacterial pathogens and testing their efficacy in transgenic plants under greenhouse conditions, it appears that the challenge is to translate the results to an effect in the field under continuous pathogen pressure. A search of the field testing release permit database in the USA reveals over 25 applications for field testing fungus-resistant transgenic soybean, corn, grape, strawberry, tobacco, lettuce, wheat, and American elm between 1993 and 2009. Both single traits such as resistance to Dutch Elm disease, ear mold, or downy mildew, were assessed as well as multiple traits against *Botrytis cinerea*, bacterial pathogens, nematodes, abiotic tolerance, glyphosate tolerance, and altered nutritional properties (oil and seed composition). Based on the FAO Biotechnology in Developing Countries Database (FAO-BioDeC), field

trials have been conducted in South Africa with transgenic strawberry expressing a phytoalexin synthesis gene and transgenic cotton with resistance to *Verticillium* and *Fusarium* in China. A few countries in Latin America, namely, Argentina, Brazil, and Cuba, are working on transgenic fungal resistance in tropical fruit trees, with some results already in the field. Apart from small projects in China, Thailand, and Pakistan addressing potato and wheat wilt, tomato wilt, and blight in rice, far fewer initiatives have been devoted to the development of transgenic cultivars with resistance against bacterial diseases.

To date, no transgenic cultivar with fungal or bacterial resistance has been released into commerce. The outcomes of field experiments have been variable and not in all cases is the resistance observed in the greenhouse extended to the field [125, 126]. In general, the constitutive expression of antimicrobial proteins in the transgenic host appears to confer partial resistance against these pathogens or increased tolerance at best, rather than absolute resistance. It is also clear that the resistance is limited to a few pathogens. The findings of Punja and Raharjo [127] further suggest that the levels of resistance in transgenic plants are influenced by interactions between endogenous and transgene defense compounds. These authors showed that transgenic carrots carrying a chitinase gene from tobacco exhibited enhanced resistance against *Botrytis cinera*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. However, the transgenic plants reacted similarly to *Alternaria radicini* and *Thielaviopsis basicola* as nontransgenic carrots. Further, improved resistance was not obtained against the same sleuth of pathogens and transgenic carrots expressing a chitinase gene from petunia. Indistinguishable disease reactions to *A. cucumerina, B. cinera, Collectotrichum lagenarium*, and *R. solani* were observed with transgenic and nontransgenic cucumber plants transformed with the chitinase gene from three sources, tobacco and petunia, as above, as well as bean.

Conclusions

Plant diseases caused by fungi, bacteria, and viruses are responsible for enormous losses in cultivated crops worldwide. Disease management relies on prevention, cultural practices, biological control, use of chemical pesticides and insecticides, antibiotic sprays, and selection of host resistant genotypes. These approaches have had limited success in the prevention or cure of diseases. Moreover, the frequent use of biocides, especially in subtherapeutic doses, is leading to the rapid development of resistance in certain fungi and bacteria. In addition, preventing fungal infection not only helps in combating yield losses but also keeps crops free of toxic compounds that are produced by some pathogenic fungi and referred to as mycotoxins. These compounds can affect the immune system and disrupt hormone balances or can be carcinogenic. Therefore, there is an urgent need to develop alternative ways to control infections caused by fungal, bacterial, and viral pathogens in agriculture.

The incorporation of specific disease-resistant traits in plants through genetic engineering offers a means to prevent disease-associated losses. For viruses, the concept of PDR [42] provided unique opportunities for innovative solutions to control virus diseases by developing resistant crops expressing genetic elements derived from a virus's own genome. Thirty years after the inception of PDR, various transgenic crops have been engineered for virus resistance and virus-resistant papaya, summer squash, sweet pepper, and tomato have been deregulated and released commercially. Applying the concept of PDR and the antiviral pathways of RNA silencing provides unique opportunities for developing virus-resistant crops and implementing efficient and environmentally sound management approaches to mitigate the impact of viral diseases. Based on the tremendous progress, the prospects of further advancing this innovative technology for practical control of virus diseases are very promising.

Engineered resistance to fungal and bacterial diseases is more complex than engineered resistance to viral diseases. This is due to the complex nature of fungi and bacteria, as well as complex interactions with their hosts. Therefore, in spite of remarkable progress and promising reports from greenhouse evaluations and field tests, no cultivar with engineered resistance to fungi and bacteria have been deregulated yet or commercially released. The fact that transgenic crops with resistance to fungi or bacteria might not have provided equivalent levels of plant protection compared to conventional management strategies in practice could account for the reluctance to deliver

transgenic plans or seeds to growers. Also, the degree of resistance to fungal and bacterial diseases achieved so far might be of limited practical use for disease management. However, new insights into pathogen–host interactions and the application of siRNA and microRNA technologies will undoubtedly identify new targets and open new horizons for the management of fungal and bacterial diseases.

Future Directions

Transgenic virus resistance is undoubtedly the most advanced of the applications of biotechnology for the management of plant pathogens. There is the documented safe release of virus-resistant transgenic crops over the past 14 years in the USA, in addition to a significant amount of evidence that these crops have little to no detrimental impact on animal health and the environment beyond those of conventional agricultural crops [6]. Given the discovery and elucidation of the antiviral pathways of RNA silencing, various new approaches have been used to develop transgenes more likely to stimulate RNA silencing via the design of transgenes that will form dsRNA structures in planta. These approaches seemingly have an advantage over a full-length coat protein gene in the sense orientation as they are generally unable to produce a functional protein, thus alleviating concerns arising from the presence of the coat protein in plant material. Some nonviral sources of virus resistance, such as host resistance genes and the silencing of host genes that are necessary for viral replication [81] have also been investigated and could theoretically alleviate concerns about synergism, recombination, and transencapsidation. Work with transcription factors, in particular the ERF subfamily transcription, provided evidence of increased resistance to bacterial, fungal, and viral pathogens as well as abiotic stress via overexpression of the soybean *GmERF3* transcription factor in tobacco [128]. The mechanism of the effect remains unknown; it is likely that a range of effects such as elevated antioxidant capacity, induced expression of chitinases, or antimicrobial compounds in *GmERF3* transgenic plants and the suppression of viral spread as a consequence of the reduced virus propagation are responsible. The commercial potential of these technologies remain largely undiscussed and untested.

On the other hand, engineering resistance to fungal and bacterial diseases has proven more recalcitrant. Whereas crops engineered for resistance against viral pathogens are in the market, no commercial transgenic product with enhanced resistance against fungal or bacterial diseases is currently available. These organisms have developed numerous survival strategies and more complex interactions with the plant host. As a result, it has proven more difficult to engineer resistance against these pathogens; far beyond designing a simple defense strategy involving the use of a single gene.

Strides in deciphering the siRNAs that are induced or repressed in response to pathogen attack will invariably contribute to new technologies for the development of transgenic resistance against fungal and bacterial pathogens. Microarray analysis with more wild-type plants and RNA-silencing mutants should reveal the relationship between the physiological processes and the associated disease-resistant phenotype. In the interim, the use of stacks or pyramids of *R* genes either from self or non-host plant species is perhaps the best approach available for the development of transgenic resistance against these pathogens. Presumably, this strategy would not affect resistance to nontarget pathogens while providing durable and broad-spectrum resistance and not interfering with the tight controls of the plants' defense systems. But the full potential of *R* gene expression to provide wide range resistance cannot be realized as long as its use is clouded by controversy over what it does and how well it works. The major limitation of the use of *R* genes in the development of transgenic resistance lies in the durability of the resistance, which is driven by the highly specific recognition of the elicitor molecule of the pathogen. Pathogens are usually able to overcome *R* gene–mediated recognition through the accumulation of mutations or through the loss of the elicitor gene [129, 130]. Moreover, not in all cases is resistance obtained after transfer to species that are not closely related [32]. Although a plethora of data is available on the identity of genes and gene products involved in plant resistance, knowledge of pathogenesis is relatively primitive. Continued sequencing of crop plant genomes as well as pathogen genome analysis will invariably lead to decoding of the functions of various defense genes as well as pathways and the isolation of more *R* genes. With this knowledge, the precise

manipulation of *R* genes to permit binding to proteins of pathogens and the activation of defense responses will be possible, and facilitate the design of synthetic *R* genes with desired specificities. For example, work with 13 alleles (*L*, *L1* to *L11*, and *LH*) from the *L* locus from flax suggests a role for the TIR region and LRR region of L proteins in the flax–flax rust interaction [131]. Most of the sequence variation between the three resistance genes, *Pi9*, *Pi2*, *and Piz*, is confined to the LRR domains, indicating that this domain plays a major role in determination of *Magnaporthe grisea* resistance specificities [30]. Further, Jia et al. [132] provided evidence that a single amino acid change in the *Pi-ta* LRD (leucine rich domain) or the *AVR-Pita*$_{176}$ protease motif disrupted interaction between the two and subsequent a loss of resistance against *M. grisea*, the etiological agent of rice blast disease. As more data become available on the structural information and on the interactions between resistance proteins and pathogen ligands, the tweaking of *R* gene domains to accommodate resistance of pathogens infecting distantly related crop species will be likely possible. Moreover, this could be extended to antimicrobial proteins that target components of the pathogen's arsenal, e.g., PGIP. Manipulation of their primary structure and hence determinants of specificity could theoretically generate novel recognition specificities.

Multiple races and range in plant hosts are major issues facing the development of durable disease resistance against fungal and bacterial pathogens. Moreover, in some instances, the expression of enhanced resistance is accompanied by reduced growth or altered morphology or development, given that the plant has been reprogrammed into defense mode. Precise control of transgene expression is thus pivotal; restricted expression at the site of infection and quick induction is crucial as well as a quick response to a wide variety of pathogens [133]. One option is to use pathogen-inducible promoters. Given that the available pathogen-inducible promoters show some patterns of background expression, trancriptomics will play an important role in the identification of more suitable promoters. Work with *Arabidopsis* has already identified potential candidates [134], as have investigations into synthetic promoters combining *cis* elements (W box and GCC-like box) that are conserved across species for restricted expression exclusively at the site of attempted pathogen invasion [135]. Other approaches are considering defensin promoters [136], while others have utilized tissue-specific promoters with some success [137]. The endogenous endothionin *Thi2.1* gene in *Arabidopsis thaliana* gene in tomato under the control of a fruit-inactive promoter (RB7) provided significant levels of enhanced resistance to bacterial and Fusarium wilt [137]. More recently, one study targeted the expression of the PR-protein in leaf trichomes, the preferential port for plant colonization by fungi. The gene encoding an exo α-1, 3-glucanase from *Trichoderma harzianum* was fused to the ATP promoter that confers high expression levels in trichome cells and transferred to *Arabidopsis* [138]. Increased resistance was obtained against *Botrytis cinerea* in needle-wounded and spray assays with transgenic plants. Up to 20% fewer total leaf infections were observed.

In summary, transgenic resistance against viral, fungal, and bacterial pathogens is being addressed on many fronts. The challenge is to develop durable, broad-spectrum resistance, given the diversity of strategies that pathogens deploy and their ability to rapidly adapt. Clearly, an integrated approach based on the knowledge of the defense system of the plant, the arsenal of the pathogen to cause disease and the key genes involved in pathogenesis is required for the design of effective transgenic management strategies. To date, tremendous progress has been made for viruses. Encouraging results have been obtained for various fungi and bacteria. The prospects of further advancing transgenic crops for practical control of fungal, bacterial, and viral diseases are very promising.

Bibliography

Primary Literature

1. Agrios G (2005) Plant pathology, 5th edn. Elsevier/Academic, San Diego, CA
2. Herrera-Estrella L, Depicker A, van Montagu M, Schell S (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303:209–213
3. Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187
4. Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803–4807

5. Murai N, Sutton DW, Murray MG, Slightom JL, Merlo DJ, Reichert NA, Sengupta-Gopalan C, Stock CA, Barker RF, Kemp JD, Hall TC (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222:476–482
6. Fuchs M, Gonsalves D (2007) Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. Annu Rev Phytopathol 45:173–202
7. Lindow SE, Hecht-Poinar EI, Elliot VJ (eds) (2002) Phyllosphere microbiology. American Phytopathological Society, St. Paul, MN
8. Matthews R (1973) Induction of disease by viruses, with special reference to *Turnip yellow mosaic virus*. Annu Rev Phytopathol 11:147–168
9. Struck C (2006) Infection strategies of plant parasitic fungi. In: Cooke BM, Gareth Jones D, Kaye B (eds) The epidemiology of plant diseases. Springer, Netherlands, pp 117–137
10. Oerke EC (2006) Crop losses to pests. J Agric Sci 144:31–43
11. Zadoks JC, Schein RD (1979) Epidemiology and plant disease management. Oxford Press, New York
12. Scorza R (2003) Breeding and molecular genetics. In: Auxt Baugher T, Singha S (eds) Concise encyclopedia of temperate tree fruit. The Haworth Press Inc, Binghamton, NY
13. Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197
14. Lorito M, Woo SL, Garcia Fernandez I, Colucci G, Harman G, Pintor-Toros J, Filippone E, Muccifora S, Lawrence C, Zoina A, Tuzun S, Scala F (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA 95:12734–12739
15. Maziah M, Sariah M, Sreeramanan S (2007) Transgenic banana rastali (AAB) with b 1, 3-glucanase gene for tolerance to fusarium wilt race 1 disease via *Agrobacterium*-mediated transformation system. Plant Pathol J 6:271–282
16. Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. Trans Res 10:533–543
17. Anand A, Zhou T, Trick H, Gill B, Bocku W, Muthukrishnan S (2003) Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. J Exp Bot 54:1101–1111
18. ÓBrien PA, MacNish GC, Milton NM (2001) Transgenic tobacco plants show different resistance to *Rhizoctonia solani* AG 4 and AG 8. Aust Plant Pathol 30:221–225
19. Epple P, Apel K, Bohlmann H (1997) Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarrium oxysporum*. Plant Cell 9:509–520
20. Chan Y, Prasad V, Sanjaya C, Chen K, Liu P, Chan M, Cheng C (2005) Transgenic tomato plants expressing an *Arabidopsis* thionin (*Thi2.1*) driven by fruit-inactive promoter battle against phytopathogenic attack. Planta 221:386–393
21. Anuradha TS, Divya K, Jami SK, Kirto PB (2008) Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens. Plant Cell Rep 27:1777–1786
22. François I, De Bolle M, Dwyer G, Goderis I, Woutors P, Verhaert P, Proost P, Schaaper W, Cammue B, Broekaert W (2002) Transgenic expression in *Arabidopsis* of a polyprotein construct leading to production of two different antimicrobial proteins. Plant Physiol 128:1346–1358
23. Sarowar S, Kim J, Deok Kim K, Kook Hwang B, Han Ok S, Sheop Shin J (2009) Overexpression of lipid transfer protein (LTP) genes enhances resistance to plant pathogens and LTP functions in long-distance systemic signaling in tobacco. Plant Cell Rep 28:419–427
24. Jayaraj J, Punja Z (2007) Combined expression of chitinase and lipid transfer protein genes in transgenic carrot plants enhances resistance to foliar fungal pathogens. Plant Cell Rep 26:1539–1546
25. Zhu YJ, Tang CS, Moore P (2005) Increased disease resistance in papaya by transforming a pathogen-inducible stilbene synthase gene. Acta Hort 692:107–114
26. Hanhineva K, Kokko H, Rogachev I, Aharoni A, Karenlampi S (2009) Stilbene synthase gene transfer caused alterations in the phenylpropanoid metabolism of transgenic strawberry (*Fragaria ananassa*). J Exp Bot 60:2093–2106
27. Solano R, Stepanova A, Chao QM, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENERESPONSE-FACTOR1. Gene Dev 12:3703–3714
28. Berrocal-Lobo M, Molina A (2004) ETHYLENE RESPONSE FACTOR 1 mediates *Arabidopsis* resistance to the soil-borne fungus *Fusarium oxysporum*. Mol Plant-Microbe Interact 17:763–770
29. Zuo K, Qin J, Zhao J, Ling H, Zhang L, Cao Y, Tang K (2007) Over-expression *GbERF2* transcription factor in tobacco enhances brown spots disease resistance by activating expression of downstream genes. Gene 391:80–90
30. Qu S, Liu G, Zhou B, Bellizzi M, Zeng L, Dai L, Han B, Wang GL (2006) The broad-spectrum blast resistance gene *Pi9* encodes a nucleotide binding site-leucine-rich repeat protein and is a member of a multigene family in rice. Genet 172:1901–1914
31. Ellis J, Dodds P, Lawrence G (2007) Flax rust resistance gene specificity is based on direct resistance-avirulence protein interactions. Annu Rev Phytopathol 45:289–306
32. Frost D, Way H, Howles P, Luck J, Manners J, Hardham A, Finnegan J, Ellis J (2004) Tobacco transgenic for the flax rust resistance gene *L* expresses allele-specific activation of defense responses. Mol Plant-Microbe Interact 17:224–232
33. Lu SF, Sun YH, Amerson H, Chiang VL (2007) MicroRNAs in loblolly pine (*Pinus taeda* L.) and their association with fusiform rust gall development. Plant J 51:1077–1098

34. Ellendorff U, Fradin EF, de Jonge R, Thomma BP (2009) RNA silencing is required for *Arabidopsis* defence against *Verticillium* wilt disease. J Exp Bot 60:591–602
35. Powell AL, Van Kan J, Ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM (2000) Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol Plant-Microbe Interact 13:942–950
36. Janni M, Sella L, Favaron F, Blechl A, De Lorenzo G, D'Ovidio R (2008) The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. Mol Plant-Microbe Interact 21:171–178
37. Desiderio A, Aracri B, Leckie F, Mattei B, Salvi G, Tigelaar H, Van Roekel JS, Baulcombe DC, Melchers LS, De Lorenzo G, Cervone F (1997) Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol Plant-Microbe Interact 10:852–860
38. Hu X, Bidney DL, Yalpani N, Duvick JP, Crasta O, Folkerts O, Lu G (2003) Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. Plant Physiol 133:170–181
39. Walz A, Zingen-Sell I, Loeffler M, Sauer M (2008) Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Plant Pathol 57:453–458
40. Liang H, Zheng J, Duan XY, Sheng BQ, Jia SG, Wang DW, Ouyang JW, Li JY, Li LC, Tian WZ, Hain R, Jia X (2000) A transgenic wheat with a stilbene synthase gene resistant to powdery mildew obtained by biolistic method. Chin Sci Bull 45:634–638
41. Schneider M, Droz E, Malnoe P, Chatot C, Bonnel E, Metrauz J (2002) Transgenic potato plants expressing oxalate oxidase have increased resistance to oomycete and bacterial pathogens. Potato Res 45:177–185
42. Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance-deriving resistance genes from the parasite's own genome. J Theor Biol 113:395–405
43. Wang GL, Song WY, Ruan DL, Sideris S, Ronald PC (1996) The cloned gene, Xa21, confers resistance to multiple *Xanthamonas oryzae* pv. *oryzae* isolates in transgenic pants. Mol Plant-Microbe Interact 9:850–855
44. Datta SK (2004) Rice biotechnology: a need for developing countries. AgBioForum 7:31–35
45. Zhang J, Li X, Jiang G, Xu Y, He Y (2006) Pyramiding of Xa7 and Xa21 for the improvement of disease resistance to bacterial blight in hybrid rice. Plant Breed 125:600–605
46. Yoshimura S, Yamanouchi U, Katayose Y, Toki S, Wang ZX, Kono I, Kurata N, Yano M, Iwata N, Sasaki T (1998) Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. Proc Natl Acad Sci USA 95:1663–1668
47. Zhao BY, Lin XH, Poland J, Trick H, Leach J, Hulbert S (2005) From the covers: A maize resistance gene functions against bacterial streak disease in rice. Proc Natl Acad Sci USA 102:15383–15388
48. Tai TH, Dahlbeck D, Clark ET, Gajiwala P, Pasion R, Whalen MC, Stall RE, Staskawicz BJ (1999) Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. Proc Natl Acad Sci USA 23:14153–14158
49. Mysore KS, D'Ascenzo MD, He X, Martin G (2003) Overexpression of the disease resistance gene *Pto* in tomato induces gene expression changes similar to immune responses in human and fruitfly. Plant Physiol 132:1901–1912
50. Arce P, Moreno M, Gutierrez M, Gebauer M, Dell'Orto P, Torres H, Acuna I, Oliger P, Venegas A, Jordana X, Kalazich J, Hluigue L (1999) Enhanced resistance to bacteria infection by *Erwinia carotovora* subsp. *atroseptica* in transgenic potato plants expression the attacin or the cecropin SB-37 gemes. Am J Potato Res 76:169–177
51. Norelli JL, Mills JZ, Jensen LA, Momol MT, Ko K, Aldwinckle HS (1998) Genetic engineering of apple for increased resistance to fire blight. Acta Hort 484:541–546
52. Mentag R, Luckevich M, Morency MJ, Seguin A (2003) Bacterial disease resistance of transgenic hybrid poplar expressing the synthetic antimicrobial peptide D4E1. Tree Physiol 23:405–411
53. Montesinos E (2007) Antimicrobial peptides and plant disease control. FEMS Microbiol Lett 270:1–11
54. Reynoird JP, Mourgues F, Norelli J, Aldwinckle HS, Brisset MN, Chevreau E (1999) First evidence for improved resistance to fire blight in transgenic pear expressing the *attacin* E gene from *Hyalophora cecropia*. Plant Sci 149:23–31
55. Malnoy M, Venissse JS, Brisset MN, Chevreau E (2003) Expression of bovine lactoferrin cDNA confers resistance to *Erwinia amylovora* in transgenic pear. Mol Breed 12:231–244
56. Norelli JL, Mills JZ, Momol MT, Aldwinckle HS (1999) effect of cecropin-like transgenes on fire blight resistance of apple. Acta Hort 489:273–278
57. Ko K, Norelli JL, Reynoird JP, Boresjza-Wysocka E, Brown SK, Aldwinckle HS (2000) Effect of untranslated leader sequence of AMV RNA 4 and signal peptide of pathogenesis-related protein 1b on attacin gene expression, and resistance to fire blight in transgenic apple. Biotech Lett 22:373–381
58. Düring K, Porsch P, Fladung M, Lörz H (1993) Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. Plant J 3:587–598
59. Ko K (1999) Attacin and T4 lysozyme transgenic 'Galazy' apple. Regulation of transgene expression and plant resistance to fire blight (*Erwinia amylovora*). PhD dissertation, Cornell University, New York
60. Mirkov TE, Fitzmaurice LC (1995) Protection of plants against plant pathogens. US Patent No 5422108
61. Allefs JJHM, de Jong ER, Florack DEA, Hoogendoors J, Stiekema WJ (1996) *Erwinia* soft rot resistance of potato cultivars expressing antimicrobial peptide tachyplesin. Mol Breed 2: 97–105
62. Wu G, Shortl BJ, Lawrence EB, Levine EB, Fitzsimmons KC, Shah DM (1995) Disease resistance conferred by expression

of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357–1368
63. Dong YH, Wang L, Xu JL, Zhang HB, Zhang XF, Zhang LH (2001) Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411:813–817
64. Herrera-Estrella L, Simpson J (1995) Genetically engineered resistance to bacterial and fungal pathogens. World J Microbiol Biotechnol 11:383–392
65. Krastanova SV, Sekiya M, Holden MR, Xue B, Pang S-Z, Momol EA, Gonsalves D, Burr TJ (2010) Transformation of grape rootstocks with a truncated *Agrobacterium* virE2 gene confers resistance to crown gall disease. Trans Res 19:949–958
66. Citovsky V, Warnick D, Zambryski P (1994) Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. Proc Natl Acad Sci USA 91:3210–3214
67. Escobar MA, Civerolo EL, Summerfelt KR, Dandekar AM (2001) RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. Proc Natl Acad Sci USA 98:13437–13442
68. Lin WC, Lu CF, Wu JW, Cheng ML, Lin YM, Yang NS, Black L, Green SK, Wang JF, Cheng CP (2004) Transgenic tomato plants expressing the Arabidopsis NPR1 gene display enhanced resistance to a spectrum of fungal and bacterial diseases. Trans Res 13:567–581
69. Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J (2006) Genetically engineered resistance to Fusarium head blight in wheat by expression of Arabidopsis NPR1. Mol Plant Microbe Interact 19:123–129
70. Meur G, Budatha M, Srinivasan T, Kumar KRR, Gupta AD, Kirti PB (2008) Constitutive expression of Arabidopsis NPR1 confers enhanced resistance to the early instars of *Spodoptera litura* in transgenic tobacco. Physiol Plant 133:765–775
71. Wally O, Jayaraj J, Punja ZK (2009) Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota* L.) expressing an Arabidopsis *NPR1* gene. Planta 231:131–141
72. Chen MS, Fitzgerald HA, Yadav RC, Canlas PE, Dong XN, Ronald PC (2001) Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J 27:101–113
73. Chen M, Fitzgerald HA, Canlas PE, Navarre DA, Ronald PC (2005) Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. Mol Plant-Microbe Interact 18:511–520
74. Malnoy M, Jin Q, Borjsze-Wysocka EE, He SY, Aldwinckle HS (2007) Overexpression of the apple MpNPR1 gene confers increased disease resistance in *Malus* x *domestica*. Mol Plant-Microbe Interact 20:1568–1580
75. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312:436–439
76. Agorio A, Vera P (2007) ARGONAUTE4 is required for resistance to *Pseudomonas syringae* in *Arabidopsis*. Plant Cell 19:3778–3790
77. Baulcombe D (2007) Amplified silencing. Science 315:199–200
78. Eamens A, Wang M-B, Smith NA, Waterhouse PM (2008) RNA silencing in plants: yesterday, today and tomorrow. Plant Physiol 147:456–468
79. Lin S-S, Henriques R, Wu H-W, Niu Q-W, Yeh S-D, Chua N-H (2007) Strategies and mechanisms of plant virus resistance. Plant Biotech Rep 1:125–134
80. Obbart DJ, Gordon KH, Buck AH, Jiggins FM (2009) The evolution of RNAi as a defence against viruses and transposable elements. Phil Trans R Soc B 364:99–115
81. Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M (2008) Strategies for antiviral resistance in transgenic plants. Mol Plant Pathol 9:73–83
82. Voinnet O (2008) Post-transcriptional RNA silencing in plant-microbe interactions: a touch of robustness and versatility. Curr Opin Plant Biol 11:464–470
83. Powell Abel P, Nelson RS, De B, Hoffman N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738–743
84. Nelson RS, McCormick SM, Delannay X, Dube P, Layton J, Anderson EJ, Kaniewskia M, Proksch RK, Horsch RB, Rogers SG, Fraley RT, Beachy RN (1988) Virus tolerance, plant growth and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. Bio/Technology 6:403–409
85. Gonsalves D, Chee P, Provvidenti R, Seem R, Slightom JL (1992) Comparison of coat protein-mediated and genetically-derived resistance in cucumbers to infection by *Cucumber mosaic virus* under field conditions with natural challenge inoculations by vectors. Bio/Technology 10:1562–1570
86. Lawson C, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer N (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance of *Potato virus X* and *Potato virus Y* in transgenic Russet Burbank. Bio/Technology 8:127–134
87. Kaniewski W, Lawson C, Sammons B, Haley L, Hart J, Delannay X, Tumer N (1990) Field resistance of transgenic Russet Burbank potato to effects of infection by *Potato virus X* and *Potato virus Y*. Bio/Technology 8:750–754
88. Tricoli DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD (1995) Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to *Cucumber mosaic virus*, *Watermelon mosaic virus 2*, and *Zucchini yellow mosaic virus*. Bio/Technology 13:1458–1465
89. Fuchs M, Gonsalves D (1995) Resistance of transgenic squash Pavo ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyviruses. Bio/Technology 13:1466–1473
90. Fuchs M, Tricoli DM, McMaster JR, Carney KJ, Schesser M, McFerson JR, Gonsalves D (1998) Comparative virus resistance and fruit yield of transgenic squash with single and multiple coat protein genes. Plant Dis 82:1350–1356
91. Lindbo JA, Dougherty WG (2005) Plant Pathology and RNAi: a brief history. Annu Rev Phytopathol 43:191–204

92. Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell 5:1749–1759
93. Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA 95:13959–13964
94. Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in post-transcriptional gene silencing in plants. Science 286:950–952
95. Hamilton AJ, Voinnet O, Chappell L, Baulcombe DC (2002) Two classes of short interfering RNA in RNA silencing. EMBO J 21:4671–4679
96. Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O (2006) Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense. Science 313:68–71
97. Hannon GJ (2002) RNA interference. Nature 418:244–251
98. Smith NA, Singh SP, Wang M-B, Stoutjesdijk P, Green A, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature 407:319–320
99. Wesley V, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590
100. Bucher E, Lohuis D, van Poppel PMJA, Geerts-Dimitriadou C, Goldbach R, Prins M (2006) Multiple virus resistance at a frequency using a single transgene construct. J Gen Virol 87:3697–3701
101. Niu Q-W, Lin S-S, Reyes JL, Chen K-C, Wu H-W, Yeh S-D, Chua N-H (2006) Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. Nat Biotechnol 11:1420–1428
102. Qu F, Ye X, Morris TJ (2008) Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. Proc Natl Acad Sci USA 105:14732–14737
103. Rudolph C, Schreier PH, Uhrig JF (2003) Peptide-mediated broad-spectrum plant resistance to tospoviruses. Proc Natl Acad Sci USA 100:4429–4434
104. Boonrod K, Galetzka D, Nagy PD, Conrad U, Krczal G (2004) Single-chain antibodies against a plant viral RNA-dependent RNA polymerase confer virus resistance. Nat Biotechnol 22:856–862
105. Nölke G, Fischer R, Shillberg S (2004) Antibody-based pathogen resistance in plants. J Plant Pathol 86:5–17
106. Nölke R, Cobanov P, Uhde-Holzem K, Reustle G, Fischer R, Shillberg S (2009) *Grapevine fanleaf virus* (GFLV)-specific antibodies confer GFLV and *Arabis mosaic virus* (ArMV) resistance in *Nicotiana benthamiana*. Mol Plant Pathol 10:41–49
107. Schillberg S, Zimmermann S, Findlay K, Fischer R (2000) Plasma membrane display of anti-viral single chain Fv fragments confers resistance to *Tobacco mosaic virus*. Mol Breed 6:317–326
108. Tavladoraki P, Benvenuto E, Trinca S, De Martinis D, Cattaneo A, Galeffi P (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature 366:469–472
109. Lodge JK, Kaniweski WK, Tumer NE (1993) Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. Proc Natl Acad Sci USA 90:7089–7093
110. Gutierrez-Campos R, Torres-Acosta JA, Saucedo-Arias LJ, Gomez-Lim MA (1999) The use of cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants. Nat Biotechnol 17:1223–1226
111. Mitra A, Higgins DW, Langenberg WG, Nie H, Sengupta DN, Silverman RH (1996) A mammalian 2–5A system functions as an antiviral pathway in transgenic plants. Proc Natl Acad Sci USA 93:6780–6785
112. Ogawa T, Hori T, Ishida I (1996) Virus-induced cell death in plants expressing the mammalian 2′, 5′, oligoadenylate system. Nat Biotechnol 14:1566–1569
113. Gonsalves D (1998) Control of *Papaya ringspot virus* in papaya: a case study. Annu Rev Phytopathol 36:415–437
114. Johnson SR, Strom S, Grillo K (2007) Quantification of the impacts on US agriculture of biotechnology-derived crops planted in 2006. National Center for Food and Agriculture policy. http://www.ncfap.org
115. James C (2010) Global status of commercialized biotech/GM crops. The first thirteen years, 1996 to 2008. http://www.isaaa.org/resources/publications/briefs/39/executivesummary/default.html
116. Stone R (2008) China plans $3.5 billion GM crops initiative. Science 321:1279
117. Kaniewski WK, Thomas PE (2004) The potato story. Agric Biol Forum 7:41–46
118. Capote N, Pérez-Panadés J, Monzó C, Carbonell E, Urbaneja A, Scorza R, Ravelonandro M, Cambra M (2007) Assessment of the diversity and dynamics of *Plum pox virus* and aphid populations in transgenic European plums under Mediterranean conditions. Trans Res 17:367–377
119. Hily JM, Scorza R, Malinowski T, Zawadzka B, Ravelonandro M (2004) Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. Transgenic Res 5:427–436
120. Malinowski T, Cambra M, Capote N, Zawadzka B, Gorris MT, Ravelonandro M (2006) Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. Plant Dis 90:1012–1018
121. Zagrai I, Capote N, Ravelonandro M, Ravelonandro M, Cambra M, Zagrai L, Scorza R (2008) *Plum pox virus* silencing of C5 transgeic plums is stable uner challenge inoculation with heterologous viruses. J Plant Pathol 90: S1.63–S1.71
122. Bech R (2007) Finding of no significant impact and decision notice. Determination of nonregulated status for C5 plum resistant to plum pox virus. Animal and Plant Health Inspection Service. http://www.aphis.usda.gov/brs/aphisdocs2/04_26401p_com.pdf

123. Davis MJ (2004) Petition for Determination of Nonregulated status for the X17-2 line of papaya: A *Papaya ringspot virus*-resistant papaya. Animal and Plant Health Inspection Service. http://www.aphis.usda.gov/brs/aphisdocs/04_33701p.pdf
124. Shea K (2009) University of Florida; determination of nonregulated status for papaya genetically engineered for resistance to the *Papaya ringspot virus*. Animal and Plant Health Inspection Service. Fed Regist 74:45163–45164. http://edocket.access.gpo.gov/2009/pdf/E9-21092.pdf
125. Anand A, Zhou T, Trick HN, Gill BS, Bockus WW, Muthukrishnan S (2003) Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. J Exp Bot 54:1101–1111
126. Chenault K, Melouk H, Payton M (2005) Field reaction to *Sclerotinia* blight among transgenic peanut lines containing antifungal genes. Crop Sci 45:511–515
127. Punja Z, Raharjo S (1996) Response of transgenic cucumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. Plant Dis 80:999–1005
128. Zhang G, Chen M, Li L, Xu Z, Chen X, Jiaming Guo J, Youzhi M (2009) Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. J Exp Bot 60:3781–3796
129. Joosten MH, Cozijnsen TJ, De Wit PJ (1994) Host resistance to fungal tomato pathogen lost by a single base pair change in an avirulence gene. Nature 367:384–386
130. Gassmann W, Dahlbeck D, Chesnokova O, Minsavage G, Jones J, Stakawicz B (2000) Molecular evolution of virulence in natural field strains of *Xanthomonas campestris* pv. *vesicatoria*. J Bacteriol 182:7053–7059
131. Ellis JG, Lawrence GJ, Luck JE, Dodds PN (1999) Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell 11:495–506
132. Jia Y, McAdams S, Bryan E, Hershey H, Valent B (2000) Direct interaction of the resistance gene and avirulence gene products confers rice blast resistance. EMBO J 14:4004–4014
133. Gurr SJ, Rushton PJ (2005) Engineering plants with increased disease resistance: how are we going to express it? Trends Biotechnol 23:283–290
134. Eulgen T (2005) Regulation of the *Arabidopsis* defence transcriptome. Trends Plant Sci 10:71–78
135. Rushton P, Reinstädler A, Lipka V, Lippok B, Somssich I (2002) Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. Plant Cell 14:749–762
136. Kovalchuk N, Li M, Wittek F, Reid N, Singh R, Shirley N, Ismagul A, Eliby S, Johnson A, Milligan A, Hrmova M, Langridge P, Lopato S (2010) Defensin promoters as potential tools for engineering disease resistance in cereal grains. Plant Biotechnol J 8:47–64
137. Chan Y, Prasad V, Sanjaya K, Chen K, Liu P, Chan M, Cheng C (2005) Transgenic tomato plants expressing an *Arabidopsis* thionin (*Thi2.1*) driven by fruit-inactive promoter battle against phytopathogenic attack. Planta 221:386–393
138. Calo L, Garcia I, Gotor C, Romero LC (2006) Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a Trichoderma a-1, 3-glucanase. J Exp Bot 57:3911–3920

Books and Reviews

Charkowski AO (2007) Potential disease control strategies revealed by genome sequencing and functional genetics of plant pathogenic bacteria. In: Punja ZK, De Boer SH, Sanfaçon H (eds) Biotechnology and plant disease management. CAB International, Cambridge, MA, pp 402–497

Csroba T, Pantaleo V, Burgyán J (2009) RNA silencing: an antiviral mechanism. In: Loebenstein G, Carr JP (eds) Advances in virus research, vol 75, natural and engineered resistance to plant viruses. Academic, San Diego, CA, pp 35–72

Diaz-Pendón JA, Ding S-W (2008) Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annu Rev Phytopathol 46:303–326

Knox RE, Clarke FR (2007) Molecular breeding approaches for enhanced resistance against fungal pathogens. In: Punja ZK, De Boer SH, Sanfaçon H (eds) Biotechnology and plant disease management. CAB International, Cambridge, MA, pp 321–357

Misra S, Bhargava A (2007) Application of cationic antimicrobial peptides for management of plant diseases. In: Punja ZK, De Boer SH, Sanfaçon H (eds) Biotechnology and plant disease management. CAB International, Cambridge, MA, pp 301–320

Rommens CM, Kishore GM (2000) Exploiting the full potential of disease-resistance genes for agricultural use. Curr Opin Biotechnol 11:120–125

Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. Annu Rev Plant Biol 60:485–510

Stuiver MH (2006) Engineering fungal resistance in crops. In: Halford N (ed) Plant biotechnology: current and future applications of genetically modified crops. Wiley, West Sussex, England, pp 225–239

Stuiver MH, Custers JH (2000) Engineering disease resistance in plants. Nature 411:865–868

Suzuki JY, Tripathi S, Gonsalves D (2007) Virus-resistant transgenic papaya: commercial development and regulatory and environmental issues. In: Punja ZK, De Boer SH, Sanfaçon H (eds) Biotechnology and plant disease management. CAB International, Cambridge, MA, pp 436–461

Xing T (2007) Signal transduction pathways and disease resistant genes and their applications to fungal disease control. In: Punja ZK, De Boer SH, Sanfaçon H (eds) Biotechnology and plant disease management. CAB International, Cambridge, MA, pp 1–15

Yang CH, Yang S (2007) Modulating quorum sensing and type III secretion systems in bacterial plant pathogens for disease management. In: Punja ZK, De Boer SH, Sanfaçon H (eds) Biotechnology and plant disease management. CAB International, Cambridge, MA, pp 16–57

Transgenic Crops, Environmental Impact

Angharad M. R. Gatehouse[1], Martin G. Edwards[1], Natalie Ferry[1,2], Micheal D. K. Owen[3]
[1]School of Biology, Newcastle Institute for Research on Sustainability, Newcastle University, Newcastle upon Tyne, UK
[2]School of Environment and Life Sciences, University of Salford, Salford, UK
[3]Department of Agronomy, Iowa State University, Ames, IA, USA

Article Outline

Glossary

***Bt*-expressing transgenic crops**
Insect resistance crops
Novel mechanisms for insect resistance
Herbicide-tolerant transgenic crops
Food security
Sustainability
Risk assessment
Environmental impact
Impact on beneficial insects
Gene flow

Definition of the Subject

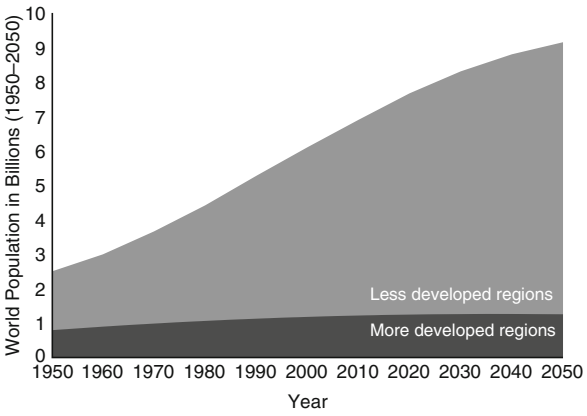
Agriculture is the production of food and commodities through farming. Since its dawn some 10,000 years ago, humankind has had an ever increasing impact on the environment. With the increasing intensification of agriculture, particularly during the twentieth century, this impact has become even more pronounced, often with undesirable or unacceptable consequences, including water pollution, soil erosion, and loss of habitat, often accompanied by a loss of biodiversity. Pests, particularly weedy plants, demonstrate a remarkable ability to adapt to agricultural production systems. The practice of growing monocultures, typically used in intensive agriculture, increases the number of pests; these are currently predominantly controlled through use of pesticides. However, with increasing exposure to pesticides, many pest populations are evolving resistance to these compounds. An additional problem encountered with many pesticides, and particularly insecticides, is their non-target effects on beneficial insects. Transgenic crops expressing genes conferring either resistance to insect pests and/or herbicides are becoming increasingly more widely grown. However, in many parts of the world, before such crops are commercialized, they have to be assessed for their potential environmental impact which measures the impact they will have on non-target species and their ability to outcompete native species. Environmental risk assessments cover both the transgenic crop concerned and the potential impacted environment. The assessment process includes evaluation of the characteristics of the crop and its effect and stability in the environment, combined with ecological characteristics of the environment in which the introduction will take place. The assessment also includes unintended consequences that could result from the insertion of the new gene. For all transgenic crops, the direct and indirect effects they have on non-target organisms must be considered. Furthermore, issues relating to potential gene flow to other non-transgenic cultivars and/or wild near-relatives also must be addressed.

Introduction

Food security figures are high on both the political and social agenda [1]. This is not surprising given that the global population increased fourfold during the last century, with current estimates suggesting that it will reach approximately 9.2 billion by 2050 (Fig. 1). However, this increase has primarily been seen in the developing regions of the world, with population remaining relatively static in the developed world. As expected, it is those countries least able that need to significantly

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Transgenic Crops, Environmental Impact. Figure 1
Project world population increase from 1950 to 2050 showing an overall fourfold increase in population. During this period, population growth in the more developed regions of the world are predicted to remain moderately static

increase food production, with an estimated increase in productivity of 300% for Africa and 80% for Latin America; even so, it is estimated that North America will need to increase productivity by some 30% [2]. Without an increase in "farm productivity," this required increase in food production equates to an additional 1.6 billion hectares of arable land by 2050, a scenario that is becoming increasingly less sustainable or, indeed, achievable. An immediate priority for agriculture is thus to achieve increased crop yields in a sustainable and cost-effective way in order to avoid a Malthusian disaster.

The concept of utilizing a transgenic (biotech) approach was realized in the mid-1990s with the commercial introduction of genetically modified crops. In 2009, it was estimated that some 14 million farmers planted 134 million hectares (330 million acres) of biotech crops in 25 countries, representing a 7% increase over the preceding year [3]. Interestingly, 90% of these farmers were small and resource-poor farmers from developing countries. In economic terms, the global market value for biotech crops in 2009 was US$10.5 billion; this figure represents 20% of the US$52.2 billion global crop protection market and 30% of US$34 billion commercial seed market. Of this US$10.5 billion biotech crop market, approximately 50% was accounted for by biotech maize, with soybean, cotton, and canola (oilseed rape) accounting for 37.2%, 10.5%, and 3%, respectively.

Although transgenic crops have only been available since the 1990s, they have dramatically changed the face of world agriculture and are likely to represent the most rapidly adopted technology in agriculture [4]. This trend is largely attributed to one herbicide, glyphosate (*N*-(phosphonomethyl)glycine), and crops that are genetically modified to have selectivity (resistance) to the glyphosate when the herbicide is applied topically to them. However, many new transgenic crops have been introduced that include multiple transgenic traits, thus providing greater value to agriculture. The reported increased economic profitability attributable to transgenic crops is a major factor that provides the impetus for the increasing adoption of these crops worldwide, both in developing as well as industrialized nations [5]. Despite their benefits, not least in increasing crop yield in a more sustainable and environmentally benign way compared to intensive agriculture (Tables 1 [6] and 2 [6–20]), neither the technology, nor the crops, are universally perceived as of benefit to humankind. There are fears that all questions of risk attributable to the wide-scale growing of such crops have not been adequately addressed, or perhaps even identified [21]. While many of these fears may be unfounded, public debate concerning potential environmental and public health risks is crucial.

This chapter will provide a brief overview of first-generation transgenic crops. Specifically, it will discuss the environmental impact of the wide-scale growing of such crops both in terms of their potential non-target effects and the potential for gene flow.

Insect-Resistant Transgenic Crops

Insect-resistant transgenic crops, also known as Biotech crops, were first commercialized in the USA during the mid-1990s with the introduction of genetically modified corn (maize), potato, and cotton plants expressing genes encoding the entomocidal δ-endotoxin from *Bacillus thuringiensis* (*Bt*; also known as crystal/Cry proteins). In terms of *Bt*-expressing maize and cotton,

Transgenic Crops, Environmental Impact. Table 1 Average farm level agronomic and economic effects of *Bt* crops

Country	Insecticide reduction (%)	Increase in effective yield (%)	Increase in gross margin (US$/ha)
	Bt cotton	*Bt cotton*	*Bt cotton*
Argentina	47	33	23
Australia	48	0	66
China	65	24	470
India	41	37	135
Mexico	77	9	295
South Africa	33	22	91
USA	36	10	58
	Bt maize	*Bt maize*	*Bt maize*
Argentina	0	9	20
Philippines	5	34	53
South Africa	10	11	42
Spain	63	6	70
USA	8	5	12

Source: Zilberman et al. (2010) [6] (Courtesy of *Choices* and the Agricultural & Applied Economics Association)

Transgenic Crops, Environmental Impact. Table 2 Summary of primary studies on the effects of herbicide-tolerant (HT) crops on yields

Crop/Reference	Data source	Effect on yields
Herbicide-tolerant soybeans		
[7]	Experiments	Same
[8]	Experiments	Increase
[9]	Experiments	Increase
[10]	Survey	Increase
[11]	Survey	Small increase
[12]	Survey	Small decrease
[13]	Survey	Same
[14]	Survey	Increase
[15]	Survey	Same
Herbicide-tolerant cotton		
[16]	Experiments	Same
[17]	Experiments	Same
[18]	Experiments	Same
[19]	Experiments	Same
[20]	Survey	Increase

Source: Zilberman et al. (2010) [6] (Courtesy of *Choices* and the Agricultural & Applied Economics Association)

the current market is estimated at US$2.3 billion and US$0.9 billion, respectively. Detailed knowledge on the mode of action of these Cry proteins is not only essential to optimize their efficacy against target insects, but also to predict the potential for non-target effects on beneficial insects. On ingestion, *Bt* toxins are solubilized in the midgut where they are proteolytically cleaved at the N-terminal to a 65–70-kDa truncated (active) form. The active molecules then exert their pathological effects by binding to a specific receptor (s) in the midgut epithelial cells and insert into the membrane where they form pores, this results in cell death by colloid osmotic lysis, followed by death of the insect [22]. In most commercial crop varieties, these Cry proteins are expressed in the active form and as such differ from those used in biopesticide formulations where the Cry proteins are present as protoxins.

Early commercial varieties of insect-resistant transgenic crops expressed single crystal (Cry) proteins with specific activity against lepidopteran pests as illustrated by Bollgard® cotton expressing Cry1Ac developed by Monsanto and Attribute® maize expressing Cry1Ab developed by Syngenta. Subsequently, other lepidopteran-active *Bt* toxins, such as Cry1F and Cry2Ab2, were introduced, and often presented as pyramided genes in a single variety (Widestrike® cotton expressing both Cry1F + Cry1Ac developed by Dow Agrosciences and Bollgard II® cotton expressing Cry1Ac + Cry2Ab2 developed by Monsanto). Transgenic crops, particularly maize, expressing Cry3 proteins to protect against coleopteran pests such as chrysomelid root-worms have also been commercialized (e.g., Monsanto's Yieldgard Rootworm® maize expressing Cry3Bb1, Dow Agrosciences' Herculex RW® maize expressing Cry34Ab1 and Cry35Ab1, stacked with a HT gene, and Syngenta's Agrisure RW® maize expressing a modified version of Cry3A). In 2009, SmartStax, a novel biotech maize containing eight different

genes for insect and herbicide resistance, was granted approval in the USA. Furthermore, Syngenta has recently launched Agrisure Viptera trait-stacked corn, the first commercially available variety to exploit a non-cry *Bt* protein (Vip3) for the provision of multiple pest resistance. In China, *Bt* cotton cultivars expressing Cry1Ac together with a modified cowpea trypsin inhibitor (CpTI) were commercially released in 2000 [23], and in 2005, accounted for approximately 15% of the cotton crop [24]. The major reason for co-expressing *Bt* and CpTI in cotton was to reduce the likelihood of the insects becoming resistant to this cultivar, thus extending its effective life.

While it is clear that *Bt*-expressing crops have made a significant beneficial impact to global agriculture, not least in terms of pest reduction, improved quality, and increased yield (Table 1 [6]), it is also becoming increasingly evident that there is a need to develop alternative strategies, not least because of the potential for pest populations to evolve resistance [25], and to the lack of effective control of homopteran pests. Recently, crops expressing vegetative insecticidal proteins (VIPs) have been commercialized (see above). However, alternative approaches based on the use of plant-derived or animal-derived genes, including those from insects (such as those encoding immunosuppressive proteins), are being investigated and developed. More recently, the potential to identify and exploit endogenous resistance genes using functional genomics and the use of RNAi are actively being investigated [26–30]. However, if these novel approaches are to play a useful role in crop protection, it is desirable that they do not have a negative impact on beneficial organisms at higher trophic levels since this would inevitably affect agro-ecosystem function. This is equally true for *Bt*-expressing crops.

Herbicide-Tolerant/Resistant Transgenic Crops

From the first commercialization of transgenic crops, to date, herbicide tolerance has consistently been the dominant trait and, as a consequence of its rapid and widespread adoption, has made an immeasurable change to global agriculture. In 2009, herbicide tolerance deployed in soybean, maize (corn), canola (oilseed rape), cotton, sugarbeet, and alfalfa occupied 62% (83.6 million hectares) of the global biotech area of 134 million hectares. Furthermore, in 2009, the stacked double and triple traits occupied an area of 28.7 million hectares (equivalent to 21% of global biotech crop area), much larger than the insect-resistant varieties which occupied 21.7 million hectares (15%). Interestingly, stacked trait products and herbicide-tolerant products grew at the same rate of 6%, while insect resistance as a trait grew at 14% [31].

While there are a number of herbicide-tolerant transgenic crops that have been developed to several herbicides with different modes of phytotoxic action, the primary influence in world agriculture is glyphosate [32, 33]. This preference for glyphosate is based on (1) the target site, (2) the ability of this compound to translocate in plants, and (3) the inability of plants to rapidly detoxify it. Glyphosate controls weeds by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), a key enzyme in the shikimate biosynthetic pathway which is necessary for the production of the aromatic amino acids, auxin, phytoalexins, folic acid, lignin, plastoquinones, and many other secondary plant products. Over 30% of the carbon fixed by plants may pass through this pathway. Inhibition of EPSPS by glyphosate deregulates the pathway, leading to even more carbon flowing through the pathway with accumulation of shikimate and shikimate-3-phosphate. Up to 16% of the plant's dry matter can accumulate as shikimate. Glyphosate occupies the binding site on EPSPS for phosphoenolpyruvate, a substrate of EPSPS, by mimicking an intermediate state of the enzyme-substrate complex. There are two forms of EPSPS in nature, EPSPS I, which is found in plants, fungi, and most bacteria, and is sensitive to glyphosate. The second form is EPSP II, which is found in glyphosate-resistant bacteria and is not inhibited by glyphosate. It is thus the gene encoding an EPSPS II that has been used to genetically engineer crops to confer resistance/tolerance to this particular herbicide [34].

Environmental Impact of Transgenic Crops

The adoption of transgenic crops, and in particular herbicide-tolerant crops, has resulted in significant changes in agronomic practices. Many clear benefits to both the grower and consumer can be identified with transgenic crops that are currently grown, not least in the often significant increases in productivity

accompanied by reductions in synthetic pesticide usage (Tables 1 [6] and 2 [7–20, 35–37]), thus making transgenic crops more environmentally sustainable. Despite the fact that such crops have now been commercialized for some 15 years, alongside these benefits, concerns are still being expressed as to their safety, particularly in terms of their impact on human health and the environment. It is imperative that these concerns are addressed in a scientifically sound and appropriate manner. Thus, assessing the environmental consequences of transgenic crops is an important prerequisite to their commercialization.

In many regions of the world, regulatory frameworks are in place to ensure that all pre-commercial transgenic crops are evaluated for potential impacts on human health, animal health, and the environment according to established standards of risk assessment and current scientific knowledge, prior to authorizations for import or planting being granted. The environmental risk assessment for such crops follows the same fundamental principles as other risk assessment schemes, i.e., risk is a function of hazard and exposure. However, one of the main differences that sets risk assessment of transgenic crops apart from other risk assessment procedures is that it is highly dependent on the crop and the introduced trait; hence, a case-by-case approach is required [38].

Impact of Insect-Resistant Transgenic Crops on Beneficial Insects

For any technology to be acceptable to the public at large, the perceived benefits have to outweigh any potential/perceived risk – this requirement is equally true for transgenic crops [39]. Common to all transgenic crops currently grown, general environmental concerns relate to the potential for horizontal gene transfer, gene flow, and invasiveness to occur both in managed and natural environments. However, in the case of insect-resistant transgenic crops, perhaps, the greatest concerns relate to their non-target effects and, in particular, their effects on beneficial insects such as natural enemies (predators and parasitoids) and pollinators.

An important consideration is the likelihood of exposure of the transgene product to the non-target insect, and indeed the different routes of exposure. The most obvious exposure route for non-target herbivores is through direct ingestion of plant material, although this will be influenced by the mode of insect feeding and spatial and temporal expression patterns of the transgene product within the plant. While exposure to pollinators is via the nectar and pollen, exposure routes to natural enemies are more diverse. Although many predators and parasitoids, particularly in the adult stage, are facultative herbivores and thus can be exposed to transgene products directly from consuming plant tissues (pollen, nectar), the larval stages are more likely to be exposed from consuming insects that have themselves fed on plant tissues where the transgene product has been expressed and accumulated. It is pertinent to point out that in some cases, particularly when the host is a sap-sucking insect such as aphids, that the natural enemy is rarely exposed to the transgene product; however, the scenario is quite different when the host is a chewing insect, in which case the likelihood of the natural enemy being exposed is very high.

Impact on Natural Enemies Agro-ecosystems consist of complex trophic interactions, with many aspects of the physiology, ecology, and behavior of the organisms present being governed by interactions with organisms from the same or different trophic levels. Since plants are the basis of these food webs on which all organisms at higher trophic levels depend, and since herbivorous insect species at the second trophic level have an important position in food webs, together with the fact that approximately 50% of all insect species are plant feeders, the potential for exposure to non-targets, either directly (bi-trophic) or indirectly (tri-trophic), is high. This is particularly true for predators and parasitoids which play an important role in suppressing insect pest populations both in the field and under specialized cultivation systems. Because of this important role and because expression of transgenes that confer enhanced levels of resistance to insect pests is of particular relevance (since they are aimed at manipulating the biology of organisms in a different trophic level to that of the plant), these beneficial insects have been the focus of numerous major studies to evaluate the non-target effects of transgenic crops. The nature of these investigations has been very varied ranging from detailed studies at the molecular/biochemical level

under controlled environmental conditions, to glasshouse trials using deliberately infested plants and released natural enemies, to effects at the population level in the field. Further, they have involved delivering the transgene product both in artificial diet and *in planta* and either directly (at the bi-trophic level) or via a tri-trophic (plant-pest-natural enemy) interaction. Recent studies relating to both the effects of *Bt*-expressing crops and crops expressing molecules still under development are summarized in Tables 3 [40–72] and 4 [50, 51, 73–102] for predators and parasitoids, respectively.

As mentioned above, prior to their commercialization, all transgenic crops have to undergo rigorous risk assessment. However, despite a global commodities market, the risk assessment process itself varies from region to region, as does the non-target organisms selected for testing. Obviously, the latter will vary depending upon geographical and climatic regions; however, there are concerns that there should be greater uniformity in the species selected; these should at least be representative of the major ecosystem functions. In an attempt to address this short-coming, Romeis et al. [103] have recently refined the 3-tier risk assessment to evaluate potential adverse impacts of insect-resistant transgenic crops on non-target arthropods, with specific recommendations for early-tier laboratory studies used in the risk assessment process. Although this risk assessment protocol has been primarily developed for crops expressing *Bt* toxins, the concepts put forward apply to other arthropod-active proteins. Typically, the risk assessment follows a tiered approach that starts with laboratory studies under worst-case exposure conditions; such studies have a high ability to detect adverse effects on non-target species, if present. Clear guidance on how such data are produced in laboratory studies assists the product developer and risk assessors. The need for a high level of reproducibility and clearly defined risk hypotheses contribute to the robustness of, and confidence in, the environmental risk assessments of transgenic plants. Further, these authors emphasized that confidence in the results of early-tier laboratory studies is a precondition for the acceptance of data across regulatory jurisdictions and should encourage agencies to share useful information and thus avoid redundant testing. When evaluating potential risks of a given technology, it is important to use relevant comparators. This holds true for risk assessment of transgenic crops. Few studies have actually been designed to directly compare recombinant DNA technology with conventional pest control strategies, although recent studies by Mulligan et al. [68, 69] directly compared the non-target effects of the synthetic pesticide cypermethrin with oilseed rape plants expressing a cysteine protease inhibitor against two predators. While neither form of pest control treatment negatively affected the carabid, the effects of the transgenic crop on the lacewing were significantly lower than with the commonly used pesticide.

Recent studies have performed meta-analyses on a modified public database [104] to synthesize current knowledge about the effects of *Bt* cotton, maize, and potato on the abundance and interactions of arthropod non-target functional guilds [105]. Overall, they show no uniform effects of *Bt* cotton, maize, and potato on the functional guilds of non-target arthropods. In fact, use of and type of insecticides influenced the magnitude and direction of effects; and insecticide effects were much larger than those of *Bt* crops. Similarly, Duan et al. [106] performed meta-analyses comparing results for non-target invertebrates exposed to *Bacillus thuringiensis* (*Bt*) Cry proteins in laboratory studies with results derived from independent field studies examining effects on the abundance of non-target invertebrates. In this case, the findings support the assumption that laboratory studies of transgenic insecticidal crops show effects that are either consistent with, or more conservative than, those found in field studies.

In combination with robust field data [107], the evidence strongly supports *Bt* crops as an environmentally neutral technology, especially in comparison to insecticides. These findings are thus in agreement with those of Mulligan et al. [68, 69].

Impact on Pollinators Many of the world's crops depend on insects for pollination and it is critically important that agricultural biotechnology does not disrupt this essential "ecosystem service" – as such, pollinators are critical to agriculture, in addition to their vital role in helping maintain biodiversity. Thus, representative pollinators form an important part of the risk assessment process and much of what has been stated for natural enemies applies to these species too. In common with natural enemies, transgenic plants can

Transgenic Crops, Environmental Impact. Table 3 Impacts of transgenic crops and transgene products on predators

Protein [1]	Transgenic plant or diet	Pest	Natural enemy	Effects on natural enemy	Reference
Bt	Corn (Cry1Ab)	Direct feeding (pollen)	Several (Coleoptera, Heteroptera, Neuroptera)	No effects on predators in both laboratory and field experiments	[40]
	Corn (Cry3Bb1)	*Diabrotica* spp. (Col: Chrysomelidae)	Several (Araneae, Carabidae, Staphylinidae)	No consistent negative effect	[41]
	Corn (Cry1Ab)	*Ostrinia nubilalis*; *Spodoptera littoralis* (Lep: Noctuidae)	*Caladenia carnea*	*Bt*-fed prey increased predator mortality and development times	[42]
		S. littoralis	*C. carnea*	Negative effects, as observed previously, determined to be due to reduced prey quality	[43]
		Tetranychus urticae (Acari)	*Stethorus punctillum* (Col: Coccinellidae)	No effects in both laboratory and field experiments	[44]
		Direct feeding (pollen)	Spiders (Araneae)		[45, 46]
	Corn (Cry3Bb1)	*Rhopalosiphum maidis* (Hom: Aphididae)	*Coleomegilla maculate* (Col.: Coccinellidae)	No effects when fed non-target aphid prey	[47]
	Corn (VIP3A + Cry1Ab)	Lepidoptera pests	Several (13 arthropod orders)	Large-scale study showed no negative effects of stacked traits over conventional corn	[48]
	Cotton (Cy1Ac)	*Aphis gossypii* (Hem. Aphididae)	*Chrysopa pallens* (Neu: Chrysopidae)	No effect	[49]
	Cotton (Cry1Ac)	Several	Predators (several)	Minor reductions in predator density in the field	[50, 51]
	Cotton (Cry1Ac/ Cry2Ab)	Lepidopterous pests		Predator numbers similar or higher in *Bt* cotton field plots	[52,53]
	Cotton (Cry1Ac)	*Spodoptera exigua*, *Helicoverpa zea* (Lep: Noctuidae)	*Geocoris punctipes* (Het: Lygaeidae)	No effects in field experiments	[54]
		S. exigua	*Podisus maculiventris*	No effects	[55]
		Lepidopterous pests	*C. carnea*; *Orius tristicolor* (Het: Anthocoridae)	No effects in field experiments	[56]
	Potato (Cry3Aa)	*Leptinotarsa decemlineata*	Several heteropteran predators and spiders	No effect of predator densities in the field	[57]

Transgenic Crops, Environmental Impact. Table 3 (Continued)

Protein [1]	Transgenic plant or diet	Pest	Natural enemy	Effects on natural enemy	Reference
	Potato (Cry3Aa)	*L. decemlineata*	Coleoptera, Araneae	No effects on field pitfall trap capture numbers	[58]
	Potato (Cry3)	*Myzus persicae*	*Hippodamia convergens* (Col: Coccinellidae)	No effect	[59]
	Potato (Cry3A)	Lep. Hem.	Several (Heteroptera)	No effects on development time	[60]
	Potato		*Harmonia axyridis*, *Nebria brevicollis*	No effects	[61]
CpTI	Injected prey	*L. oleracea*	*P. maculiventris* (Het: Pentatomidae)	Reduced growth of predators	[62]
	Potato		*P. maculiventris*	No effects	[62]
	Strawberry	*Otiorhynchus sulcatus* (Col: Curculionidae)	Carabids and others	Field abundance not affected	[63]
HvCPI-1 C68 → G	Potato	*L. decemlineata*	*P. maculiventris*	No effect	[64]
		S. littoralis			[64]
MTI-2	Oilseed rape	*Plutella xylostella*	*Pterostichus madidus* (Col: Carabidae)	No effects on reproductive fitness; female weight gain reduced at first but compensated for later	[65]
Aprotinin (bovine pancreatic or bovine spleen trypsin inhibitor) (BPTI/BSTI)	Diet	*Helicoverpa armigera*	*Harpalus affinis* (Col: Carabidae)	Beetles consumed less prey after 24 h of exposure to inhibitor-fed prey	[66]
BPTI/BSTI		*H. armigera*	*Nebria brevicollis* (Col: Carabidae)	Transient minor changes in adult beetle weights	[67]
OCI		Direct feeding	*C. carnea*	No effect via contaminated pollen	[68]
	Oilseed rape	*Deroceras reticulatum* (Mollusca)	*Pterostichus melanarius* (Col. Carabidae)	No effects on beetle mortality, weight gain, or food consumption	[69]
	Oilseed rape	*P. xylostella* (Lep: Plutellidae)	*H. axyridis*	No effect on survival or development of ladybird	[70]
	Potato	*L. decemlineata* (Colorado potato beetle)	*Perillus bioculatus* (Het: Pentatomidae)	No effects	[71]
Soybean trypsin inhibitor (SBTI)	Diet	Direct feeding	*C. carnea*		[72]

Transgenic Crops, Environmental Impact. Table 4 Impacts of transgenic crops and transgene products on parasitoids

Protein [1]	Transgenic plant or diet	Pest	Natural enemy	Effects on natural enemy	Reference
Bt	Corn (Cry1Ab)	*Chilo partellus* (Lep: Crambidae)	*Diaeretiella rapae* (Hym: Braconidae)	Reduced survival due to host mortality, smaller cocoons and adults	[73]
	Corn (Cry1Ab) (field)	*Ostrinia nubilalis* (Lep: Crambidae)	*Macrocentrus cingulum* (Hym: Braconidae)	Reductions of 29–60% is numbers of wasps found in the field	[74]
	Corn (Cry1Ab)	*Spodoptera frugiperda* (Lep: Noctuidae)	*Campoletis sonorensis* (Hym: Ichneumonidae)	Wasps were significantly smaller when developing in *Bt*-fed hosts	[75]
		Eoreuma loftini (Lep: Pyralidae)	*Parallorhogas pyralophagus* (Hym: Braconidae)	Various aspects of parasitoid biology (not all) negatively affected	[76]
	Cotton (Cry1Ac)	*Helicoverpa armigera* (Lep: Noctuidae)	*Microplitis mediator* (Hym: Braconidae)	Wasp survival and development negatively affected	[77, 78]
		Pseudoplusia includens (Lep: Noctuidae)	*Cotesia marginiventris* (Hym: Braconidae), *Copidosoma floridanum* (Hym: Encyrtidae)	Development times for both wasp species negatively affected. Adult longevity for *C. marginiventris* reduced	[79]
	Cotton (Cry1Ac) (field)	Hemipteran pests	Aphelinid parasitoids	Small reduction of parasitoid population density relative to non-*Bt* cotton	[50, 51]
	Diet (Cry1Ac)	*Spodoptera litura*/*H. armigera* (Lep: Noctuidae)	*Meteorus pulchricornis* (Hym: braconidae); *Cotesia kazak* (Hym: Braconidae)	Survival of both unaffected in *Bt*-fed *S. litura*. *M.pulchricornis* negatively affected by *Bt* –fed *H. armigera*	[80]
	Oilseed rape (Cry1Ac)	*Plutella xylostella* (Lep: Plutellidae)	*Cotesia plutellae* (Hym: Braconidae)	No effect when *Bt*-resistant hosts were parasitized	[81]
	Broccoli	*P. xylostella*	*Diadegma insulare* (Hym: Ichneumonidae)	No effect on parasitoid when exposed by *Bt*-resistant hosts	[82]
	Pine (Cry1Ac)	*Pseudocoremia suavis* (Lep: Geometridae)	*M. pulchricornis*	No effect on parasitoid developmental parameters	[83]
	Diet (Cry9Aa)	*Galleria mellonella* (Lep: Gelechiidae)	*Exorista larvarum* (Dip: Tachinidae)	No effect on fly parasitoid when exposed to facticious *Bt*-fed hosts	[84]
	Tobacco (Cry1Ab) (field)	*Heliothis virescens* (Lep. Noctuidae)	*C. sonorensis*	Rates of parasitism increased	[85]
CpTI	Diet or potato	*Lacanobia. oleracea* (Lep: Noctuidae)	*Eulophus pennicornis* (Hym: Eulophidae)	Fewer hosts parasitized, no effects on parasitoids	[86]

Transgenic Crops, Environmental Impact. Table 4 (Continued)

Protein [1]	Transgenic plant or diet	Pest	Natural enemy	Effects on natural enemy	Reference
CpTI	Diet	Direct feeding (adult wasps)	*E. pennicornis*	No effects	[87]
CpTI and *Bt*	Cotton	*H. armigera*	*Microplitis mediator* (Hym: Braconidae)	No greater effects than with *Bt*-cotton	[78]
	Cotton pollen	Direct feeding	*Trichogramma chilonis* (Hym: Trichogrammatidae)	No adverse effects due to CpTI	[88]
OC1	Diet	*Macrosiphum euphorbiae* (Hom: Aphididae)	*Aphelinus abdominalis* (Hym: Braconidae)	Parasitoid fitness impaired	[89]
	Potato	*M. euphorbiae*	*Aphidius nigripes* (Hym: Braconidae)	Wasp size and fecundity increased on OC1 line	[90]
	Oilseed rape	*Myzus persicae* (Hom: Aphididae)	*Diaeretiella rapae* (Hym: Braconidae)	No consistent effects on adult wasp emergence and sex ratio; no effects on control of aphids	[91]
OC1 IΔD86 (for nematode control)	Potato (field)	*M. euphorbiae*, *M. persicae*	*Aphidius ervi* (Hym: Braconidae)	No effects on percent parasitism, adult wasp emergence, parasitoid communities more diverse on GM plants	[92]
Con A	Diet	Direct feeding	*E. pennicornis*	Higher doses reduced adult longevity and reproductive fitness	[87], [93]
GNA		*M. euphorbiae*	*A. abdominalis*	No effect via host feeding on aphids Reduced size and longevity of adults when exposed via host	[94, 95]
		Direct feeding (adult wasps)	*Aphidius colemani* (Hym: Braconidae)	Higher doses reduced adult longevity	[96]
	Honeydew	Direct feeding (adult wasps)	*A. ervi*	Indirect negative affect potentially due to altered honeydew composition	[97]
	Potato/diet	*M. persicae*	*A. ervi*	No effects via potato, dose-dependent effects on development via diet	[94, 95]
	Sucrose diet	Direct feeding	*Cotesia glomerata* (Hym: Braconidae)	Higher doses reduced longevity	[96]
	Diet/ Sugarcane	*Diatraea saccharalis* (sugarcane borer)	*Cotesia flavipes* (Hym: Braconidae)	Small negative effects on parasitism. No effects on host location of prey or parasitism	[98, 99]
	Potato/Diet	*L. oleracea*	*E. pennicornis*	No effect on parasitism success	[100]
	Diet	Direct feeding		Reduced adult longevity and reproductive fitness	[87], [93]

Transgenic Crops, Environmental Impact. Table 4 (Continued)

Protein [1]	Transgenic plant or diet	Pest	Natural enemy	Effects on natural enemy	Reference
	Host diet/host injection	*L. oleracea*	*E. pennicornis*	Negative effects on the survival of parasitoid larvae	[93]
	Sugarcane	*E. loftini*	*P. pyralophagus*	Reduced size and longevity of adult wasps	[101]
	Tomato/ potato/diet	*L. oleracea*	*Meteorus gyrator* (Hym: Braconidae)	No effects	[102]
	Sucrose diet	Direct feeding (adult wasps)	*Trichogramma brassicae* (Hym: Trichogrammatidae)	Antifeedant; high dose reduced longevity	[96]

impact on pollinators in two ways, either directly (via the transgenic plant itself posing a hazard to the pollinator), or indirectly (via the use of a transgenic crop affecting other ecological requirements of the pollinator). Of the extensive field trials carried out to date with either *Bt*-expressing crops, or HT transgenic crops, no deleterious effects on pollinators have been reported [108]. Furthermore, results from the many studies carried out to date on transgenic plants still under development suggest that only a few of the lectins and protease inhibitors tested could present dose-dependent hazards to bees, and then only if expressed in the pollen at sufficiently high levels; most current promoters used, albeit constitutive promoters, only express at very low levels, if at all, in the pollen.

In addition to evaluating the potential impacts of transgenic crops on pollinator species, it is also important to address issues relating to the ability of these species to collect and transport pollen between transgenic and non-transgenic crops, i.e., their role in pollen dispersal and hence gene flow. Recombinant proteins are usually expressed at low levels in the pollen of transgenic plants due to the use of specific "constitutive" promoters in the gene constructs. However, the transgenes themselves are present in DNA contained in the pollen, and therefore may be transferred to other related plants via the activities of the pollinator species.

Impact of Herbicide-Tolerant Transgenic Crops

The wide-scale growing of glyphosate-tolerant crops has resulted in an increase in glyphosate usage at the expense of other herbicides [4, 109–111]. However, despite this increase in glyphosate use, overall, the use of HT crops has resulted, overall, in a significant reduction in pesticides. This has been accompanied by a reduction in tillage which has an additional benefit of reducing the use of petroleum-based fuels as well as an implicit gain in time-use efficiency by growers [112]. Furthermore, HT crops have dramatically changed the crop cultivars selected by growers and has hastened the development of new transgenic crops for commercial distribution worldwide [32, 113].

Weed control based on HT crops is efficient and cost effective and provides considerable savings in terms of both time and labor [114]. However, despite this and other obvious benefits of the technology (see above), there is much opposition in some quarters to these crops – possibly more so than for insect-resistant transgenic crops. One of the reasons for this opposition stems from a lack of effective communication and understanding between the general public and the scientific and agricultural communities [21, 115]. Designing a risk assessment to test specific questions to also address societal concerns may help address this issue since it would provide an opportunity for society to participate in the regulatory decisions affecting HT crops [116]. It is possible that a major part of the concern expressed about HT crops is that the technology is associated with herbicide use, which in itself is perceived as risky by the public sector [117]. The mode of action of glyphosate is well understood and in fact was one of the first commercially successful herbicides to have an identified enzymatic site of action in plants.

The selective and specific interaction of glyphosate with EPSPS accounts for its potent herbicidal properties and low toxicity to other life forms; as such, this will significantly reduce the potential for direct non-target effects and should negate many of these concerns.

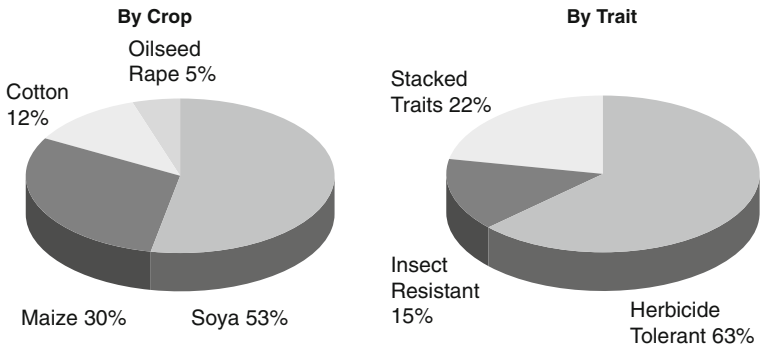
Despite their undoubted success and major contribution to global agriculture, potential risks associated with the technology must be assessed, not least in terms of the increased use of glyphosate [112, 118–121]. However, this apart, the primary environmental risk associated with HT crops relates to their impact on weed population shifts, whether expressed as the rise in economic prominence of a new weedy species or the evolution of glyphosate-tolerant weed biotypes. This position is supported by the herbicide resistance risk analysis which suggests that glyphosate-based systems are at high risk of selecting for glyphosate-tolerant weeds [122], although not all research supports this view point [123].

Impact on Biodiversity Agriculture itself has had a major impact on biodiversity, often manifested in a marked decline in the abundance of species [124–126]. While it is suggested that the ecosystem effects of HT crops have been minimal [127], the indirect impact of these crops on the agro-ecosystem, particularly as a result of changes in tillage and weed management tactics, is important [128]. There is conflicting evidence in the literature with some reports suggesting an increase in species diversity in HT-based systems while other reports suggest a reduction [129–133]. These apparent contradictions may be due to the fact that effects are often specific to the crop in question and dependent on the different weed management tactics used for HT crops compared to non-transgenic crops [134, 135]. For example, deployment of HT crops was shown to have a negative impact on butterfly population densities as an indirect effect of good weed control, reflecting a lack of nectar availability [136]. In contrast, little effect was observed for bees, gastropods, and other invertebrates. Furthermore, the effects of such crops on the soil biota were found to be negligible [132]. Thus, there appears to be both favorable and unfavorable data on the effects of HT crops on biological diversity [32]. The critical consideration is that these effects are highly dependent on specific crop and management tactics. It is likely that any unfavorable effect on biological diversity could be ameliorated by subtle manipulation of the HT-based system.

Gene Flow Another pervasive problem with HT crops is their coexistence with non-GM crops. Three important considerations have to be addressed: (1) introgression of the trait via pollen (pollen drift); (2) containment of plant products during the production year (grain segregation); and (3) volunteer HT plants in following years [115]. While HT crops and their non-transgenic counterparts can, and do, coexist, and while grain segregation and controlling volunteers is feasible [115, 137], controlling/preventing introgression of the HT trait via pollen movement in open-pollinated crops such as maize is considerably more difficult [138–141]. A number of factors affect the success of maize pollen movement and subsequent pollination, and generally, the greater the distance between the pollen source and donor, the less likely is the introgression of the transgenic trait [139, 140]. Given the tolerance levels established for some transgenic traits in non-transgenic crops, the isolation distances required to mitigate the risks of gene flow may be too great to be realistic in commercial maize production systems [142]. Other open-pollinated crops have also been scrutinized with significant legal ramification [143–146]. However, because of the possibility of "contamination" as a consequence of gene flow, many countries require buffer zones between fields growing transgenic crops and their non-transgenic counter parts; the size of these buffer zones will vary, depending upon a number of different factors including the crop itself. It is suggested that the issues of the coexistence of such crops with non-transgenic crops will continue to be a concern as long as there are economic differences between the crop cultivar types [147–149].

Future Directions

The major traits currently commercialized predominantly confer resistance to biotic stress, with herbicide-tolerant (HT) crops occupying the market share (Fig. 2). In 2009, SmartStax, a novel biotech maize containing eight different genes for insect and



Transgenic Crops, Environmental Impact. Figure 2
Global status of biotech crops for 2008

herbicide resistance, was approved for commercialization in the USA. These so-called first-generation transgenic crops, expressing improved agricultural traits are often perceived as being of benefit to the grower rather than to the consumer *per se*. Although the seed is more expensive, such crops lower the costs of production by reducing inputs of machinery, fuel, and chemical pesticides. In addition, due to more effective pest control, crop yields are often higher (Tables 1 [6] and 2 [7–20]). However, it is important to appreciate the environmental and health benefits of growing these first-generation transgenic crops, many of which are associated with reduced spraying of highly toxic chemical insecticides and herbicides. In terms of environmental benefits, these include controlling farm runoff that otherwise pollutes water systems and reduced mechanical weeding, so reducing loss of topsoil [150], while the major health benefits are a consequence of reduced pesticide exposure for farmers and rural laborers, and lower pesticide residues for consumers.

In addition to transgenic crops with increased tolerance to biotic stress, drought-tolerant maize is expected to be deployed in the USA in 2012 and sub-Saharan Africa in 2017. Other transgenic crops on the horizon include adoption of Golden Rice by the Philippines in 2012 and Bangladesh and India before 2015. Other smaller hectarage crops are also expected to be approved by 2015, including potatoes with pest and/or disease resistance, sugarcane with quality and agronomic traits, and disease-resistant bananas [3]. Interestingly, wheat remains the last major staple crop without approved biotech traits. However, political will for the crop is growing globally and many authorities suggest that China may be the first country to approve biotech wheat as early as 5 years from now.

As the science develops, so does the technology, but irrespective of which particular generation of transgenic crops is being considered, their environmental impact is of prime importance. Any risk assessment process must thus keep pace with the changing technology and the development of novel crops expressing novel traits.

Conclusion

Although one of the major concerns of recombinant DNA technology relates to its impact on non-target organisms, and thus on biodiversity, these fears have not, in the main, been realized, although there have been some well-publicized cases to the contrary. Concern over the potential for *Bt*-expressing maize to have negative effects on the Monarch Butterfly (*Danaus plexippus*) population was voiced following the publication of lab-based studies that demonstrated that unrealistically high levels of pollen from such plants had a deleterious effect [151]. However, subsequent large-scale field trials demonstrated this not to be the case, one factor being that when the maize was in flower, the Monarchs were not present [152–157]. Thus, in this instance, while the potential hazard was high, exposure was negligible resulting in effectively zero risk. Other examples include reports of initial studies concerning toxicity of *Bt* maize–fed hosts toward the predator *Chrysoperla carnae* (the green

lacewing) via a tri-trophic interaction [42]. However, subsequent studies demonstrated that *Bt* Cry1Ab was not toxic to the larvae but that the effects reported were mediated by prey quality [158]. Studies of this type emphasize the need not only to place them within an ecological context, but also to use appropriate comparators in the risk assessment process. Studies such as those carried out by Hilbeck [42] and colleagues emphasize the importance of scientific rigor and the need for demonstration of "cause and effect."

While numerous studies have now been carried out to evaluate the environmental safety of transgenic crops on beneficial insects such as natural enemies that play an important role in biological control and pollinators, in the vast majority of cases, few negative effects have actually been demonstrated. Interestingly, in the majority of cases studied to date regarding natural enemies, it is apparent that the predator/parasitoid is often able to avoid the toxic effects of the different insecticidal proteins being expressed, despite exposure at physiologically relevant levels. Further, there is evidence that the transgene product is diluted as it passes through the different trophic levels. For pollinators, this lack of toxicity is attributed to lack of receptors, in the case of *Bt*, and lack of exposure when expressed in the transgenic plant.

In addition to their potential impact on biodiversity, other environmental concerns relating to the deployment of transgenic crops are the potential for gene flow, particularly in the case of HT crops. However, the potential for gene flow is highly dependent upon the plant species [159]. Gene flow in crops can occur via pollen and via seed, the latter potentially affecting agriculture temporally and on a much larger scale than gene flow attributable to pollen [160]; in general, it is acknowledged that the risks of unintended trait movement are difficult to assess [161]. It is, however, important to point out that gene flow is no different in transgenic crops than in non-transgenic cultivars and that gene flow from transgenic crops is a reality [162]. To expect compliance of zero gene flow is neither reasonable nor realistic, and an acceptable tolerance must be established for the coexistence of transgenic crops with their non-transgenic counterparts.

Bibliography

1. Royal Society Policy Document 11/09 (2009) Reaping the benefits: science and the sustainable intensification of global agriculture. Royal Society, London
2. FAO (2000) Agriculture: Towards 2015/30. Technical interim report, April. Economic and social department. Rome, Italy
3. James C (2008) Global status of commercialized biotech/GM crops: 2008. The First Thirteen Years, 1996 to 2008. ISAAA Brief 39-2008: Executive Summary. ISAAA, New York
4. Service RF (2007b) A growing threat down on the farm. Science 316:1114–1117
5. Meyer B (2008) Biotech crops gaining favor around the globe. Learfield Communications, Inc., Jefferson City
6. Zilberman D, Sexton SE, Marra M, Fernandez-Cornejo J (2010) The economic impact of genetically engineered crops. http://www.choicesmagazine.org/
7. Delannay X, Bauman TT, Beighley DH, Buettner MJ, Coble HD, DeFelice MS, Derting CW, Diedrick TJ, Griffin JL, Hagood ES, Hancock FG, Hart SE, LaVallee BJ, Loux MM, Lueschen WE, Matson KW, Moots CK, Murdock E, Nickell AD, Owen MDK, Paschall EH II, Prochaska LM, Raymond PJ, Reynolds DB, Rhodes WK, Roeth FW, Sprankle PL, Tarochionc LJ, Tinius CN, Walker RH, Wax LM, Weigelt HD, Padgette SR (1995) Yield evaluation of a glyphosate-tolerant soybean line after treatment with glyphosate. Crop Sci 35:1461–1467
8. Roberts RK, Pendergrass R, Hayes RM (1998) Farm-level economic analysis of roundup ReadyTM soybeans. Paper presented at the Southern Agricultural Economics Association meeting, Little Rock, 1–4 Feb 1998
9. Arnold JC, Shaw DR, Medlin CR (1998) Roundup ReadyTM programs versus conventional programs: efficacy, varietal performance, and economics. Proc South Weed Sci Soc 51:272–273
10. Marra M, Carlson G, Hubbell B (1998) Economic impacts of the first crop biotechnologies. Available at http://www.ag.econ.ncsu.edu/faculty/marra/firstcrop/imp001.gif
11. Fernandez-Cornejo J, Klotz-Ingram C, Jans S (2002) Farm-level effects of adopting herbicide-tolerant soybeans in the USA. J Agric Appl Econ 34:149–163
12. Duffy M (2001) Who benefits from biotechnology? Paper presented at the American Seed Trade Association meeting. Chicago, 5–7 Dec 2001
13. Marra MC (2004) Review of the economic and environmental impacts of agbiotech: a global perspective. AgBioForum 6:144–145, Nicholas GK (ed)
14. Bernard JC, Pesek JD Jr, Fan C C (2004) Delaware farmers adoption of GE soybeans in a time of uncertain U.S. adoption. Agribus Int J 20:81–94
15. Qaim M, Traxler G (2005) Roundup Ready soybean in Argentina: farm level and aggregate welfare effects. Agric Econ 32:73–86
16. Vencill WK (1996) Weed management systems utilizing herbicide-resistant cotton. Proceedings of the Beltwide Cotton Conferences Cotton Weed Science Research Conference 2:1532–1533

17. Keeling W, Dotray P, Jones C, Sunderland S (1996) Roundup™ ready cotton: a potential new weed management tool for the Texas high plains. Proceedings of the Beltwide Cotton Conferences Cotton Weed Science Research Conference 2:1529
18. Goldman IL (1998) From out of old fields comes all this new corn: An historical perspective on heterosis in plant improvement. Concepts and breeding of heterosis in crop plants. Book series: CSSA Special Publications Issue: 25, pp 1–12
19. Culpepper AS, York AC (1998) Weed management in glyphosate-tolerant cotton. J Cotton Sci 4:174–185
20. Fernandez-Cornejo J, Klotz-Ingram C, Jans S (2000) Farm-level effects of adopting genetically engineered crops in the USA. In: Lesser WL (ed) Transitions in agrobiotech: economics of strategy and policy. Food Marketing Research Center, University of Connecticut and Department of Research Economics, University of Massachusetts, pp 57–72
21. Krayer von Krauss MP, Casman EA, Small MJ (2004) Elicitation of expert judgments of uncertainty in the risk assessment of herbicide-tolerant oilseed crops. Risk Anal 24:1515–1527
22. de Maagd RA, Bravo A, Crickmore N (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet 17:193–199
23. Song XX, Wang SM (2001) Status and evaluation on the expression of cotton varieties in the production in China in the past 20 years. Cotton Sci 13:315–320
24. He KL, Wang ZY, Zhang YJ (2009) Monitoring *Bt* resistance in the field: China as a case study. In: Ferry N, Gatehouse AMR (eds) Environmental impact of genetically modified crops. Wallingford, CAB International, pp 344–359, Chap 16
25. Tabashnik BE, Carrìe Y (2009) Insect resistance to genetically modified crops. In: Ferry N, Gatehouse AMR (eds) Environmental impact of genetically modified crops. CAB International, Wallingford, pp 74–100, Chap 5
26. Christou P, Capell T, Kohli A, Gatehouse JA, Gatehouse AMR (2006) Recent developments and future prospects in insect pest control in transgenic crops. Trends Plant Sci 11:302–308
27. Gatehouse JA (2002) Plant resistance towards insect herbivores: a dynamic interaction. New Phytol 156:145–169
28. Gatehouse JA (2008) Biotechnological prospects for engineering insect-resistant plants. Plant Physiol 146: 881–887
29. Malone LA, Gatehouse AMR, Barratt BIP (2008) Beyond Bt: alternative strategies for insect-resistant crops. In: Romeis J, Shelton T, Kennedy G (eds) Integration of insect-resistant genetically modified crops within integrated pest management programs, Series on Progress in Biological Control. Springer, New York
30. Price DR, Gatehouse JA (2008) RNAi-mediated crop protection against insects. Trends Biotechnol 26:393–400
31. James C (2009) Global status of commercialized biotech/GM crops: 2009. ISAAA Brief No. 41. ISAAA: Ithaca
32. Duke SO (2005) Taking stock of herbicide-resistant crops ten years after introduction. Pest Manag Sci 61:211–218
33. Duke SO, Powles SB (2008) Glyphosate: a once-in-a-century herbicide. Pest Manag Sci 64:319–325
34. Mazur S, Falco C (1989) The development of herbicide resistant crops. Annu Rev Plant Physiol Plant Mol Biol 40:441–470
35. Betz FS, Hammond BG, Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul Toxicol Pharmacol 32:156–173
36. Cattaneo MG, Yafuso C, Schmidt C, Huang CY, Rahman M, Olson C, Ellers-Kirk C, Orr BJ, Marsh SE, Antilla L, Dutilleu P, Carriere Y (2006) Farm-scale evaluation of the impacts of transgenic cotton on biodiversity, pesticide use, and yield. Proc Natl Acad Sci USA 103:7571–7576
37. Thies JE, Devare MH (2007) An ecological assessment of transgenic crops. J Dev Stud 43:97–129
38. Tencalla FG, Nickson TE, Garcia-Alonso M (2009) Environmental risk assessment. In: Ferry N, Gatehouse AMR (eds) Environmental impact of genetically modified crops. CAB International, Wallingford, pp 61–73, Chap 4
39. Waltz E (2009) Battlefield. Nature 461:27–32
40. Pilcher CD, Obrycki JJ, Rice ME, Lewis LC (1997) Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environ Entomol 26:446–454
41. Bhatti MA, Duan J, Head G, Jiang CJ, Mckee MJ, Nickson TE, Pilcher CL, Pilcher CD (2005) Field evaluation of the impact of corn rootworm (Coleoptera: Chrysomelidae)-protected Bt corn on ground-dwelling invertebrates. Environ Entomol 34:1325–1335
42. Hilbeck A, Baumgartner M, Fried PM, Bigler F (1998) Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environ Entomol 27: 480–487
43. Dutton A, Klein H, Romeis J, Bigler F (2002) Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. Ecol Entomol 27:441–447
44. Alvarez-Alfageme F, Ferry N, Castanera P, Ortego F, Gatehouse AMR (2008) Prey mediated effects of Bt maize on fitness and digestive physiology of the red spider mite predator *Stethorus punctillum* Weise (Coleoptera: Coccinellidae). Transgenic Res 17:943–954
45. Ludy C, Lang A (2006) A 3-year field-scale monitoring of foliage-dwelling spiders (Araneae) in transgenic Bt maize fields and adjacent field margins. Biol Control 38:314–324
46. Ludy C, Lang A (2006) Bt maize pollen exposure and impact on the garden spider, *Araneus diadematus*. Entomol Exp Appl 118:145–156
47. Lundgren JG, Wiedenmann RN (2005) Tritrophic interactions among Bt (CryMb1) corn, aphid prey, and the predator *Coleomegilla maculata* (Coleoptera: Coccinellidae). Environ Entomol 34:1621–1625
48. Dively GP (2005) Impact of transgenic VIP3a x Cry1ab lepidopteran-resistant field corn on the nontarget arthropod community. Environ Entomol 34:1267–1291

49. Guo JY, Wan FH, Dong L, Lovei GL, Han ZJ (2008) Tri-trophic interactions between *Bt* cotton, the herbivore *Aphis gossypii* Glover (Homoptera: Aphididae), and the predator *Chrysopa pallens* (Rambur) (Neuroptera: Chrysopidae). Environ Entomol 37:263–270
50. Naranjo SE (2005) Long-term assessment of the effects of transgenic *Bt* cotton on the abundance of nontarget arthropod natural enemies. Environ Entomol 34: 1193–1210
51. Naranjo SE (2005) Long-term assessment of the effects of transgenic Bt cotton on the function of the natural enemy community. Environ Entomol 34:1211–1223
52. Hagerty AM, Kilpatrick AL, Turnipseed SG, Sullivan MJ, Bridges WC (2005) Predaceous arthropods and lepidopteran pests on conventional, Bollgard, and Bollgard II cotton under untreated and disrupted conditions. Environ Entomol 34:105–114
53. Head G, Moar M, Eubanks M, Freeman B, Ruberson J, Hagerty A, Turnipseed S (2005) A multiyear, large-scale comparison of arthropod populations on commercially managed *Bt* and non-*Bt* cotton fields. Environ Entomol 34:1257–1266
54. Torres JB, Ruberson JR (2006) Interactions of *Bt*-cotton and the omnivorous big-eyed bug *Geocoris punctipes* (Say), a key predator in cotton fields. Biol Control 39:47–57
55. Torres JB, Ruberson JR (2008) Interactions of *Bacillus thuringiensis* Cry1Ac toxin in genetically engineered cotton with predatory heteropterans. Transgenic Res 17:345–354
56. Sisterson M, Biggs RW, Manhardt NM, Carriere Y, Dennehy TJ, Tabashnik BE (2007) Effects of transgenic Bt cotton on insecticide use and abundance of two generalist predators. Entomol Exp Appl 124:305–311
57. Reed GL, Jensen AS, Riebe J, Head G, Duan JJ (2001) Transgenic Bt potato and conventional insecticides for Colorado potato beetle management: comparative efficacy and non-target impacts. Entomol Exp Appl 100: 89–100
58. Duan JJ, Head G, Jensen A, Reed G (2004) Effects of transgenic *Bacillus thuringiensis* potato and conventional insecticides for Colorado potato beetle (Coleoptera: Chrysomelidae) management on the abundance of ground-dwelling arthropods in Oregon potato ecosystems. Environ Entomol 33:275–281
59. Dogan EB, Berry RE, Reed GL, Rossignol PA (1996) Biological parameters of convergent lady beetle (Coleoptera: Coccinellidae) feeding on aphids (Homoptera: Aphididae) on transgenic potato. J Econ Entomol 89:1105–1108
60. Armer CA, Berry RE, Kogan M (2000) Longevity of phytophagous heteropteran predators feeding on transgenic Bt-potato plants. Entomol Exp Appl 95:329–333
61. Ferry N, Mulligan EA, Majerus MEN, Gatehouse AMR (2007) Bitrophic and tritrophic effects of Bt Cry3A transgenic potato on beneficial, non-target, beetles. Transgenic Res 16:795–812
62. Bell HA, Down RE, Fitches EC, Edwards JP, Gatehouse AMR (2003) Impact of genetically modified potato expressing plant-derived insect resistance genes on the predatory bug *Podisus maculiventris*. Biocontrol Sci Technol 13:729–742
63. Graham J, Gordon SC, Smith K, McNicol RJ, McNicol JW (2002) The effect of the cowpea trypspin inhibitor in strawberry on damage by vine weevil under field conditions. J Hortic Sci Biotechnol 77:33–40
64. Alvarez-Alfageme F, Martinez M, Pascual-Ruiz S, Castanera P, Diaz I, Ortego F (2007) Effects of potato plants expressing a barley cystatin on the predatory bug *Podisus maculiventris* via herbivorous prey feeding on the plant. Transgenic Res 16:1–13
65. Ferry N, Jouanin L, Ceci LR, Mulligan EA, Emami K, Gatehouse JA, Gatehouse AMR (2005) Impact of oilseed rape expressing the insecticidal serine protease inhibitor, mustard trypsin inhibitor-2 on the beneficial predator *Pterostichus madidus*. Mol Ecol 14:337–349
66. Jørgensen HB, Lövei GL (1999) Tri-trophic effect on predator feeding: consumption by the carabid *Harpalus affinis* of *Heliothis armigera* caterpillars fed on proteinase inhibitor-containing diet. Entomol Exp Appl 93:113–116
67. Burgess EPJ, Malone LA, Christeller JT, Lester MT, Murray C, Philip BA, Phung MM, Tregidga EL (2002) Avidin expressed in transgenic tobacco leaves confers resistance to two noctuid pests, *Helicoverpa armigera* and *Spodoptera litura*. Transgenic Res 11:185–198
68. Mulligan EA, Ferry N, Jouanin L, Romeis J, Gatehouse AMR (2010) Characterisation of adult green lacewing (*Chrysoperla carnea*) digestive physiology: comparison of the impact of genetically modified crops and conventional pest control. Pest Manag Sci 66(3):325–336
69. Mulligan EA, Ferry N, Jouanin L, Walters K, Port GR, Gatehouse AMR (2006) Comparing the impact of conventional pesticide and use of a transgenic pest-resistant crop on the beneficial carabid beetle *Pterostichus melanarius*. Pest Manag Sci 62:999–1012
70. Ferry N, Raemaekers RJM, Majerus MEN, Jouanin L, Port G, Gatehouse JA, Gatehouse AMR (2003) Impact of oilseed rape expressing the insecticidal cysteine protease inhibitor oryzacystatin on the beneficial predator *Harmonia axyridis* (Multicolored Asian Ladybeetle). Mol Ecol 12:493–504
71. Bouchard E, Cloutier C, Michaud D (2003) Oryzacystatin I expressed in transgenic potato induces digestive compensation in an insect natural predator via its herbivorous prey feeding on the plant. Mol Ecol 12:2439–2446
72. Lawo NC, Romeis J (2008) Assessing the utilization of a carbohydrate food source and the impact of insecticidal proteins on larvae of the green lacewing, *Chrysoperla carnea*. Biol Control 44:389–398
73. Prutz G, Dettner K (2004) Effect of Bt corn leaf suspension on food consumption by *Chilo partellus* and life history parameters of its parasitoid *Cotesia flavipes* under laboratory conditions. Entomol Exp Appl 111:179–187

74. Pilcher CD, Rice ME, Obrycki JJ (2005) Impact of transgenic *Bacillus thuringiensis* corn and crop phenology on five nontarget arthropods. Environ Entomol 34:1302–1316
75. Sanders CJ, Pell JK, Poppy GM, Raybould A, Garcia-Alonso M, Schuler TH (2007) Host-plant mediated effects of transgenic maize on the insect parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). Biol Control 40: 362–369
76. Bernal JS, Griset JG, Gillogly PO (2002) Impacts of developing on Bt maize-intoxicated hosts on fitness parameters of a stem borer parasitoid. J Entomol Sci 37:27–40
77. Liu XX, Zhang QW, Xu BL, Li JC (2006) Effects of Cry1Ac toxin of *Bacillus thuringiensis* and nuclear polyhedrosis virus of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) on larval mortality and pupation. Pest Manag Sci 62:729–737
78. Liu XX, Zhang QW, Zhao JZ, Cai QN, Xu HL, Li JC (2005) Effects of the Cry1Ac toxin of *Bacillus thuringiensis* on *Microplitis mediator*, a parasitoid of the cotton bollworm, *Helicoverpa armigera*. Entomol Exp Appl 114:205–213
79. Baur ME, Boethel DJ (2003) Effect of Bt-cotton expressing Cry1A(c) on the survival and fecundity of two hymenopteran parasitoids (Braconidae, Encyrtidae) in the laboratory. Biol Control 26:325–332
80. Walker GP, Cameron PJ, MacDonald FM, Madhusudhan VV, Wallace AR (2007) Impacts of *Bacillus thuringiensis* toxins on parasitoids (Hymenoptera: Braconidae) of *Spodoptera litura* and *Helicoverpa armigera* (Lepidoptera: Noctuidae). Biol Control 40:142–151
81. Schuler TH, Denholm I, Clark SJ, Stewart CN, Poppy GM (2004) Effects of Bt plants on the development and survival of the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) in susceptible and Bt-resistant larvae of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). J Insect Physiol 50:435–443
82. Chen M, Zhao JZ, Collins HL, Earle ED, Cao J, Shelton AM (2008) A critical assessment of the effects of Bt transgenic plants on parasitoids. PLoS ONE 3:1–7
83. Barraclough EI, Burgess EPJ, Philip BA, Wohlers MW, Malone LA (2009) Tritrophic impacts of Bt-expressing transgenic pine on the parasitoid *Meteorus pulchricornis* (Hymenoptera: Braconidae) via its host *Pseudocoremia suavis* (Lepidoptera: Geometridae). Biol Control 49:192–199
84. Marchetti E, Alberghini S, Battisti A, Squartini A, Baronio P, Dindo ML (2009) Effects of conventional and transgenic *Bacillus thuringiensis galleriae* toxin on *Exorista larvarum* (Diptera: Tachinidae), a parasitoid of forest defoliating Lepidoptera. Biol Control Sci Technol 19: 463–473
85. Johnson MT, Gould F (1992) Interaction of genetically engineered host plant resistance and natural enemies of *Heliothis virescens* (Lepidoptera: Noctuidae) in tobacco. Environ Entomol 21:586–597
86. Bell HA, Fitches EC, Down RE, Ford L, Marris GC, Edwards JP, Gatehouse JA, Gatehouse AMR (2001) Effect of dietary cowpea trypsin inhibitor (CpTI) on the growth and development of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) and on the success of the gregarious ectoparasitoid *Eulophus pennicornis* (Hymenoptera: Eulophidae). Pest Manag Sci 57:57–65
87. Bell HA, Kirkbride-Smith AE, Marris GC, Edwards JP, Gatehouse AMR (2004) Oral toxicity and impact on fecundity of three insecticidal proteins on the gregarious ectoparasitoid *Eulophus pennicornis* (Hymenoptera: Eulophidae). Agric Forest Entomol 6:215–222
88. Geng JH, Shen ZR, Song K, Zheng L (2006) Effect of pollen of regular cotton and transgenic Bt plus CpTI cotton on the survival and reproduction of the parasitoid wasp *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae) in the laboratory. Environ Entomol 35:1661–1668
89. Azzouz H, Cherqui A, Campan EDM, Rahbe Y, Duport G, Jouanin L, Kaiser L, Giordanengo P (2005) Effects of plant protease inhibitors, oryzacystatin I and soybean Bowman-Birk inhibitor, on the aphid *Macrosiphum euphorbiae* (Homoptera, Aphididae) and its parasitoid *Aphelinus abdominalis* (Hymenoptera, Aphelinidae). J Insect Physiol 51:75–86
90. Ashouri A, Michaud D, Cloutier C (2001) Recombinant and classically selected factors of potato plant resistance to the Colorado potato beetle, *Leptinotarsa decemlineata*, variously affect the potato aphid parasitoid *Aphidius nigripes*. Biocontrol 46:401–418
91. Schuler TH, Denholm I, Jouanin L, Clark SJ, Clark AJ, Poppy GM (2001) Population-scale laboratory studies of the effect of transgenic plants on nontarget insects. Mol Ecol 10:1845–1853
92. Cowgill SE, Danks C, Atkinson HJ (2004) Multitrophic interactions involving genetically modified potatoes, nontarget aphids, natural enemies and hyperparasitoids. Mol Ecol 13:639–647
93. Wakefield ME, Bell HA, Gatehouse AMR (2010) Longevity and fecundity of *Eulophus pennicornis*, an ectoparasitoid of the tomato moth *Lacanobia oleracea*, is affected by nutritional state and diet quality. Agric Forest Entomol 12:19–27
94. Couty A, de la Vina G, Clark SJ, Kaiser L, Pham-Delegue MH, Poppy GM (2001) Direct and indirect sublethal effects of *Galanthus nivalis* agglutinin (GNA) on the development of a potato-aphid parasitoid, *Aphelinus abdominalis* (Hymenoptera: Aphelinidae). J Insect Physiol 47:553–561
95. Couty A, Poppy GM (2001) Does host-feeding on GNA-intoxicated aphids by *Aphelinus abdominalis* affect their longevity and/or fecundity? Entomol Exp Appl 100:331–337
96. Romeis J, Babendreier D, Wackers FL (2003) Consumption of snowdrop lectin (*Galanthus nivalis* agglutinin) causes direct effects on adult parasitic wasps. Oecologia 134:528–536
97. Hogervorst PAM, Wackers FL, Woodring J, Romeis J (2009) Snowdrop lectin (*Galanthus nivalis* agglutinin) in aphid honeydew negatively affects survival of a honeydew- consuming parasitoid. Agric Forest Entomol 11:161–173

98. Setamou M, Bernal JS, Legaspi JC, Mirkov TE (2002) Effects of snowdrop lectin (*Galanthus nivalis* agglutinin) expressed in transgenic sugarcane on fitness of *Cotesia flavipes* (Hymenoptera: Braconidae), a parasitoid of the nontarget pest *Diatraea saccharalis* (Lepidoptera: Crambidae). Ann Entomol Soc Am 95:75–83
99. Setamou M, Bernal JS, Legaspi JC, Mirkov TE, Legaspi BC (2002) Evaluation of lectin-expressing transgenic sugarcane against stalkborers (Lepidoptera: Pyralidae): Effects on life history parameters. J Econ Entomol 95:469–477
100. Bell HA, Fitches EC, Down RE, Marris GC, Edwards JP, Gatehouse JA, Gatehouse AMR (1999) The effect of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on *Eulophus pennicornis* (Hymenoptera: Eulophidae), a parasitoid of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae). J Insect Physiol 45:983–991
101. Tomov BW, Bernal JS, Vinson SB (2003) Impacts of transgenic sugarcane expressing GNA lectin on parasitism of Mexican rice borer by *Parallorhogas pyralophagus* (Marsh) (Hymenoptera: Braconidae). Environ Entomol 32:866–872
102. Wakefield ME, Bell HA, Fitches EC, Edwards JP, Gatehouse AMR (2006) Effects of *Galanthus nivalis* agglutinin (GNA) expressed in tomato leaves on larvae of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) and the effect of GNA on the development of the endoparasitoid *Meteorus gyrator* (Hymenoptera: Braconidae). Bull Entomol Res 96:43–52
103. Romeis J, Hellmich RL, Candolfi MP, Carstens K, De Schrijver A, Gatehouse AMR, Herman RA, Huesing, JE, McLean MA, Raybould A, Shelton AM, Waggoner A (2010) Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. Transgenic Res 1–22
104. Marvier M, McCreedy C, Regetz J, Kareiva P (2007) A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316:1475–1477
105. Wolfenbarger LL, Naranjo SE, Lundgren JG, Bitzer RJ, Watrud LS (2008) Bt crop effects on functional guilds of non-target arthropods: a meta-analysis. PLoS ONE 3(5):e2118. doi:10.1371/journal.pone.0002118
106. Duan JJ, Lundgren JG, Naranjo S, Marvier M (2010) Extrapolating non-target risk of *Bt* crops from laboratory to field. Biol Lett 23(6):74–77
107. Sanvido O, Romeis J, Bigler F (2007) Ecological impacts of genetically modified crops: Ten years of field research and commercial cultivation. In: Green Gene Technology: Research in an Area of Social Conflict, vol 107. Advances in Biochemical Engineering/Biotechnology. Springer-Verlag Berlin, Berlin, pp 235–278
108. Malone MA, Burgess EPJ (2009) Impact of genetically modified crops on pollinators. In: Ferry N, Gatehouse AMR (eds) Environmental impact of genetically modified crops. CAB International, Wallingford, pp 199–224
109. Young BG (2006) Changes in herbicide use patterns and production practices resulting from glyphosate-resistant crops. Weed Technol 20:301–307
110. Service RF (2007) Glyphosate – the conservationist's friend? Science 316:1116–1117
111. Foresman C, Glasgow L (2008) US grower perceptions and experiences with glyphosate-resistant weeds. Pest Manag Sci 64:388–391
112. Sankula S (2006) Quantification of the impacts on US agriculture of biotechnology-derived crops planted in 2005. Executive Summary. National Center for Food and Agricultural Policy, Washington, DC
113. Dill GM, CaJacob CA, Padgette SR (2008) Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag Sci 64:326–331
114. Gianessi LP, Reigner NP (2007) The value of herbicides in U.S. crop production. Weed Technol 21:559–566
115. Owen MDK (2005) Maize and soybeans – controllable volunteerism without ferality. In: Gressel J (ed) Crop ferality and volunteerism. CRC, Boca Raton, pp 149–165
116. Johnson KL, Raybould AF, Hudson MD, Poppy GM (2006) How does scientific risk assessment of GM crops fit within the wider risk analysis? Trends Plant Sci 12:1–5
117. Madsen KH, Sandoe P (2005) Ethical reflections on herbicide-resistant crops. Pest Manag Sci 61:318–325
118. Dill GM (2005) Glyphosate-resistant crops; history, status and future. Pest Manag Sci 61:219–224
119. Gianessi LP (2005) Economic and herbicide use impacts of glyphosate-resistant crops. Pest Manag Sci 61:241–245
120. Rissler J, Mellon M (1996) The ecological risks of engineered organisms. MIT, Cambridge
121. Altieri MA (2000) The ecological impacts of transgenic crops on agroecosystem health. Agroecology in action. University of California: Berkeley
122. Vidal RA, Trezzi MM, De Prado R, Ruiz-Santaella JP, Vial-Aiub M (2007) Glyphosate resistant biotypes of wild poinsettia (*Euphorbia heterophylla* L.) and its risk analysis on glyphosate-resistant soybeans. J Food Agric Environ 5:265–269
123. Werth JA, Preston C, Taylor IN, Charles GW, Roberts GN, Baker J (2008) Managing the risk of glyphosate resistance in Australian glyphosate-resistant cotton production systems. Pest Manag Sci 64:417–421
124. Krebs JR, Wilson JD, Bradbury RB, Siriwardena GM (1999) The second Silent Spring? Nature 400:611–612
125. Schutte G (2003) Herbicide resistance: Promises and prospects of biodiversity for European agriculture. Agric Hum Values 20:217–230
126. Benton TG (2007) Managing farming's footprint on biodiversity. Science 315:341–342
127. Davis LC (2006) Genetic engineering, ecosystem change, and agriculture: an update. Biotechnol Mol Biol Rev 1:87–102
128. Norris RF (2005) Ecological bases of interactions between weeds and organisms in other pest categories. Weed Sci 53:909–913
129. Scursoni J, Peterson D, Forcella F, Gunsolus J, Arnold RB, Owen M, Smeda OR, Vidrine R (2001) Weed diversity in glyphosate-tolerant soybean from Minnesota to Louisiana.

In: Hartzler RG (ed) CD-ROM Computer File. North Central Weed Science Society, Champaign, Milwaukee
130. Bitzer RJ, Buckelew LD, Pedigo LP (2002) Effects of transgenic herbicide-resistant soybean varieties and systems on surface-active springtails (Entognatha: Collembola). Environ Entomol 31:449–461
131. Freckleton RP, Stephens PA, Sutherland WJ, Watkinson AR (2004) Amelioration of biodiversity impacts of genetically modified crops: predicting transient versus long-term effects. Proc R Soc Biol 271:325–331
132. Cerdeira AL, Duke SO (2006) The current status and environmental impacts of glyphosate-resistant crops: a review. J Environ Qual 35:1633–1658
133. Scursoni JA, Forcella F, Gunsolus J (2007) Weed escapes and delayed emergence in glyphosate-resistant soybean. Crop Protect 26:212–218
134. Heard MS, Hawes C, Champion GT, Clark SJ, Firbank LG, Haughton AJ, Parish AM, Perry JN, Rothery JN, Scott P, Skellern RJ, Squire MP, Hill GR (2003) Weeds in fields with contrasting conventional and genetically modified herbicide-tolerant crops. I. Effects on abundance and diversity. Philos Trans R Soc Lond B 358:1819–1832
135. Heard MS, Clark SJ, Rothery P, Perry JN, Bohan DA, Brooks DR, Champion GT, Dewar AM, Hawes C, Haughton AJ, May MJ, Scott RJ, Stuart RS, Squire GR, Firbank LG (2006) Effects of successive seasons of genetically modified herbicide-tolerant maize cropping on weeds and invertebrates. Ann Appl Biol 149:249–254
136. Roy DB, Bohan DA, Haughton AJ, Hill MO, Osborne JL, Clark SJ, Perry JN, Rothery P, Scott RJ, Brooks DR, Champion GT, Hawes C, Heard MS, Firbank LG (2003) Invertebrates and vegetation of field margins adjacent to crops subject to contrasting herbicide regimes in the Farm Scale Evaluations of genetically modified herbicide-tolerant crops. Philos Trans R Soc Lond B 358:1879–1898
137. Owen MDK (2000) Current use of transgenic herbicide-resistant soybean and corn in the USA. Crop Prot 19: 765–771
138. Luan V, Figueroa SJ, Baltazar MB, Gomez MR, Townsend LR, Schoper JB (2001) Maize pollen longevity and distance isolation requirements for effective pollen control. Crop Sci 41:1551–1557
139. Palmer RG, Gai J, Sun H, Burton JW (2001) Production and evaluation of hybrid soybean. In: Janick J (ed) Plant breeding reviews. Wiley, New York, pp 263–308
140. Westgate ME, Lizaso J, Batchelor W (2003) Quantitative relationship between pollen shed density and grain yield in maize. Crop Sci 43:934–942
141. Abud SPI, Moreira MS, Farias Neto CT, Vianna AL, Andrade GR, Nunes SRM, Guerzoni J, Monteiro RA, Assuncao PMFO, Rech MS, Aragao EL (2004) Distance of flow in transgenic BROO-69515 and the non transgenic soybean in the Cerrado Region, Brasil. In: Moscardi F, Hoffmann-Campo CB, Saraiva OF, Galerani PR, Krzyzanowski FC, Carrao-Panizzi MC (eds) VII World Soybean Research Conference. Brasilian Agricultural Research Corporation, Foz do Iguassu, Brazil, pp 110–111
142. Matus-Cadiz PH, Horak MJ, Blomquist LK (2004) Gene flow in wheat at the field scale. Crop Sci 44:718–727
143. Charles D (2007) U.S. courts say transgenic crops need tighter scrutiny. Science 315:1069
144. Fisher L (2007) Growers continue to grow and use Roundup Ready alfalfa but Monsanto Company is disappointed with preliminary injunction affecting purchase and planting. http://www.monsanto.com Accessed 8 March 2007
145. Harriman P (2007) Roundup Ready concerns land alfalfa seed in court. http://4.26.159.139/static/news/NEWSID_8242.php. Accessed 8 March 2007
146. Weise E (2007) Effect of genetically engineered alfalfa cultivate a debate, USA Today. http://www.columbia.org/pdf_files/centerforfoodsafety20.pdf. Accessed 08 December 2010
147. Hurburgh JCR (2000) The GMO controversy and grain handling for 2000. GMO and Grain, Iowa State University, Ames
148. Hurburgh JCR (2003) Constraints for isolation and traceability of grains. Iowa State University, Ames
149. Ginder RG (2001) Channelling, identity preservation and the value chain: Lessons from the recent problems with starlink corn, Staff General Research Papers, Iowa State University, Department of Economics, http://econpapers.repec.org/RePEc:isu:genres:11480
150. Qaim M (2010) The benefits of genetically modified crops—and the costs of inefficient regulation. Resources for the future. www.rff.org/Publications/WPC/Pages/The-Benefits-of-Genetically-Modified-Crops-and-the-Costs-of-Inefficient-Regulation.aspx
151. Losey JE, Rayor LS, Carter ME (1999) Transgenic pollen harms monarch larvae. Nature 399:214
152. Stanley-Horn DE, Dively GP, Hellmich RL, Mattila HR, Sears MK, Rose R, Jesse LCH, Losey JE, Obrycki JJ, Lewis L (2001) Assessing the impact of Cry1Ab-expressing corn pollen on monarch butterfly larvae in field studies. Proc Natl Acad Sci USA 98:11931–11936
153. Zangerl AR, McKenna D, Wraight CL, Carroll M, Ficarello P, Warner R, Berenbaum MR (2001) Effects of exposure to event 176 *Bacillus thuringiensis* corn pollen on monarch and black swallowtail caterpillars under field conditions. Proc Natl Acad Sci USA 98:11908–11912
154. Pleasants JM, Hellmich RL, Dively GP, Sears MK, Stanley-Horn DE, Mattila HR, Foster JE, Clark P, Jones GD (2001) Corn pollen deposition on milkweeds in and near cornfields. Proc Natl Acad Sci USA 98:11919–11924
155. Oberhauser KS, Prysby MD, Mattila HR, Stanley-Horn DE, Sears MK, Dively G, Olson E, Pleasants JM, Lam WKF, Hellmich RL (2001) Temporal and spatial overlap between monarch larvae and corn pollen. Proc Natl Acad Sci USA 98:11913–11918
156. Sears MK, Hellmich RL, Stanley-Horn DE, Oberhauser KS, Pleasants JM, Mattila HR, Siegfried BD, Dively GP (2001) Impact of *Bt* corn pollen on monarch butterfly populations: a risk assessment. Proc Natl Acad Sci USA 98:11937–11942

157. Gatehouse AMR, Ferry N, Raemaekers RJM (2002) The case of the monarch butterfly: a verdict is returned. Trends Genet 18:249–251
158. Obrist LB, Dutton A, Romeis J, Bigler F (2006) Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. Biocontrol 51:31–48
159. Ellstand NC (2003) Current knowledge of gene flow in plants: implications for transgene flow. Philos Trans R Soc Lond B 358:1163–1170
160. Hall LM, Good A, Beckie HJ, Warwick SI (2003) Gene flow in herbicide-resistant canola (*Brassica napus*): the *Canadian experience*. In: Lelley T, Balazs E, Tepfer M (eds) Ecological impact of GMO dissemination in agroecosystems. Facultas Verlagsun Buchhandels AG, Austria, pp 57–66
161. Clark EA (2006) Environmental risks of genetic engineering. Euphytica 148:47–60
162. Mallory-Smith C, Zapiola M (2008) Gene flow from glyphosate-resistant crops. Pest Manag Sci 64:428–440

Transgenic Crops, Next Generation

Martina Newell-McGloughlin
Department of Plant Pathology, University of California Davis, Davis, CA, USA

Article Outline

Glossary

Abiotic stress External (nonliving) factors which can cause harmful effects to plants, such as soil conditions, drought, and extreme temperatures.

Acclimatization Adaptation of an organism to a new environment.

Adaptation In the evolutionary sense, some heritable feature of an individual's phenotype that improves its chances of survival and reproduction in the existing environment.

Additive genetic variance Genetic variance associated with the average effects of substituting one allele for another.

Agronomic performance/trait Pertains to practices of agricultural production and its costs and the management of crop land. Examples of agronomic traits include yield, input requirements, stress tolerance.

Agrobacterium tumefaciens A bacterium normally responsible for causing crown gall disease in a variety of plants. A plasmid has been isolated from this bacterium that is useful in plant genetic engineering. This plasmid, called the Ti plasmid, has been modified so that it does not cause disease but can carry foreign DNA into susceptible plant cells.

Aldolase An enzyme, not subject to allosteric regulation, that catalyzes in a reversible reaction the cleavage of fructose 1,6-biphosphate to form dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The enzyme catalyzing the fourth reaction in the glycolytic pathway, which splits a monosaccharide into two 3-carbon units.

Allele Any of several alternative forms of a given gene.

Allele frequency Often called gene frequency. A measure of how common an allele is in a population the proportion of all alleles at one gene locus that are of one specific type in a population.

Allelic exclusion A process whereby only one immunoglobulin light chain and one heavy chain gene are transcribed in any one cell; the other genes are repressed.

Allogenic Of the same species, but with a different genotype.

Allopolyploid Polyploid produced by the hybridization of two species.

Allopolyploid Plants Plants having more than two sets of haploid chromosomes inherited from different species.

Allosteric Regulation Regulation of an enzyme's activity by binding of a small molecule at a site that does not overlap the active site region.

Anabolic The part of metabolism that is concerned with synthetic reactions.

Aneuploid Having a chromosome number that is not an exact multiple of the haploid number, caused by one chromosome set being incomplete or chromosomes being present in extra numbers.

Aneuploidy The condition of a cell or an organism that has additions or deletions of a small number of whole chromosomes from the expected balanced diploid number of chromosomes.

Antibiotic Chemical substance formed as a metabolic by-product in bacteria or fungi and used to treat bacterial infections. Antibiotics can be produced naturally, using microorganisms, or synthetically.

Antibody A protein produced by the immune system in response to an antigen (a molecule that is perceived to be foreign). Antibodies bind specifically to their target antigen to help the immune system destroy the foreign entity.

Antinutrients Substances that act in direct competition with or otherwise inhibit or interfere with the use or absorption of a nutrient.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Antisense RNA RNA produced by copying and reversing a portion of an RNA-encoding DNA, usually including a protein-specifying region, and placing it next to a transcription-control sequence. This cassette can be delivered to the target cell, resulting in genetic transformation and production of RNA that is complementary to the RNA that is produced from the original, not reversed, DNA segment. This complementary, or antisense, RNA is able to bind to the complementary sequences of the target RNA, resulting in the inhibition of expression of the target gene.

Antiserum Blood serum containing specific antibodies against an antigen. Antisera are used to confer passive immunity to diseases and as analytical and preparative reagents for antigens, for example, to determine potential allergenicity.

Avirulent Unable to cause disease.

Bacillus thuringiensis (Bt) A naturally occurring microorganism which produces a toxin protein that only kills organisms with alkalineing stomachs, such as insect larvae. As and when delivered as a part of the whole killed organism, this toxin protein has been used for biological control for decades. The genetic information that encodes the toxin protein was identified and was moved into plants to make them insect tolerant.

Bioconversion Chemical restructuring of raw materials by using a biocatalyst.

Biodegradable Capable of being broken down by the action of microorganisms, usually by microorganisms and under conditions generally in the environment.

Bioinformatics The discipline encompassing the development and utilization of computational facilities to store, analyze, and interpret biological data.

Biomass The totality of biological matter in a given area. As commonly used in biotechnology, it refers to the use of cellulose, a renewable resource, for the production of chemicals that can be used to generate energy or as alternative feedstocks for the chemical industry to reduce dependence on nonrenewable fossil fuels.

Bioprocess A process in which living cells, or components thereof, are used to produce a desired end product.

Biosynthesis Production of a chemical by a living organism.

Biotechnology Development of products by a biological process. Production may be carried out by using intact organisms, such as yeasts and bacteria, or by using natural substances (e.g., enzymes) from organisms.

Biosynthetic The formation of complex compounds from simple substances by living organisms.

Biotic Stress Living organisms which can harm plants, such as viruses, fungi, and bacteria, and harmful insects. (See Abiotic stress).

Callus A cluster of undifferentiated plant cells that can, for some species, be induced to form the whole plant.

Calvin Cycle A series of enzymatic reactions, occurring during photosynthesis, in which glucose is synthesized from carbon dioxide.

Catalyst An agent (such as an enzyme or a metallic complex) that facilitates a reaction but is not itself changed at the completion of the reaction.

Catabolic The part of metabolism that is concerned with degradation reactions.

Chloroplast A chlorophyll-containing photosynthetic organelle, found in eukaryotic cells that can harness light energy.

Cistron A length of chromosomal DNA representing the smallest functional unit of heredity, essentially identical to a gene.

Clone A group of genes, cells, or organisms derived from a common ancestor. Because there is no combining of genetic material (as in sexual reproduction), the members of the clone are genetically identical or nearly identical to the parent.

Codon A sequence of three nucleotide bases that in the process of protein synthesis specifies an amino acid or provides a signal to stop or start protein synthesis (translation).

Coenzyme An organic compound that is necessary for the functioning of an enzyme.Coenzymes are smaller than the enzymes themselves and may be tightly or loosely attached to the enzyme protein molecule.

Cofactor A nonprotein substance required for certain enzymes to function. Cofactors can be coenzymes or metallic ions.

Comparative genomics The comparison of genome structure and function across different species for further understanding of biological mechanisms and evolutionary processes.

Composition analysis The determination of the concentration of compounds in a plant. Compounds that are commonly quantified are proteins, fats, carbohydrates, minerals, vitamins, amino acids, fatty acids, and antinutrients.

Conventional breeding Breeding of plants carried out by controlled transfer of pollen from one plant to another followed by selection of progeny through multiple generations for a desirable phenotype. This method has also often included irradiation or mutation of plants or seeds to induce extra variation in the donor material.

Complementary DNA (cDNA) DNA synthesized from an expressed messenger RNA through a process known as reverse transcription. This type of DNA is used for cloning or as a DNA probe for locating specific genes in DNA hybridization studies.

Coumarins White vanilla-scented crystalline esters used in perfumes and flavorings and as an anticoagulant. Formula: C9H6O2.

Crossbreeding Interbreeding (of animals or plants) using parents of different races, varieties, breeds, etc.

Cyto- A prefix referring to cell or cell plasm.

Cytokines Intercellular signals, usually protein or glycoprotein, involved in the regulation of cellular proliferation and function.

Diet A specific allowance or selection of food or feed that a person or animal regularly consumes.

Diploid A cell with two complete sets of chromosomes. Cf. Haploid.

DNA sequencing Technologies through which the order of base pairs in a DNA molecule can be determined.

Enzyme A protein catalyst that facilitates specific chemical or metabolic reactions necessary for cell growth and reproduction. Cf Catalyst.

Epigenetics The study of changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence – hence the name epi- (Greek: επί- over, above, outer) -genetics. Examples of such changes might be DNA methylation or histone deacetylation, both of which serve to suppress gene expression without altering the sequence of the silenced genes.

Event The term used to describe a plant and its offspring that contain a specific insertion of DNA. Such events will be distinguishable from other events by their unique site of integration of the introduced DNA.

Exposure assessment The qualitative and/or quantitative evaluation of the likely exposure to biological, chemical, and physical agents via different sources.

Feedstock The raw material used in chemical or biological processes.

Flavonoids Any of a group of organic compounds that occur as pigments in fruit and flowers.

Food additive Any substance not normally consumed as a food by itself and not normally used as a typical ingredient of food, whether or not it has nutritive value, the intentional addition of which to a food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport, or holding of such food results, or may be expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include "contaminants" or substances added to food for maintaining or improving nutritional qualities.

Fructan A type of polymer of fructose, present in certain fruits.

Functional foods The Institute of Medicine's Food and Nutrition Board defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains."

Functional genomics The development and implementation of technologies to characterize the mechanisms through which genes and their products function and interact with each other and with the environment. The science that is usually applied to the studies of gene and the expression (mRNA) of usually large numbers of genes simultaneously.

Gene expression The process through which a gene is activated at particular time and place so that its functional product is produced.

Gene flow The exchange of genetic traits between populations by movement of individuals, gametes, or spores. It involves the spread of new variants among different populations through dispersal.

Gene silencing (See RNAi) A method usually performed by the expression of an mRNA of complementary or the same nucleotide sequence in a cell such that the expression of the mRNA causes the downregulation of the protein which is being targeted.

Gene transfer The transfer of genes to an organism. Usually used in terms of transfer of a gene to an organism other that the original organism, through the tools of biotechnology.

Gene A segment of chromosome that encodes the necessary regulatory and sequence information to direct the synthesis of a protein or RNA product. (*See also* Operator Regulatory g. Structural g. Suppressor g).

Gene mapping Determination of the relative locations of genes on a chromosome.

Gene sequencing Determination of the sequence of nucleotide bases in a strand of DNA.

Genetic engineering A technology used to alter the genetic material of living cells in order to make them capable of producing new substances or performing new functions.

Genetic map A Map showing the positions of genetic markers along the length of a chromosome relative to each other (genetic map) or in absolute distances from each other (physical map).

Genome The total hereditary material of a cell, comprising the entire chromosomal set found in each nucleus of a given species.

Genomics Science that studies the genomes (i.e., the complete genetic information) of living beings. This commonly entails the analysis of DNA sequence data and the identification of genes.

Genotype Genetic makeup of an individual or group. Cf. Phenotype.

Germplasm The total genetic variability, represented by germ cells or seeds, available within a particular population of organisms.

Gene pool The total genetic information contained within a given population.

Glycoalkaloid toxins Steroid-like compounds produced by plant members of the botanical family Solanaceae, most notably "solanine" present in potato tubers.

Golden rice In 1999, Swiss and German scientists announced the development of a genetically engineered rice crop that produces beta-carotene, a substance which the body converts to vitamin A. This improved nutrient rice was developed to treat individuals suffering from vitamin A deficiency, a condition that afflicts millions of people in developing countries, especially children and pregnant women.

Haploid A cell with half the usual number of chromosomes, or only one chromosome set. Sex cells are haploid. Cf. Diploid.

Hazard characterization The qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical, and physical agents. For chemical agents, a dose-response assessment should be performed if the data are obtainable.

Hazard identification The identification of biological, chemical, and physical agents capable of causing adverse health or environmental effects.

Hazard A biological, chemical, or physical agent, or condition, with the potential to cause an adverse health or environmental effect.

Hereditary Capable of being transferred as genetic information from parent cells to progeny.

Heterozygote With respect to a particular gene at a defined chromosomal locus, a heterozygote has a different allelic form of the gene on each of the two homologous chromosomes.

Homologous Corresponding or alike in structure, position, or origin.

Homologous recombination Rearrangement of related DNA sequences on a different molecule by crossing over in a region of identical sequence.

Homozygote With respect to a particular gene at a defined chromosomal locus, a homozygote has the same allelic form of the gene on each of the two homologous chromosomes.

Hormone A chemical that acts as a messenger or stimulatory signal, relaying instructions to stop or start certain physiological activities. Hormones are synthesized in one type of cell and then released to direct the function of other cell types.

Horizontal gene transfer Transmission of DNA between species, involving close contact between the donor's DNA and the recipient, uptake of DNA by the recipient, and stable incorporation of the DNA into the recipient's genome.

Host A cell or organism used for growth of a virus, plasmid, or other form of foreign DNA, or for the production of cloned substances.

Hybridization Production of offspring, or hybrids, from genetically dissimilar parents. The process can be used to produce hybrid plants (by cross-breeding two different varieties) or hybridomas (hybrid cells formed by fusing two unlike cells, used in producing monoclonal antibodies). The term is also used to refer to the binding of complementary strands of DNA or RNA.

Hybrid The offspring of two parents differing in at least one genetic characteristic (trait). Also, a heteroduplex DNA or DNA-RNA molecule.

Identity preservation The segregation of one crop type from another at every stage from production and processing to distribution. This process is usually performed through audits and site visits and provides independent third-party verification of the segregation.

Immunoassay Technique for identifying substances based on the use of antibodies.

Immunogen Any substance that can elicit an immune response, especially specific antibody production. An immunogen that reacts with the elicited antibody may be called an antigen.

Inbred Progeny produced as a result of inbreeding.

Inducer A molecule or substance that increases the rate of enzyme synthesis, usually by blocking the action of the corresponding repressor.

Inserted DNA The segment of DNA that is introduced into the chromosome, plasmid, or other vectors using recombinant DNA techniques.

Introgressed Backcrossing of hybrids of two plant populations to introduce new genes into a wild population.

Inulins A fructose polysaccharide present in the tubers and rhizomes of some plants. Formula: $(C_6H_{10}O_5)_n$.

In vitro Literally, "in glass." Performed in a test tube or other laboratory apparatus.

In vivo In the living organism.

Invertase activity Enzyme activity occurring in the intestinal juice of animals and in yeasts, that hydrolyses sucrose to glucose and fructose.

Isoflavones Water-soluble chemicals, also known as phytoestrogens, found in many plants and so named because they cause effects in the mammalian body somewhat similar to those of estrogen. The most investigated natural isoflavones, genistein and daidzen, are found in soy products and the herb red clover.

Knock in Replacement of a gene by a mutant version of the same gene using homologous recombination.

Knock out Inactivation of a gene by homologous recombination following transfection with a suitable DNA construct.

Linkage The tendency for certain genes to be inherited together due to their physical proximity on the chromosome.

Locus (Plural loci) The position of a gene, DNA marker, or genetic marker on a chromosome. (See gene locus).

Macronutrient Any substance, such as carbon, hydrogen, or oxygen, that is required in large amounts for healthy growth and development.

Marker Any genetic element (locus, allele, DNA sequence, or chromosome feature) which can be readily detected by phenotype, cytological or molecular techniques, and used to follow a chromosome or chromosomal segment during genetic analysis.

Marker assisted selection or marker aided selection (MAS) A process whereby a marker (morphological, biochemical, or one based on DNA/RNA variation) is used for indirect selection of a genetic determinant or determinants of a trait of interest (i.e., productivity, disease resistance, abiotic stress tolerance, and/or quality). This process is used in plant and animal breeding.

Mass spectrometry Analytical technique by which compounds in a vacuum compartment are ionized, eventually fragmented, accelerated, and detected based upon the mass-dependent behavior of the ionized compounds or their fragments in response to the application of a magnetic or electric field in a vacuum.

Messenger RNA (mRNA) Nucleic acid that carries instructions to a ribosome for the synthesis of a particular protein.

Metabolism All biochemical activities carried out by an organism to maintain life.

Metabolite A substance produced during or taking part in metabolism.

Metabolomics "Open-ended" analytical techniques that generate profiles of the metabolites, that is, chemical substances within a biological sample. Commonly differences between profiles of different (groups of) samples are determined and the identity of the associated metabolites elucidated. Contrary to targeted analysis, these techniques are indiscriminate in that they do not require prior knowledge of every single substance that is present.

Microarray A microscopic, ordered array of nucleic acids, proteins, small molecules, cells, or other substances that enables parallel analysis of complex biochemical samples. There are many different types of microarrays both from a biological and production system perspective. The generic terms "DNA array," "GeneChipTM," or "hybridization array" are used to refer broadly to all types of oligonucleotide-based arrays. The two most common are cDNA arrays and genomic arrays. cDNA array: A microarray composed of grid of nucleic acid molecules of known composition linked to a solid substrate, which can be probed with total messenger RNA from a cell or tissue to reveal changes in gene expression relative to a control sample.

Micronutrient Any substance, such as a vitamin or trace element, essential for healthy growth and development but required only in minute amounts.

Mini-chromosome Contains only centromeres and telomeres with little additional DNA. This provides the ability to accept multiple genes coding for stacked traits. They are particularly useful because they allow scientists to add numerous genes onto one mini-chromosome and manipulate those genes easily because they are all in one place.

mRNA Messenger RNA.

Multigenic Of hereditary characteristics one that is specified by several genes.

Mutant A cell that manifests new characteristics due to a change in its DNA.

Mutation A structural change in a DNA sequence resulting from uncorrected errors during DNA replication.

Mutation Breeding Genetic change caused by natural phenomena or by use of mutagens. Stable mutations in genes are passed on to offspring unstable mutations are not.

Nitrogen fixation A biological process (usually associated with plants) whereby certain bacteria convert nitrogen in the air to ammonia, thus forming a nutrient essential for growth.

Nucleic acid Large molecules, generally found in the cell nucleus and/or cytoplasm, that are made up of nucleotide bases. The two kinds of nucleic acid are DNA and RNA.

Nucleotides The building blocks of nucleic acids. Each nucleotide is composed of sugar, phosphate, and one of four nitrogen bases. If the sugar is ribose, the nucleotide is termed a "ribonucleotide," whereas deoxyribonucleotides have deoxyribose as the sugar component (i.e., adenine, cytosine, guanine, and thymine in the case of DNA). The sequence of the nucleotides within the nucleic acid determines, for example, the amino acid sequence of an encoded protein.

Nucleus In eukaryotic cells, the centrally located organelle that encloses most of the chromosomes. Minor amounts of chromosomal substance DNA are found in some other organelles, most notably the mitochondria and the chloroplasts.

Nutritionally improved Improving the quantity, ratio, and/or bioavailability of essential macro- and micronutrients and other compounds for which the clinical and epidemiological evidence is clear that they play a significant role in the maintenance of optimal health and are limiting in diets.

Nutraceutical The term was coined by the Foundation for Innovation in Medicine in 1991 and is defined as "any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease."

Organoleptic Able to perceive a sensory stimulus such as taste.

Operon Sequence of genes responsible for synthesizing the enzymes needed for biosynthesis of

a molecule. An operon is controlled by an operator gene and a repressor gene.

Pathogen Disease-causing organism.

Peptide Two or more amino acids joined by a linkage called a peptide bond.

Pesticide Any substance intended for preventing, destroying, attracting, repelling, or controlling any pest including unwanted species of plants or animals during the production, storage, transport, distribution and processing of food, agricultural commodities, or animal feeds, or which may be administered to animals for the control of ectoparasites. The term includes substances intended for use as a plant-growth regulator, defoliant, desiccant, fruit-thinning agent, or sprouting inhibitor, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport. The term normally excludes fertilizers, plant and animal nutrients, food additives, and animal drugs.

Phenotype Observable characteristics, resulting from interaction between an organism's genetic makeup and the environment. Cf. Genotype

Phenylpropanoids Especially the derivatives of the cinnamyl alcohols and of cinnamic acids, isolated from medicinal plants due to the interest as the source for the preparation of the remedies.

Photosynthesis Conversion by plants of light energy into chemical energy, which is then used to support the plants' biological processes.

Phytate (phytic acid) A phosphorus-containing compound in the outer husks of cereal grains that, in addition to limiting the bioavailability of phosphorous itself, binds with minerals and inhibits their absorption.

Phytochemicals Small molecule chemicals unique to plants and plant products.

Plasmid Circular extrachromosomal DNA molecules present in bacteria and yeast. Plasmids replicate autonomously each time the organism, a bacterium, divides and are transmitted to the daughter cells. DNA segments are commonly cloned using plasmid vectors.

Plasticity The quality of being plastic or able to be molded, changed.

Plastid Any of various small particles in the cytoplasm of the cells of plants and some animals that contain pigments (see chromoplast), starch, oil, protein, etc.

Pleiotropic Genes or mutations that result in the production of multiple effects at the phenotypic level. It is the consequence of the fact that biochemical pathways starting from different genes intersect in many places, inhibiting, deflecting, and variously modifying each other. Introduced genes may also insert into sites that effect phenotypic changes other than the one desired.

Polyclonal Derived from different types of cells.

Polymer A long molecule of repeated subunits.

Polypeptide Long chain of amino acids joined by peptide bonds. *Post-Transcriptional Gene Silencing* Post-transcriptional gene silencing (PTGS) is a sequence-specific RNA degradation system designed to act as an antiviral defense mechanism. A form of PTGS triggered by transgenic DNA, called co-suppression, was initially described in plants and a related phenomenon, termed quelling, was later observed in the filamentous fungus *Neurospora crassa*.

Posttranscriptional modification A series of processes through which protein molecules are biochemically modified within a cell following their synthesis by translation of messenger RNA. A protein may undergo a complex series of modifications in different cellular compartments before its final functional form is produced.

Profiling Creation of indiscriminate patterns of the substances within a sample with the aid of analytical techniques, such as functional genomics, proteomics, and metabolomics. The identity of the compounds detectable within the pattern need not be known.

Promoter A DNA sequence that is located near or even partially within encoding nucleotide sequences and which controls gene expression. Promoters are required for binding of RNA polymerase to initiate transcription.

Protein Proteins are biological effector molecules encoded by an organism's genome. A protein consists of one or more polypeptide chains of amino acid subunits. The functional action of a protein depends on its three-dimensional structure, which

is determined by its amino acid composition and any posttranscriptional modifications.

Proteomics The development and application of techniques used to investigate the protein products of the genome and how they interact to determine biological functions. This is an "Open ended" analytical technique that generates profiles of the proteins within a biological sample. The technique is commonly used to find differences between profiles of different (groups of) samples are and determined and the identity of the associated proteins elucidated. Contrary to targeted analysis, these techniques are indiscriminate in that they do not require prior knowledge of every single substance protein present that is analyzed beforehand.

Protoplast fusion The fusion of two plant protoplasts that each consists of the living parts of a cell, including the protoplasm and cell membrane but not the vacuoles or the cell wall.

Protoplast The cellular material that remains after the cell wall has been removed. A plant cell from which the cell wall has been removed by mechanical or enzymatic means. Protoplasts can be prepared from primary tissues of most plant organs as well as from cultured plant cells.

Quantitative trait loci The locations of genes that together govern a multigenic trait, such as yield or fruit mass.

Recombinant DNA Any DNA molecule formed by joining DNA segments from different sources (not necessarily different organisms). This may also be a strand of DNA synthesized in the laboratory by splicing together selected parts of DNA strands from different organic species, or by adding a selected part to an existing DNA strand.

Regeneration Laboratory technique for forming a new plant from a clump of plant cells.

Regulatory gene A gene that acts to control the protein-synthesizing activity of other genes.

Regulatory sequence A DNA sequence to which specific proteins bind to activate or repress the expression of a gene.

Replication Reproduction or duplication, as of an exact copy of a strand of DNA.

Rhizobium A class of microorganisms that converts atmospheric nitrogen into a form that plants can utilize for growth. Species of this microorganism grow symbiotically on the roots of certain legumes such as peas, beans, and alfalfa.

Ribonucleic acid (RNA) A molecule similar to DNA that functions primarily to decode the instructions for protein synthesis that are carried by genes. (*See also* Messenger RNA Transfer RNA).

Ribosome A cellular component, containing protein and RNA, that is involved in protein sythesis.

Ribozyme Any of the RNA molecules possessing catalytic activity and acting as biological catalysts.

Risk A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s).

Risk analysis A process consisting of three components: risk assessment, risk management, and risk communication.

Risk assessment A scientific process consisting of the following steps: (1) hazard identification, (2) hazard characterization, (3) exposure assessment, and (4) risk characterization.

Risk characterization The qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization, and exposure assessment.

Risk communication The interactive exchange of information and opinions throughout the risk analysis process concerning hazards and risks, risk-related factors, and risk perceptions, among risk assessors, risk managers, population, industry, the academic community, and other parties, including the explanation of risk assessment findings and the basis of risk management decisions.

Risk management The process, distinct from risk assessment, of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of population and for the promotion of fair practices, and if needed, selecting appropriate prevention and control options.

RNAi RNA Interference (RNAi), a term coined by Fire et al. in 1998, is a phenomenon whereby small double-stranded RNA (referred as small

interference RNA or siRNA) can induce efficient sequence-specific silence of gene expression.

SAFOTEST EU project on new methods for the safety testing of transgenic food.

Scale-up Transition from small-scale production to production of large industrial quantities.

Secondary metabolites Chemical substances within a biological organism sample that are not necessary for, or concerned with, primary cellular functions. Examples of secondary metabolites are Secondary metabolism proceeds by modification of the primary metabolites of photosynthesis, respiration, etc., by four main pathways. The malonate/polyketide pathway leads to the production of fatty acids and naphthoquinones. The mevalonate/isoprenoid pathway leads to the various terpenes (such as menthol), carotenoids and steroids. The shikimate pathway leads to aromatic amino acids and the phenolics and the final group of metabolites is a nonspecific mix of amino-acid derivatives including the and alkaloids (such as solanine) and others of mixed biogenesis.

Selectable marker A gene, often encoding resistance to an antibiotic or an herbicide, introduced into a group of cells to allow identification of those cells that contain the gene of interest from the cells that do not. Selectable markers are used in genetic engineering to facilitate identification of cells that have incorporated another desirable trait that is not easy to identify in individual cells.

Selective breeding Making deliberate crosses or matings of organisms so the offspring will have particular desired characteristics derived from one or both of the parents.

Selective medium Nutrient material constituted such that it will support the growth of specific organisms while inhibiting the growth of others.

Sequence homology The measurable likeness degree of identity or similarity between two nucleotides or amino acid sequences.

Sera-binding tests Immunological assays that evaluate for the presence of antigen-specific IgE in blood serum obtained from individuals allergic to food, pollen, or other environmental antigens. Sera-binding tests include assays such as western blotting, ELISA, ELISA-inhibition, RAST, and RAST-inhibition techniques.

Shikimate pathway Pathway in microorganisms and plants involved in the biosynthesis of the aromatic amino acid family (phenylalanine, tyrosine, tryptophan) with a requirement for chorismate as well as shikimate. Secondary metabolites such as lignin, pigments, UV light protectants, phenolic redox molecules, and other aromatic compounds, such as folic acid and ubiquinone, are postscript products of the shikimate pathway.

Signal transduction The molecular pathways mechanism through which a cell senses changes in its external environment and changes its gene expression patterns in response.

Signal sequence The N-terminal sequence of a secreted protein, which is required for transport through the cell membrane.

Small interfering RNA (siRNA) Small Interfering RNA (siRNA) is 21–23-nt double-stranded RNA molecules. It guides the cleavage and degradation of its cognate RNA.

Site-specific recombination A crossover event, such as the integration of phage lambda, that requires homology of only a very short region and uses an enzyme specific for that recombination. Recombination occurring between two specific sequences that need not be homologous, mediated by a specific recombination system.

Somaclonal selection Epigenetic or genetic changes, sometimes expressed as a new trait, resulting from in vitro culture of higher plant cells. Somatic (vegetative nonsexual) plant cells can be propagated in vitro in an appropriate nutrient medium. The cells which multiply by division of the parent somatic cells are called somaclones and, theoretically, should be genetically identical with the parent. In fact this process occasionally in vitro cell culture of somatic cells, whether from a leaf, a stem, a root, a shoot, or a cotyledon, frequently generates cell plants which are significantly different, epigenetically and/or genetically, from the parent in a stable fashion and such progenies are called somaclonal variants and may provide a useful source of genetic variation.

Stilbenes A colorless or slightly yellow crystalline water-insoluble unsaturated hydrocarbon used in the manufacture of dyes trans-1,2-diphenylethene. Formula: C6H5CH:CHC6H5. It forms the

backbone structure of several compounds with estrogenic activity. Trans-3,4′,5-trihydroxy-stilbene also known as resveratrol, has been found in some experiments to inhibit cell mutations, and stimulate at least one enzyme that can inactivate certain carcinogens, and may contribute to a low incidence of cardiovascular disease.

Structural gene A gene that codes for a protein, such as an enzyme.

Substantial equivalence In the report of the 1996 FAO/WHO Expert Consultation, substantial equivalence was identified as being "established by a demonstration that the characteristics assessed for the genetically modified organism, or the specific food product derived therefrom, are equivalent to the same characteristics of the conventional comparator. The levels and variation for characteristics in the genetically modified organism must be within the natural range of variation for those characteristics considered in the comparator and be based upon an appropriate analysis of data." In the Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (2003), the concept of substantial equivalence is described as "a key step in the safety assessment process. However, it is not a safety assessment in itself rather it represents the starting point which is used to structure the safety assessment of a new food relative to its conventional counterpart. This concept is used to identify similarities and differences between the new food and its conventional counterpart. It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods derived from recombinant-DNA plants. The safety assessment carried out in this way does not imply absolute safety of the new product; rather it focuses on assessing the safety of any identified differences so that the safety of the new product can be considered relative to its conventional counterpart."

Substrate Material acted on by an enzyme.

Synteny All loci on one chromosome are said to be syntenic (literally on the same ribbon). Loci may appear to be unlinked by conventional genetic tests for linkage but still be syntenic.

Systems biology A biology-based interdisciplinary study field that focuses on complex interactions in biological systems claiming that it uses a new perspective (holism instead of reduction). Particularly, from year 2000 onward, the term is used widely in the biosciences, and in a variety of contexts. An often stated ambition of systems biology is the modeling and discovery of emergent properties, properties of a system whose theoretical description is only possible using techniques which fall under the remit of systems biology.

Tannins Any of a class of yellowish or brownish solid compounds found in many plants and used as tanning agents, mordants, medical astringents, etc. Tannins are derivatives of gallic acid with the empirical formula $C_{76}H_{52}O_{46}$.

T-DNA The segment of the Ti plasmid of *A. tumefaciens* that is transferred to the plant genome following natural infection.

Ti Plasmid A plasmid containing the gene(s) responsible for inducing plant tumor formation transfer of genes from *A. tumefaciens* to plant cells.

Tissue culture In vitro growth in nutrient medium of cells isolated from tissue.

Traditional breeding Modification of plants and animals through selective breeding. Practices used in traditional plant breeding may include aspects of biotechnology such as tissue culture and mutational breeding.

Transcription The process through which a gene is expressed to generate a complementary messenger RNA molecule. Synthesis of messenger (or any other) RNA on a DNA template.

Transcription activator–like effector nucleases (TALENs) Transcription activator–like effector (TALE) proteins from Xanthomonasare nucleases that cleave unique genomic sequences in living cells and can be used for targeted gene editing and mutagenesis.

Transcriptome The total messenger RNA expressed in a cell or tissue at a given point in time.

Transgene A gene from one source that has been incorporated into the genome of another organism.

Transgenic plant A fertile plant that carries an introduced gene(s) in its germ line.

Transformation Change in the genetic structure of an organism by the incorporation of foreign DNA.

Transgenic organism An organism formed by the insertion of foreign genetic material into the germ line cells of organisms. Recombinant DNA techniques are commonly used to produce transgenic organisms.

Translation Process by which the information on a messenger RNA molecule is used to direct the synthesis of a protein.

Transmissible spongiform encephalopathy A disease that can be transmitted from one animal to another and will produce changes in the brain that appear similar to a sponge (i.e., some of the cells are clear when seen down the microscope).

Transposon A segment of DNA that can move around and be inserted at several sites in the genome of a cell possibly altering expression. The first to be described was the Ac/Ds system in maize shown by McClintock to cause unstable mutations.

Trypsin inhibitors Antinutrient proteins present in plants such as soybeans that inhibit the digestive enzyme, trypsin if not inactivated by heating or other processing methods.

Unintended effect An effect that was not the purpose of the genetic modification or mutation. An unintended effect may be either predictable or unpredictable, based on the knowledge of, among other things, the function of the introduced DNA and of the native DNA affected by the genetic modification. A predicted unintended effect would be, for example, variations in metabolic intermediates and endpoints, and an unpredicted effect might be turning on of unknown endogenous genes.

Variety A subdivision of a species for taxonomic classification also referred to as a "cultivar." A variety is a group of individual plants that is uniform, stable, and distinct genetically from other groups of individuals in the same species.

Virulence Ability to infect or cause disease.

Virus A submicroscopic organism that contains genetic information but cannot reproduce by itself. To replicate, it must invade another cell and use parts of that cell's reproductive machinery.

Wildtype The form of an organism that occurs most frequently in nature.

Definition of the Subject

New and innovative techniques will be required to improve the efficiency of the global agriculture sector to ensure an ample supply of healthy food. To confound this situation the inequity between the affluent and developing countries will continue to grow and only a handful of technologies are sufficiently scale neutral to help with redressing this imbalance. Biotechnology is one such technology which offers efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. The first generation of biotechnology products commercialized were crops focusing largely on input agronomic traits whose value was often opaque to consumers. The coming generations of crop plants can be grouped into four broad areas each presenting what, on the surface, may appear as unique challenges and opportunities. The present and future focus is on continuing improvement of agronomic traits such as yield and abiotic stress resistance in addition to the biotic stress tolerance of the present generation; crop plants as biomass feedstocks for biofuels and "bio-synthetics"; value-added output traits such as improved nutrition and food functionality; and plants as production factories for therapeutics and industrial products. From a consumer perspective the focus on value-added traits, especially improved nutrition, is undoubtedly one of the areas of greatest interest.

Introduction

During the coming decades, food and agricultural production systems will need to be significantly enhanced to respond to a number of remarkable changes, such as a growing world population; increasing international competition; globalization; shifts to increased meat consumption in developing countries and rising consumer demands for improved food quality, safety, health enhancement, and convenience. The 2008 World Bank Development Report emphasized that "Agriculture is a vital development tool for achieving the Millennium Development Goals that call for halving by 2015 the share of people suffering from extreme

poverty and hunger [1]." The Report notes that three out of every four people in developing countries live in rural areas and most of them depend directly or indirectly on agriculture for their livelihoods. It recognizes that overcoming abject poverty cannot be achieved in sub-Saharan Africa without a revolution in agricultural productivity for resource-poor farmers in Africa, many of whom are women.

New and innovative techniques will be required to ensure that this revolution produces an ample supply of nutritious food by improving the efficiency of the global agriculture sector. Innovation is essential for sustaining and enhancing agricultural productivity. This involves new, science-based products and processes that contribute reliable methods for improving quality, productivity, and environmental sustainability. Biotechnology has introduced a new dimension to such innovation, offering efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. It has the potential to improve qualitative and quantitative aspects of food, feed, fiber, and biofuel production; reduce the dependency of agriculture on chemicals and fossil fuels; diminish overcultivation and erosion; and lower the cost of raw materials, all in an environmentally sustainable manner. Commercialization of the first generation of products of recombinant DNA technology was another facet in a long history of human intervention in nature for agricultural and food production purposes. As such, the same parameters of risk-based assessment should apply. Commercialization of products must be undertaken within a regulatory framework that ensures adequate protection of the consumer, the environment, and alternate production systems while not stymieing innovation.

In a world whose population is increasing disproportionally in disadvantaged regions, it is hard to envisage feeding and sustaining these numbers in a livable environment without the use of biotechnology. From 1800 onward, more food was simply produced by plowing up virgin land and forest. The land area used for farming increased about fivefold up to the middle of the twentieth century in step with population increases. The Green Revolution put a brake on this expansion, increasing yields threefold with limited need for further expansion. Since 1950, the proportion of the land devoted to farming has barely increased, even though the world population doubled over the same period. At least half the available good quality soil is currently used for agriculture, with the remainder under tropical forests. Coupling this with the ever diminishing nonrenewable resources and the compounding effects of climate change on the limitation of land usage leads to an obvious dilemma. Unless a second Green Revolution is carried out, increasing yield but limiting it to land currently used for farming, there will be further deterioration of natural habitats and biodiversity that may threaten more than our lifestyles.

During the most recent global food crisis in 2008 which was erroneously laid disproportionately on the shoulders of biofuel production, most especially grain ethanol, the Gates Foundation announced $306 million in grants to boost agricultural yields in the developing world, with nearly $165 million to replenish depleted soils in Africa. As noted by US News and World reports these efforts are not without controversy as they charge that critics consider that western philanthropists are violating African "food sovereignty" and promoting American at the expense of peasant farmers who are knowledgeable about local practices [2]. But local practices have yielded scarcity. A farmer in India grows three to four times as much food on the same amount of land as a farmer in Africa; a farmer in China, roughly seven times as much.

As noted, the FAO reports that global demand for food could easily double over the period 2000–2050, with a two-and-a-half- threefold increase in the poorest countries [2]. They found that biotechnology and genetic engineering of crops hold great promise for agriculture in developing countries. The report noted that more than 70% of the world's poor still live in rural areas and depend directly on agriculture for their survival. The WHO estimates that 800 million people worldwide suffer from malnutrition. It is difficult to imagine a promising alternative to biotechnology and industrial agriculture that will sustain such numbers without catastrophic consequences. As far back as 2004 the Economic and Social Council of the United Nations (ECOSOC) noted that most developing countries are unlikely to meet the Millennium Development Goals without a clear political commitment to making science and technology among top priorities in their development agenda [3]. FAO members called for

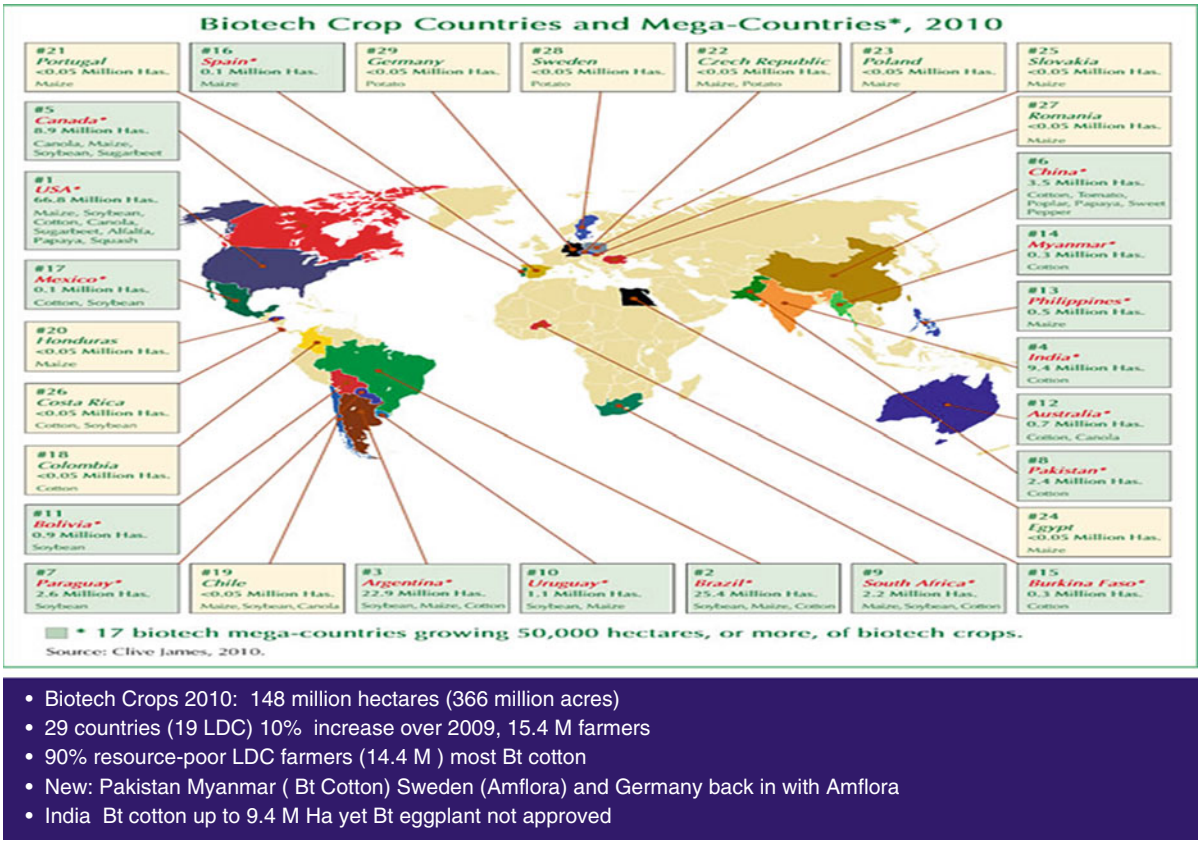
strengthening efforts in maximizing the benefits and minimizing the potential adverse consequences of biotechnology, through the Committee on Agriculture, the Council and the Conference, the development of a multidisciplinary, cross-sectoral program. In response, the Biotechnology Applications in Food and Agriculture, Forestry and Fisheries Priority Area for Interdisciplinary Action (Biotech-PAIA) was established and an Inter-Departmental Working Group on Biotechnology was set up to oversee its planning and implementation [4]. And prior to that the US National Academy of Sciences, joined by six other academies from around the world (Royal Society of London, Third World Academy of Sciences, and National Academies of Brazil, China, India, and Mexico) issued a report in 2000 declaring that biotechnology should be used to increase the production of main food staples, improve the efficiency of production, reduce the environmental impact of agriculture, and provide access to food for small-scale farmers [5]. Agricultural research of all forms holds an important key to meeting their needs, as the FAO noted biotechnology can speed up conventional breeding programs and may offer solutions where conventional methods fail. This is a positive outcome for consumers and the environment.

Progress to Date

Modifications of crop plants can be organized into two broad-based non-mutually exclusive categories: those that benefit the producer and those that benefit the consumer. Modifications that protect the crop from either biotic or abiotic stress (biotic stress being damage by predators such as insects and nematodes and disease agents such as viruses, fungi, bacteria, and weeds, and abiotic stress in the form of drought, cold, heat, and poor soils), or increase in total crop yield benefit the producer and are called "input traits." The majority of modified crops in commercial use fit in this group. Scientists have just begun to tap the large potential of biotechnology to produce varieties of plants that confer a wide spectrum of advantages to consumers. These varieties are modified with "output traits." Developing and commercializing plants with these improved traits involves overcoming a variety of technical, regulatory, and perception challenges inherent in perceived and real challenges of complex modifications. Both the panoply of traditional plant breeding tools and modern biotechnology-based techniques will be required to produce plants with the desired quality traits. In addition to the older gene transfer technology where mostly single genes were modified, newer techniques such as the use of RNA interference to manipulate endogenous genes and especially the use of transcription factors to modulate whole suites of genes and metabolic networks will become increasingly important tools in the effort to introduce valuable traits. The later approach is already a major focus in multigenic and quantitative traits such as developing stress tolerance crops and modifying paths for improving nutritional characteristics.

Since the first biotech crop was commercialized in 1996, genetically modified (GM) crops are now grown commercially by 15.4 million farmers in 29 countries on 366 million acres [6] (Fig. 1). More than half of the 63 countries engaged in biotech research, development, and production are developing countries. While North America still leads with US acreage accounting for about 45% of the total acreage worldwide, nevertheless, 19 of the 29 countries are developing countries and of the 15.4 million farmers that grew these crops, a full 14.4 million (90%) are resource-poor LDC farmers. The most recent countries to join this group were, in 2009, South America (Uruguay, Paraguay, and Bolivia) and Africa (Egypt and Burkina Fosa) and in 2010, three countries planted approved biotech crops for the first time and Germany resumed planting. Pakistan planted Bt cotton, as did Myanmar, and notably Sweden, the first Scandinavian country to plant a biotech crop, planted "Amflora," a potato with high amylase starch for industrial applications. Germany also resumed adoption of biotech crops by planting "Amflora" for a net gain of four countries in 2010 [6]. The first generation of such crops focused largely on input agronomic traits, and the next generation will focus more on value-added output traits. In the next decade, some studies estimate, the global value of biotech crops will increase nearly fivefold to $210 billion [7].

Agricultural biotechnology has helped farmers around the world boost their productivity and grow crops in more ecologically healthy fields while allowing much more efficient use of resources. This technology allows reduced tillage, which cuts down on greenhouse gas emissions, water runoff, machinery use, and soil



Transgenic Crops, Next Generation. Figure 1
Global map of biotech crop countries and mega-countries in 2010

erosion. Meanwhile, the benefits experienced by larger-scale farmers in both industrialized nations and lesser-developed countries are already considerable [6]. Research by Brookes and Barfoot [8, 9] shows in the first 11 years of GM crop cultivation that global net farm income increased by $33.8 billion since 1996; the environmental footprint associated with pesticide use was reduced by 15.4%; and there was a reduction in carbon dioxide emissions in 2006 equivalent to taking nearly 6.6 million cars off the road for a year.

An earlier study by researchers at Denmark's National Environmental Research Institute (NERI) monitored the fields of conventional and glyphosate-tolerant sugar beet. They found that the GM plots supported more plant species and insects than the conventional plots, thus providing more food for birds, and other types of wildlife use of transgenic crops increased biodiversity compared to traditional herbicide treatments [10]. Proper measurements in the UK indicate that no-till, (directly compared with plowed organic fields on the same farm and using the same farmer) uses only one third fossil fuel, uses land much more efficiently, reduces nitrate (and pesticide) runoff by at least half, and increases soil carbon which is lost when plowed. In addition, bird territories are orders of magnitude higher, soil erosion almost vanishes, and soil invertebrates such as earthworms soar in numbers, as do predatory arthropods to keep pests down. Organic fields in the UK see a threefold rise in weeds on conversion that necessitates use of the plow [11].

Therefore, reduced-till agriculture means healthier soil, with reduced erosion and far less carbon dioxide release. Soil carbon sequestration will be an important part of any international strategy to mitigate the increase in atmospheric CO_2 concentrations. By

adopting more sustainable management practices, agriculture may play a large part in enhancing soil carbon sequestration across the globe. One way is by reducing the amount of conventional tillage, after long-term tillage soil carbon stocks are depleted. In general, cultivation is not a sustainable practice. It is energy intensive and exposes soil to wind and water erosion. It allows rain to compact the soil and increases the oxygen content thus allowing organic matter to oxidize away. In turn, lower organic matter in the soil allows more compaction and more nutrient loss. Additionally in warmer and drier climates, evaporative water loss may be reduced as residue remains on the soil surface creating a wetter and cooler soil microclimate.

The Brooks and Barfoot study indicates that pesticide use fell by over 286 million kg (−7.8%: equivalent to about 40% of the annual volume of pesticide-active ingredient applied to arable crops in the European Union). Less spraying means fewer tractor passes, contributing to lower carbon dioxide emissions. Insect-resistant maize also has a collateral effect – less insect damage results in much less infection by fungal molds which reduces mycotoxins that are known health risks causing such problems as liver cancer to humans and animals. Bt corn resulted in a 90% reduction in mycotoxin fungal fumonisins [12]. In addition to the obvious health benefits, the total US economic benefit is estimated to be approximately $23million annually [6]. The only "natural" way to control those fungi is the use of copper sulfate which has one of the highest toxic hazard ratings of acceptable pesticides and selects for antibiotic-resistant bacteria in the soil.

A 2005 paper from the Royal Society suggests that intensive high-yield farming on less land is better for wildlife than "wildlife-friendly" less-efficient farming [13]. They provide convincing evidence that without yield increase, land use will double by 2050 and that this effect will be especially significant in developing countries where, without greater productivity China and India will need four times the land area to support their expanding populations. They show that in Latin America, where increased productivity was achieved, there was a significant decrease in deforestation; those producers with greatest yield increase had lower land use.

While North America remains the epicenter for cutting edge GM research, other regions, namely, China are emerging as contenders on the global stage. Agricultural science is now China's fastest-growing research field with China's share of global publications in agricultural science growing from 1.5% in 1999 to 5% in 2008 [6]. China's early experience with Bt cotton demonstrated the direct and indirect benefits of its investment in plant biotechnology research and product development. In 2002, Bt cotton was grown in 2.1 million hectares by around five million farmers. At that time the average Bt cotton farmer had reduced pesticide sprayings for the Asian bollworm from 20 to 6 times per year, reduced applications by 59–80% compared to conventional cotton (assessed in 3 years of use), and produced a kilogram of cotton for 28% less cost than the farmer using non-Bt varieties. Net revenues increased by 357–549 USD/ha compared to conventional cotton (assessed in 3 years of use) [14]. Ultimately, however, it is the social benefits from reducing exposure to insecticides and saving lives which is the real payoff.

The demand for productivity-enhancing technologies by farmers and for cost savings by consumers, the rate of increase in research investments, and success with Bt cotton suggest that products from China's research program will 1 day become widespread inside China. Indeed China is emerging as one of the trend-setters in the adoption of novel traits as more recently China is setting the pace for new approvals, with Bt rice and phytase maize approved on November 27, 2009. Rice is the principal staple for much of the world and maize is the largest animal feed source. Bt rice has the potential to increase yields up to 8%, decrease pesticide use by 80% (17 kg/ha), and generate US$4 billion in benefits annually [6]. The phytase approval is a major step forward in approvals as it is the first since the FLAVR SAVR tomato focusing on a "quality" trait. However, it is far more than this, both literally and figuratively since this single trait addresses several issues from nutritional to environmental as expanded on later.

The first GM crop to be released for commercial cultivation in India was Bt cotton, developed by the Maharashtra Seed Company (Mahyco) in partnership with Monsanto. The approval, which was given in 2002, came after several years of field trials following the

biosafety procedures laid down by the government. Three cotton hybrids were granted permission for field sowing in six states for 3 years. For the first season, farmer demand for Bt cotton seed was very high; it is estimated that 44,500 ha of certified Bt cotton were planted by nearly 55,000 farmers. However, the initial events thrived in regions that resembled the area in which they were originally developed but did not perform as well in growing regions with disparate climate challenges. It was not until the trait was introgressed into locally adapted varieties that Bt cotton thrived in all growing regions. Between 2005 and 2006 the biggest impact of this approach was realized. From 3 Bt cotton hybrids in 2002 to 62 in 2006 the rapid deployment of Bt cotton hybrids based on different agro-climatic conditions resulted in decreased insecticide sprays by 39%, and increased yield of 31%, resulting in increased profit per hectare of 88% or $ 250. Over this period of rapid deployment the average cotton yields increased from 308 to 450 kg/ha of lint (of this increase 50% could be attributed to Bt technology). Over the same period raw cotton exports rose from 0.9 million bales in 2005 to 4.7 million in 2006 and had achieved 5.9 million by 2007 [15]. By 2009, 5.6 million resource-poor farmers in India planted 8.4 million hectares of Bt cotton, equivalent to 87% of the 9.6 million hectare national cotton crop. The increase from 50,000 ha when Bt cotton was first commercialized in 2002 to 8.4 million hectares in 2009 represents an unprecedented 168-fold increase in 8 years. Between 2002 and 2008, Bt cotton generated economic benefits for farmers valued at US$5.1 billion, halved insecticide requirements, contributed to the doubling of yield and transformed India from a cotton importer to a major exporter. Choudary contends that the deployment of Bt cotton over the last 8 years has resulted in India becoming the number one exporter of cotton globally as well as the second largest cotton producer in the world [15].

However, despite the success of Bt cotton the expected successful commercialization of Bt eggplant never materialized as an effective opposition managed to scupper its approval. Bt eggplant, or brinjal as it is referred to in India, was found to be effective against fruit and shoot borer (FSB), with 98% insect mortality in shoots and 100% in fruits compared to less than 30% mortality in non-Bt counterparts. The multi-location research trials confirmed that Bt brinjal required, on average, 77% less insecticides than non-Bt counterparts for control of FSB, and 42% less for the control of all insect pests of brinjal. The benefits of Bt brinjal translate to an average increase of 116% in marketable fruits over conventional hybrids, and 166% increase over popular open-pollinated varieties [16]. Furthermore, the significant decrease in insecticide usage reduced the farmers' exposure to insecticides and resulted in a substantial decline in pesticide residues on brinjal fruits. Scientists have estimated that Bt brinjal will deliver farmers a net economic benefit ranging from $330 to $397 per acre with national benefits to India exceeding $400 million per year. However in February, 2010, the environmental minister announced a 6-month moratorium citing that "There is no overriding food security argument for Bt brinjal. Our objective is to restore public confidence and trust in Bt brinjal" [17], clearly articulating the fact that the decision was not based on scientific analysis or risk assessment.

A number of other multi-institutional projects have also been launched, including the development of transgenic plants for resistance to geminiviruses in cotton, mungbean, and tomato, resistance to rice tungro disease, development of a nutritionally enhanced potato with a balanced amino acid composition, and development of molecular methods for heterosis breeding. Other transgenic crops that are awaiting approval for commercial cultivation include transgenic herbicide-tolerant mustard hybrids and nutritionally enhanced potato varieties. However, despite the resounding success of Bt cotton, given the experience with Bt brinjal it is difficult to be optimistic about the prospects for commercialization of food crops.

A somewhat similar but even more insidious situation was experienced by Egypt. In 2008, Egypt became the first country in the Arab world (and only one of three in Africa) to commercialize biotech crops by planting 700 ha of Bt yellow maize. The variety commercialized, Ajeeb-YG, is a cross between MON 810 and an Egyptian maize variety with resistance to three corn borer pests. The reason that the amount was so low can in part be attributed to political pressure from their EU market.

The Next Generation

The vast majority of products approved to date are in the area of agronomic traits, most specifically biotic stress. The principal focus in the immediate future will remain on agronomic traits especially the area of pest control but with an increasing interest in abiotic stress tolerance which is gaining prominence as external pressures from climate change to land use change.

On the biotic stress tolerance side the focus is expanding to multi-tiered control systems. This in theory serves a double advantage, primarily expanding the effectiveness of the broad-based resistance events but also allowing more effective management of the resistance trait since there is less selective pressure when genes are stacked. SmartStax, an eight-trait event developed through collaboration between Monsanto and Dow takes advantage of multiple modes of insect protection and herbicide tolerance against above and below ground insects and provides broad herbicide tolerance, including Yieldgard VT Triple (Monsanto), Herculex Xtra (Dow), Roundup Ready 2 (Monsanto), and Liberty Link (Dow). It is currently available for corn, but cotton, soybean, and specialty crop variations are to be released [18]. It is estimated that this should require only 5% refuge acres as opposed to the 20% mandated for older technologies to mitigate against pest tolerance [15].

On the second area of agronomic traits, namely abiotic stress, there is a meta-issue that overlays much of the individual efforts, which is climate change. This poses a real challenge in terms of available agricultural land and freshwater use. Apart from the obvious effects of climate change, the decline of crop yields, ocean acidification, poor nutrition and abiotic stress, population displacement, and threatened ecosystems are effects underlined by the Stern Report [19] as potential consequences of climate change. In addition there are also broader, more systemic effects of drought beyond food insecurity such as decreased household income, the loss of assets due to slaughter of livestock, health threats due to the lack of water for hygiene and household uses, environmental degradation, and less-sustainable land management.

These effects should be considered in the light of growing population levels. In order to feed the overall population, the world will have to double its rate of agricultural production over the next 25 years, despite having already quadrupled it in the last 50 years. Severe drought accounts for half the world's food emergencies annually [1]. In 2003, the World Food Program spent US$565 million in response to drought in sub-Saharan Africa (SSA). In this context, solutions must be developed to adapt crops to the existing but also evolving conditions, such as marginal soils or harsher conditions such as cold, heat, drought, and salinity. The agriculture sector is both a contributor and provider of potential solutions to this phenomenon. It impacts two of the principal components of climate change greenhouse gases and water. Agriculture is a major source of the former emissions. Practices – such as deforestation, cattle feedlots, and fertilizer use – currently account for about 25% of greenhouse gas emissions. When broken down this amounts to 14% of carbon dioxide emission, 48% of methane, and 52% of nitrous oxide emissions [19]. In addition, this sector uses a significant amount of available freshwater – approximately 70% of the water currently consumed by humans is used in agriculture – and this is likely to increase as temperatures rise.

Given the potential impacts of climate change on the range and extent of agricultural productivity and the impact of agriculture practices itself on global warming, techniques should play a substantial part in mitigating against climate change. Green biotechnology offers a set of tools which can help producers limit greenhouse gas emissions as well as adapting their agricultural techniques to shifting climates. The three major contributions of green biotechnology to the mitigation of the impact of climate change are greenhouse gas reduction, crop adaptation (environmental stress, changing niches) and protection, and yield increase in less desirable and marginal soils.

On the first of these issues greenhouse gas reduction in addition to carbon dioxide agriculture contributes two of the other major gases indeed one of them nitrous oxide has a global warming potential of about 300 times that of carbon dioxide. In addition, nitrous oxides stay in the atmosphere for a considerable period. Nitrous oxide is produced through bacterial degradation of applied nitrogen fertilizer. In addition, fertilizer can contribute to eutrophication at ground level so its reduction is desirable on several levels. However, nitrogen is essential for crop production since it is quantitatively the most essential nutrient for plants and

a major factor limiting crop productivity. One of the critical steps limiting the efficient use of nitrogen is the ability of plants to acquire it from applied fertilizer. Therefore, the development of crop plants that absorb and use nitrogen more efficiently can serve both the plant and the environment. Arcadia Biosciences of Davis, CA, developed nitrogen-efficient crops by introducing a barley AlaAT (alanine aminotransferase) into both rice and canola. Arcadia's Nitrogen Use Efficiency (NUE) technology produces plants with yields that are equivalent to conventional varieties but which require significantly less nitrogen fertilizer because the AlaAT gene allows more efficient use. Compared with controls, transgenic plants also demonstrated significant changes in key metabolites and total nitrogen content, confirming increased nitrogen uptake efficiency. This technology has the potential to reduce the amount of nitrogen fertilizer that is lost by farmers every year due to leaching into the air, soil, and waterways. In addition to environmental pressures, nitrogen costs can represent a significant portion of a farmer's input costs and can significantly impact farmer profitability. Farmers spend $60 billion annually for 150 million tons of fertilizer [20]. The technology has been licensed to Dupont for maize and Monsanto for application in canola.

The second area where green technology can help in a changing climate is crop adaptation to environmental stress and changing niches. Under stress plants will divert energy into survival instead of producing biomass and reproduction, so addressing this impact should have substantial effect on yield. In addition, improved stress tolerance allows expanded growing season especially earlier planting and further reduces yield variability and grower financial risk. The most critical of these stresses is water. One of the most effective methods of addressing water limitation problems, namely, irrigation, unfortunately is also one of the major causes of arable land degradation. It is estimated that 24.7 million acres of farmland worldwide is lost each year due to salinity build up resulting from over irrigation. In fact crops are now limited by salinity on 40% of the world's irrigated land (25% of the USA). To address the extreme end of irrigation impact Eduardo Blumwald at UC Davis used AtNHX1, the most abundant vacuolar Na+/H+ antiporter in Arabidopsis thaliana which mediates the transport of Na+ and K+ into the vacuole. By overexpressing this vacuolar Na+/H+ antiporter, transgenic tomatoes were able to grow, flower, and produce fruit in the presence of 200 mM sodium chloride [21]. Arcadia Biosciences had now introduced this gene into economically important crops.

Even at a more moderate level of impact it is estimated that about 70–80 million acres in USA suffer yield losses due to moderate water stress. The most critical time for water stress is near-pollination and flowering where yields with or without irrigation can vary by up to 100%. This effect is clearly demonstrable in dry land production where yields can be cut in half in the absence of irrigation. At this time about 15% of US maize acres are irrigated. Given the negative effective and cost of irrigation it is estimated 20 million acres in USA would benefit from a drought tolerance gene that gives a 10% yield increase. It would also allow shifting of high-value crops into production on more marginal land.

One of the first commercialized products to have included a "yield gene" is Monsanto's second generation Roundup Ready 2 Yield® Soybeans which include not only the glyphosate-tolerant trait but also that which was developed using extensive gene mapping to identify specific DNA regions that segregated with yield increase. It is a perfect example of the power of combining recombinant DNA technology with genomics tools. The company claims that following 4 years of field trials across six US states showed 7–11% higher yields, compared to the first generation of Roundup Ready soybeans. At the National Technical Biosafety Committee (CTNBio) meeting in Brazil in August 2010, the committee approved the Bt enhanced version of this product for planting in Brazil [18].

As noted transcription factors are some of the most versatile tools being employed in developing stress-tolerant plants. One of the most versatile classes of transcription factors in so far as environmental response is concerned is the DREB (dehydration-responsive element binding protein) transcription factors which are involved in the biotic stress signaling pathway and can activate as many as 12 resistant functional genes relying on DREmembers of cis regulation under adverse conditions, for instance, rd29, cor15, and rd17, cause proline content to rise so as to enable plants to improve in many resistances such as drought, freezing, and salt tolerance. It has been possible to engineer stress tolerance in transgenic plants by

manipulating the expression of DREBs [22]. One isolated from Arabidopsis has improved drought tolerance increasing productivity by at least twofold during severe water stress. In Monsanto field trials using this approach, maize yields have increased under-water stress by up to 30% [22].

Other approaches include modification of individual genes involved in stress response and cell signaling. For example, drought-tolerant canola engineered to reduce the levels of PARP [poly(ADP-ribose) polymerase], a key stress-related protein in many organisms, shows relative yield increases of up to +44% compared to control varieties. A subset of the transcription factors homeodomain leucine zipper proteins (HDZip) play a role in regulating adaptation responses including developmental adjustment to environmental cues such as water stress in plants [23]. One of these effectors is abscisic acid (ABA), an important plant regulator controlling many environmental responses including stomata movement which is itself modulated by the DREB elements. Some work is being done on modifying HDZip directly and others are working indirectly, for example, down regulating farnesyltransferase, a signaling system in the production of abscisic acid and stomata control, which results in stomata closure and water retention.

Investigators are also working on modifying basic acid to enhance the tolerance of plants to water-deficit by delaying the drought-induced leaf senescence and abscission during the stress episode. Using tobacco plants expressing an isopentenyltransferase (IPT) gene under the control of a stress- and maturation-induced promoter (PSARK) it was shown that delayed drought-induced leaf senescence resulted in remarkable drought-tolerant phenotypes, as well as minimal yield loss when plants were watered with only 30% of the water used under controlled conditions [24]. This is now being introduced into rice among other crops. This work is being done in conjunction with Arcadia Biosciences. In addition, Bayer CropScience, Pioneer Hi-Bred, BASF and Dow among others are conducting research on maize, cotton, canola, and rice, to develop a new generation of stress-tolerant, high-performance crop varieties. Clearly stress-tolerant traits are of paramount importance in LDCs especially sub-Saharan Africa and Asia. Major efforts are already underway on this front. The partnership, known as Water Efficient Maize for Africa (WEMA), was formed in response to a growing call by African farmers, leaders, and scientists to address the devastating effects of drought on small-scale farmers. Frequent drought leads to crop failure, hunger, and poverty. Climate change can only aggravate this situation [25].

On the other end of the spectrum of climate change impact is flooding due to changing rain patterns and rising sea levels. This is already a major cause of rice crop loss. It is estimated that four million tons of rice are lost every year because of flooding which is sufficient to feed 30 million people. Rice is not grown in flooded fields through necessity but rather to control weeds, however, most rice varieties die after more than 3 days of complete submergence. Researchers know of at least one rice variety FR13A that can tolerate flooding for longer periods, but conventional breeding failed to create an event that was acceptable to farmers. The Ronald laboratory at UC Davis cloned the submergence tolerance (Sub1) locus from this resistance variety using a map-based cloning approach. The Sub1 locus encodes three putative transcription regulators one of which increases dramatically in response to oxygen deprivation in sub1 seedlings, whereas Sub1C levels decrease. Transgenic lines over-expressing the Sub1A-1 gene have been introgressed into a submergence-intolerant line and display-enhanced submergence tolerance [26].

There is also some research in the final abiotic stress focus area, namely, expansion of crops into and increased yield in less desirable and marginal soils. For example, a gene that produces citric acid in roots can protect plants from soils contaminated with aluminum as it binds to the contaminant preventing uptake by the root system [27]. Genes such as these can allow crops to be cultivated in hostile soils and temperatures increasing geographic range while reducing potential impact on fragile ecosystems.

While exciting and very relevant, research in abiotic stress tolerance is still an input trait. The first generation of biotechnology crops focused largely on those input agronomic traits; the next generation will focus more on value-added output traits. This will include identifying and isolating genes and metabolites that will make possible the enhancement of valuable traits, with some of the later compounds being produced in mass quantities

for niche markets. Two of the more promising markets are improved nutrition including nutraceuticals, or so-called functional foods, and plants developed as bioreactors (production factories) for the commercial-level production of valuable proteins and compounds, a field known as plant molecular farming [28]_ENREF_24.

While the correlative link between food and health, beyond meeting basic nutrition requirements, has only been unequivocally proven in a number of cases, a growing body of evidence indicates that food components can influence physiological processes at all stages of life [29]. Nutrition intervention from a functionality perspective has a personal dimension. Determining individual response is at least as complex a challenge as the task of increasing or decreasing the amount of a specific protein, fatty acid, or other components of the plant itself. There is also evidence that early food regimes can effect later life health, for example, some children that survived famine conditions in certain regions of Africa grew into adults battling obesity and related problems presumably due to the selective advantage of the thrifty gene in their early food-stressed environment becoming a hazard during more abundant times especially if later diets are calorie-dense.

Functional foods are defined as any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. Scientific evidence is accumulating to support the role of phytochemicals and functional foods in the prevention and treatment of disease. Functional food components are of increasing interest in the prevention and/or treatment of a number of the leading causes of death including but not limited to cancer, diabetes, cardiovascular disease, and hypertension. Many food components are known to influence the expression of both structural genes and transcription factors in humans. Examples of these phytochemicals are listed in Table 2. The large diversity of phytochemicals suggests that the potential impact of phytochemicals and functional foods on human and animal health is worth examining as targets of biotechnology efforts. Developing plants with improved quality traits involves overcoming a variety of technical challenges inherent to metabolic engineering programs. Both traditional plant breeding and biotechnology techniques are needed to produce plants carrying the desired quality traits [29] (Table 1).

From a health perspective, plant components of dietary interest can be broadly divided into four main categories, which can be further broken down into positive and negative attributions for human nutrition, macronutrients (proteins, carbohydrates, lipids [oils], and fiber), micronutrients (vitamins, minerals, phytochemicals), anti-nutrients (substances such as phytate that limit bioavailability of nutrients), allergens, intolerances, and toxins [29]. Developing and commercializing plants with these improved traits involves overcoming a variety of technical, regulatory, and perception challenges inherent in perceived and real challenges of complex modifications. Both the panoply of traditional plant breeding tools and modern biotechnology-based techniques will be required to produce plants with the desired quality traits. Table 2 presents examples of crops that have already been genetically modified with macro- and micronutrient traits that may provide nutritional benefits.

In addition to functional foods, this area has the potential to address both nutrition and environmental impact. A good example of this is the addition of transgenic phytase enzymes to crops to reduce the need to add phosphate to feed [6]. Most of the phosphate is added to counteract the non-bioavailability of phosphorus in phytic acid and the sequestering effect of phytic acid on uptake of divalent mineral ions such as iron, calcium, and zinc. Unfortunately excess phosphate is excreted causing major environmental impact through eutrophication and fish kills in regions with intense pig and poultry farming [28, 29]. In addition, in humans, such mineral deficiencies due to phytate binding are estimated to afflict two to three billion people, primarily in the developing world. Several studies have shown that Aspergillus-derived phytases can be produced in large quantities in a range of plants including cereals with clear-cut positive effects on phytate degradation, and phosphate and mineral bioavailability in animal-feeding trials [104]. It is thus conceivable that genetic engineering of staples for increased phytase expression could have potential for improving iron and zinc bioavailability alleviating the need for supplementation in all monogastrics and consequent reduction in polluting runoff in non-ruminant animals [105]. As noted earlier China has led the way

Transgenic Crops, Next Generation. Table 1 Examples of crops in research with nutritionally improved traits[a]

Trait	Crop (trait detail)	References
Protein and amino acids		
Protein quality and level	Bahiagrass (protein↑)	Luciani and Wofford [30]
	Canola (amino acid composition)	Roesler et al. [31]
	Maize (amino acid composition; protein↑)	Cromwell et al. 1969, [32], Yang et al. [33], O'Quinn et al. [34], Young et al. [35]
	Potato (amino acid composition; protein↑)	Chakraborty et al. [36], Li et al. [37], Yu and Ao [38], Atanassov et al. [39]
	Rice (protein↑; amino acid)	Katsube et al. [40]
	Soybean (amino acid balance)	Rapp [41]; Dinkins et al. [42]
	Sweet Potato (protein↑)	Prakash and Jaynes [43]
	Wheat (protein↑)	Uauy et al. [44]
Essential amino acids	Canola (lysine↑)	Falco et al. [45]
	Lupin (methionine↑)	White et al. [46]
	Maize (lysine↑; methionine↑)	Agbios 2006, Lai [47]
	Potato (methionine↑)	Zeh et al. [48]
	Sorghum (lysine↑)	Zhao et al. 2003
	Soybean (lysine↑; tryptophan↑)	Falco et al. [45], Galili et al. [49]
Oils and fatty acids		
	Canola (lauric acid↑; γ-linolenic acid↑; + ω-3 fatty acids; 8:0 and 10:0 fatty acids↑; lauric + myristic acid↑; oleic acid↑)	Del Vecchio [50], Froman [51], James et al. [52], Ursin [53], Dehesh et al. 1996, Agbios 2006, Roesler et al. [31]
	Cotton (oleic acid↑; oleic acid + stearic acid↑)	Chapman et al. [54], Liu et al. [55]
	Linseed (+ ω-3 and -6 fatty acids)	Abbadi et al. [56]
	Maize (oil↑)	Young et al. [35]
	Oil Palm (oleic acid↑ or stearic acid↑; oleic acid↑ + palmitic acid↓)	Parveez [57], Jalani et al. [58]
	Rice (α-linolenic acid↑)	Anai et al. [59]
	Soybean (oleic acid↑; γ-linolenic acid↑)	Kinney [60], Reddy and Thomas [61]
	Safflower (γ Linoleic Acid GLA↑)	Arcadia [62]
Carbohydrates		
Fructans	Chicory, (fructan↑; fructan modification)	Smeekens [63], Sprenger et al. [64], Sevenier et al. [65]
	Maize (fructan↑)	Caimi et al. [66]
	Potato (fructan↑)	Hellwege et al. [67]
	Sugar beet (fructan↑)	Smeekens [63]
Frustose, Raffinose, Stachyose	Soybean	Hartwig et al. [68]

Transgenic Crops, Next Generation. Table 1 (Continued)

Trait	Crop (trait detail)	References
Inulin	Potato (inulin↑)	Hellwege et al. [69]
Starch	Rice (amylase ↑)	Chiang et al. [70], Schwall et al. [71]
Micronutrients and functional Metabolites		
Vitamins and Carotenoids	Canola (vitamin E↑)	Shintani and DellaPenna [72]
	Maize (vitamin E↑; vitamin C↑; beta-carotene↑; folate↑)	Rocheford et al. [73], Cahoon et al. [74], Chen et al. [75], Naqvi et al. [76]
	Mustard (+β-carotene)	Shewmaker et al. [77]
	Potato (β-carotene and lutein↑)	Ducreux et al. [78]
	Rice (+β-carotene)	Ye et al. [79]
	Strawberry (vitamin C↑)	Agius et al. [80]
	Tomato (folate↑; phytoene and β-carotene↑; lycopene↑; provitamin A↑)	Della Penna 2007, Diaz de la Garza et al. [81], Enfissi et al. [82] Mehta et al. 2002, Fraser et al. [83], Rosati et al. [84]
Functional secondary metabolites	Apple (+stilbenes)	Szankowski et al. [85]
	Alfalfa (+resveratrol)	Hipskind and Paiva [86]
	Kiwi (+resveratrol)	Kobayashi et al. [87]
	Maize (flavonoids↑)	Yu et al. [88]
	Potato (anthocyanin and alkaloid glycoside↓; solanin↓)	Lukaszewicz et al. [89]
	Rice (flavonoids↑; +resveratrol)	Shin et al. 2006, Stark-Lorenzen et al. [90]
	Soybean (flavonoids↑)	Yu et al. [91]
	Tomato (+resveratrol; chlorogenic acid↑; flavonoids↑; stilbene↑anthocyanins↑)	Giovinazzo et al. [92], Niggeweg et al. [93], Muir et al. [94], Rosati et al. [84], Gonzali et al. [95]
	Wheat (caffeic and ferulic acids↑; +resveratrol)	UPI [96]
Mineral availabilities	Alfalfa (phytase↑)	Austin-Phillips et al. [97]
	Lettuce (iron↑)	Goto et al. [98]
	Rice (iron↑)	Lucca et al. [99]
	Maize(phytase↑, ferritin↑)	Drakakaki et al. [100], Han [101]
	Soybean (phytase↑)	Denbow et al. [102]
	Wheat (phytase↑)	Brinch-Pedersen et al. [103]

[a]Excludes protein/starch functionality, shelf life, taste/aesthetics, fiber quality and allergen reduction traits. Modified from Newell-McGloughlin [29] (25)

in the approval of this "output trait" in Maize being the first country to approve commercialization in November 2009 [6]. Continuing improvements in molecular and genomic technologies are contributing to the acceleration of such product development. One estimate states that foods that are used for functional purposes made up 10% of the $503 billion total US retail food market [106].

In addition to being a source of nutrition, plants have been a valuable wellspring of therapeutics for

Transgenic Crops, Next Generation. Table 2 Examples of plant components with suggested functionality[a]

Class/components	Source[b]	Potential Health Benefits
Carotenoids		
Alpha-carotene	Carrots	Neutralizes free radicals that may cause damage to cells
Beta-carotene	Various fruits, vegetables	Neutralizes free radicals
Lutein	Green vegetables	Contributes to maintenance of healthy vision
Lycopene	Tomatoes and tomato products (ketchup, sauces)	May reduce risk of prostate cancer
Zeaxanthin	Eggs, citrus, maize	Contributes to the maintenance of healthy vision
Dietary fiber		
Insoluble fiber	Wheat bran	May reduce risk of breast and/or colon cancer
Beta glucan[c]	Oats	May reduce risk of cardiovascular disease (CVD)
Soluble fiber[c]	Psyllium	May reduce risk of CVD
Whole grains[c]	Cereal grains	May reduce risk of CVD
Collagen Hydrolysate	Gelatin	May help improve some symptoms associated with osteoarthritis
Fatty acids		
Omega-3 fatty acids – DHA/EPA	Tuna; fish and marine oils	May reduce risk of CVD and improve mental, visual functions
Conjugated linoleic acid (CLA)	Cheese, meat products	May improve body composition, may decrease risk of certain cancers
Gamma linolenic acid	Borage, evening primrose	May reduce inflammation risk of cancer, CVD disease and improve body composition
Flavonoids		
Anthocyanidins: cyanidin	Berries	Neutralize free radicals, may reduce risk of cancer
Hydroxycinnamates	Wheat	Antioxidant-like activities, may reduce risk of degenerative diseases
Flavanols: catechins, tannins	Tea (green, catechins), (black, tannins)	Neutralize free radicals, may reduce risk of cancer
Flavanones	Citrus	Neutralize free radicals, may reduce risk of cancer
Flavones: quercetin	Fruits/vegetables	Neutralize free radicals, may reduce risk of cancer
Glucosinolates, indoles, isothiocyanates		
Sulphoraphane	Cruciferous vegetables (broccoli, kale), horseradish	Neutralizes free radicals, may reduce risk of cancer
Phenolics		
Stilbenes – resveratrol	Grapes	May reduce risk of degenerative diseases; heart disease; cancer. May have longevity effect
Caffeic acid, ferulic acid	Fruits, vegetables, citrus	Antioxidant-like activities; may reduce risk of degenerative diseases; heart disease, eye disease
Epicatechin	Cacao	Antioxidant-like activities; may reduce risk of degenerative diseases; heart disease

Transgenic Crops, Next Generation. Table 2 (Continued)

Class/components	Source[b]	Potential Health Benefits
Plant stanols/sterols		
Stanol/sterol ester[c]	Maize, soy, wheat, wood oils	May reduce risk of coronary heart disease (CHD) by lowering blood cholesterol levels
Prebiotic/probiotics		
Fructans, inulins, fructo-oligosaccharides (FOS)	Jerusalem artichokes, shallots, onion powder	May improve gastrointestinal health
Lactobacillus	Yogurt, other dairy	May improve gastrointestinal health
Saponins	Soybeans, soy foods, soy protein-containing foods	May lower LDL cholesterol; contains anticancer enzymes
Soybean protein	Soybeans and soy-based foods	25 g/day may reduce risk of heart disease.
Phytoestrogens		
Isoflavones – daidzein, genistein	Soybeans and soy-based foods	May reduce menopause symptoms, such as hot flashes, reduce osteoporosis, CVD
Lignans	Flax, rye, vegetables	May protect against heart disease and some cancers; may lower LDL cholesterol, total cholesterol, and triglycerides
Sulfides/thiols		
Diallyl sulfide	Onions, garlic, olives, leeks, scallions	May lower LDL cholesterol, helps to maintain healthy immune system
Allyl methyl trisulfide, dithiolthiones	Cruciferous vegetables	May lower LDL cholesterol, helps to maintain healthy immune system
Tannins		
Proanthocyanidins	Cranberries, cranberry products, cocoa, chocolate, black tea	May improve urinary tract health May reduce risk of CVD, and high blood pressure

[a]Examples are not an all-inclusive list

[b]US Food and Drug Administration approved health claim established for component. Modified from Newell-McGloughlin [29] (25)

centuries. During the past decade, however, intensive research has focused on expanding this source through rDNA biotechnology and essentially using plants and animals as living factories for the commercial production of vaccines, therapeutics, and other valuable products such as industrial enzymes and biosynthetic feedstocks [28].

More pressingly, with the increasing costs in economic and environmental terms of our dependency on fossil fuels, biotechnology offers innovative means to improve plant material for biomass conversion and enzymes to do the converting. There are two principal classes of biofuels: bio-alcohol (initially bio-ethanol but with increasing interest in higher-energy alcohols such as bio-butanol) and bio-diesel.

The first generation of biofuels was fermented from easy sources of simple sugars such as sugarcane and simple polymers primarily from grain starch. This source of bio-ethanol is unsustainable on many levels including the fact that its sources compete with food and especially feed grains for markets, land, and water. The focus for second generation bio-alcohols is mostly on complex polymers, primarily cellulosic ethanols and what are being termed third generation bio-alcohols such as bio-butanol [107]. From a biotechnology perspective work is being carried out on the biomass component focusing on increased production in such sources as switchgrass and miscanthus by among other things modifying photoperiodicity genes to switch energy to vegetative tissue production and improved biomass conversion by such

approaches as reducing lignin composition and incorporating self-activating enzyme digestion upon harvesting. On the enzyme component companies including Novozymes (Davis, CA) and Danisco (Palo Alto, CA) are making considerable strides in improving the effectiveness, specificity, and cost of cellulosic enzymes and increasing the conversion range especially for the more difficult pentose sugars such as xylose. Protein engineers are taking a synthetic biology approach with recent progress in engineering more stable and effective enzymes such as cellobiohydrolases by researchers at Caltech [108] and completely novel metabolic pathways by the Berkeley company, Amyris [109].

Biodiesel is defined by the National Biodiesel Board as a mono-alkyl ester. It is basically vegetable oil or animal fat–based diesel fuel consisting of long-chain alkyl (methyl, propyl, or ethyl) esters. Biodiesel is typically made via a trans-esterification process reacting lipids (vegetable oil, animal fat) with an alcohol.

Biodiesel per se can be used by standard diesel engines and is therefore qualitatively distinct from the vegetable, animal, and other waste oils used to fuel-converted diesel engines. Biodiesel can be used alone, or blended with petrodiesel. Biodiesel has better lubricating properties and much higher cetane ratings than today's lower sulfur diesel fuels but is still not economical as an alternate stand-alone fuel. Although still carbon-based, it is suggested by some that in terms of biofuels the algal biodiesel approach is much more sustainable than either the cellulosic or other land-based sources with DOE claiming that algae fuel yields have not yet been accurately determined, but DOE is reported as saying that algae yield 30 times more energy per acre than land crops such as soybeans [110].

From a biotechnology perspective the main focus for expanding interest in this area is increasing lipid production and modifying lipid composition for optimum performance. Work is being done to modify algae for increased production of desirable medium chain fatty acids (MCFA) which abrogates the requirement for cracking and isomerizing of long chain fatty acids (LCFAs). The advantages of MCFAs over LCFAs are high energy density, low fuel viscosity, low flash point, and low freezing point. The real issue with algal production systems is the scale-up step for commercial-level production where contamination, dewetting, and lipid isolation are still economically prohibitive. In February 2010, the Defense Advanced Research Projects Agency announced that the US military was about to begin large-scale production of jet fuel from algal pond isolates [111].

Barriers to Introduction

Most of the crops approved to date do appear to support the notion that the deregulation process is prohibitive for any but well-financed companies whose focus is primarily on the large commodity crops as just discussed. Worldwide there is clear asymmetry and lack of consensus in regulatory systems [112]. This discourages research on anything but the most mundane of crops and traits and is a real disincentive to creative research. For all intent and purposes there is just one trait from a public institution that has successfully traversed the regulatory minefields and been translated into a commercially viable commodity and that is the viral coat protein protection system initially developed for the papaya ringspot virus pandemic in Hawaii. This crop, papaya is a major tropical fruit crop in the Asian region. However, production in many Asian countries is set back by the prevalence of the PRSV disease as well as post-harvest losses. The PRSV-resistant papaya, based on RNAi suppression of the coat protein expression, literally saved the $17 M economy in Hawaii and is of significant importance in Taiwan and other SE Asian countries. Coat protein-based resistance is a demonstration of what is known as post-transcriptional gene silencing (PTGS). RNA interference (RNAi) in animals and basal eukaryotes, quelling in fungi, and PTGS in plants are examples of a broad family of phenomena collectively called RNA silencing. This system has now been applied to many species. A 5-year effort to combat plum pox virus disease through PTGS resistance paid off. In 1990, USDA/Agricultural Research Service (ARS) scientists began their efforts with a papaya ringspot virus coat protein gene obtained from Dennis Gonsalves [113]. This gene shows 70% homology to the plum pox gene and has been used to control other viruses similarly related to papaya ringspot. However, irrespective of the mechanism, it is important that resistance based on a single gene is managed well and alternate control mechanisms are introduced to reduce pressure on the development of viral resistance. Other approaches

include expression of the RNA replicating enzymes of the virus, expression of satellite RNA, replicating RNA molecules that are molecular parasites of the virus, or the use of protease inhibitors to interfere with processing of the viral proteins.

While translation of biotech research into value-added products for producers and consumers is a challenge in the USA, it is exponentially more difficult in LDCs [112]. A problem facing Africa in particular is the lack of a dynamic private sector to take technologies to the farmer. It has also been estimated that regulatory costs might exceed the costs of research and experimentation needed to develop a given GM crop, which is a major problem in releasing such crops to the market. A way to reduce the costs of generating food and environmental safety data is to develop regional "centers of excellence" with complementary facilities where food safety testing can be done reliably and regulatory costs could be reduced. The economic gains from using genetically modified crop technology in sub-Saharan Africa (SSA) are potentially large according to the World Bank Group [114]. The results suggest that the welfare gains are potentially very large, especially from golden rice and nutritionally enhanced GM wheat, and that those benefits are diminished only slightly by the presence of the de facto European Union's current ban on imports of GM foods.

The authors used the global economy-wide computable general equilibrium model known as GTAP. They specifically noted that if SSA countries impose bans on GM crop imports in deference to EU market demand for non-GM products, the domestic consumer loss net of that protectionism boost to SSA farmers would be more than the small gain derived from greater market access to the EU.

Problems cited for the slow passage of GM crops from experimental, to trial, to commercial stage especially in LDCs include the lack of capacity to negotiate licenses to use genes and research techniques patented by others, especially for crops with export potential [28]. In addition, there are difficulties in meeting regulatory requirements and a lack of effective public commercialization modalities and working extension networks. Biosafety and IPR regulations still have to be enforced in many countries for an effective and safe use of genetically engineered crops, especially if their production is meant for the export market.

Scientific, civic, and religious opinion leaders from all over the world have expressed support for the value of this technology. Florence Wambugu (CEO, Africa Harvest Biotech Foundation International, Kenya) states that the great potential of biotechnology to increase agriculture in Africa lies in its "packaged technology in the seed," which ensures technology benefits without changing local cultural practices [115]. For example, over 120 million children worldwide are deficient in vitamin A. In the late 1990s, Potrykus [108] group engineered rice to accumulate provitamin A (b-carotene). Incorporation of this trait into rice cultivars and widespread distribution of this "packaged technology in the seed" could prevent one to two million deaths each year. She observes that in the past, many foreign donors funded high-input projects, which have not been sustainable because they have failed to address social and economic issues such as changes in cultural practice [115]. In concurrence with this, Ismail Serageldin, former Chairman of the CGIAR (Consultative Group on International Agricultural Research) noted that, a priori, biotechnology could contribute to food security by helping to promote sustainable agriculture centered on smallholder farmers in developing countries [104].

US Consumer attitudes also tend to be relatively positive on the whole about agricultural biotechnology. In a 2010 IFIC survey, consumers were determined to be largely familiar with the term "biotechnology" [116]: More than two thirds of consumers (69%) have read or heard at least "a little" about the concept. Half (51%) of consumers say they are favorable toward farmers using biotechnology to grow more crops that would help meet food demand. In addition, significantly more consumers this year (28% vs. 23% in 2008) believe foods produced through biotechnology are available in the supermarket today, although this figure is still relatively low. Certain benefits of biotechnology are found to resonate better with consumers than others. These tend to be consumer-facing qualities such as improved health or better taste. For example, the majority of consumers say they are somewhat or very likely to purchase foods produced through biotechnology to provide more healthful fats like Omega-3 s (76%), to avoid trans fat (74%), and to make foods taste better/fresher (67%). This is consistent from 2008. Additionally, more than three quarters (77%) of

consumers say they would be likely to purchase foods produced through biotechnology for their ability to reduce pesticide use, and 73% of consumers said they would be likely to purchase bread, crackers, cookies, cereal, or pasta made with flour from wheat that had been modified by biotechnology to use less land, water, and/or pesticides. Of the 18% who would like to see additional information on the FDA label, only 3% mentioned anything about biotechnology.

But what of the context in which these crops are grown? Can all cropping systems co-exist in harmony? According to Brookes and Barfoot, [8, 117] it is important to determine the relative importance of different crop production systems based on planted area, production, and economic value to the region in question. The issue is what, if any, are the economic consequences of adventitious presence of material from one crop system within another based on the notion that farmers should be able to cultivate freely the crops of their choice using whichever production system works best in any given context (GM, conventional, or organic). It is never a food or environmental safety issue but rather a production and marketing matter. The heart of the issue is assessing the likelihood of adventitious presence of material from one production system affecting another and the potential impacts. This requires consistency when dealing with adventitious presence of any unwanted material including, but most definitely not limited to, biotech-derived material. Adventitious presence is simply the unintended incidence of something other than the desired crop such as small quantities of weed seeds, seeds from other crops, dirt, insects, or foreign material (e.g., stones). It is unrealistic to expect 100% purity for any crops, or products derived therefrom, so thresholds that are consistent across all materials should be set and should not discriminate (e.g., thresholds for adventitious presence of biotech material should be the same as applied to thresholds for other unwanted material and vice versa). All measures should be proportionate, non-discriminatory, and science-based.

The issue of economic liability provisions that compensate growers for adventitious presence of biotech material is often raised [117]. Historically, worldwide the market has adequately addressed economic liability issues relating to the adventitious presence of unwanted material in any agricultural crop. For example, for certified seed the onus is on the producers, who require isolation from undesired pollination for the purity of their product, to insure such purity; this is not their neighbor's problem. By extension the onus is on growers of any specialty crops to take action to protect the purity of their crops since these are self-imposed standards for and by that market. Growers who have themselves chosen a more stringent standard than that established in EU legislation should not expect their neighbors to bear the special management costs of meeting that self-imposed standard; to do so would reverse fundamental freedoms of economic activity and would establish a dangerous precedent. To allow specialty operators to formulate unrealistic standards for GM in their own produce would impose impossibly high standards on neighbors and would effectively impose a ban on the choice of other producers. Such growers usually are rewarded by higher prices and niche markets for taking such actions. Their neighbors enjoy no such advantage.

Existing legislation in North America and the EU is more than adequate to protect all grower and consumer interests but if new regulations were considered to address economic liability provisions for any negative economic consequences of adventitious presence of unwanted material, the same principle should apply to all farmers regardless of their chosen production methods. On equity grounds, biotech growers should have equal access to compensation for adventitious presence of material from conventional or organic crops (such as fungal contamination) as conventional and organic producers have from biotech growers. No one sector should be able to unfairly prohibit another – access and choice work both ways. All co-existence measures should be based on legal, practical, and scientific realities and not on commercial or niche marketing objectives. Where unintended presence has occurred on a number of occasions to date such as the presence of minute levels of Bayer Crop Sciences–regulated material LLRICE 604 found in Clearfield 131 (CL131) rice seed in 2007 and Mycogen's event "32" in maize in 2008, the agencies cooperated and determined that these events did not prove any risk as they carried similar constructs to those already having achieved non-regulated status.

According to Brookes and Barfoot, [8, 117] biotech crops co-exist successfully with conventional and

organic crops in North America (where, as noted, biotech crops account for the majority of acreage of important arable crops like soybeans, cotton, and maize) Spain, and more recently the Czech Republic. The market has developed practical, proportionate, and workable coexistence measures without new regulations or indeed any government intervention. Where isolated instances of adventitious presence of biotech material have been found in conventional or organic crops these have usually been caused by inadequate implementation of good coexistence practices (e.g., inefficient segregation of crops in storage and transport, nonuse of tested, certified seed). Under civil liability (i.e., tort damages) and for intellectual property infringement (except for the unauthorized StarLink), there have been no lawsuits brought by any parties for adventitious presence. Every case brought by a seed company for infringement has involved a claim that the farmer charged with infringement was an intentional infringer (i.e., adventitious presence was not the issue). And, to date, each of these cases was upheld by the courts. Indeed, all except one notable exception in North America has conceded to this claim. The exception is Percy Schmeiser who famously was found by a number of courts to have infringed Monsanto's glyphosate-tolerant patent by deliberately spraying and subsequently saving seeds from resistant sport canola plants found growing near his property. He initially claimed adventitious contamination but upon losing all the way to the Canadian Supreme court he changed tact to try to take advantage of Canadian patent law which prohibits patenting of higher organisms. While the court upheld this they determined that the construct within the plant was subject to IP protection and so Schmeiser was found to have infringed a patentable article under Canadian law.

Virtually all EU member states have transcribed EU Directive 2001/18 and implement EU regulations on traceability and labeling. Within the EU, provision has been made for a de minimis threshold for unavoidable presence of GMOs but no actual threshold has been set. Therefore, the default state of the 0.9% on labeling and traceability is the one enforced. In the USA, organic products cannot be (legally) downgraded or the producer decertified by unintentional presence when all required measures and best practices are adhered to and no producer has been so impacted to date [118].

Going forward there are four major stanchions to the furtherance of co-existence and all of them are incumbent on cooperation.

1. Monitoring: Verify the models and predictions about cost, isolation standards, and generally to learn how the farming community copes with the requirements for keeping the product streams separated.
2. Dialog: Strategy development takes place in a dialog between the scientific and technical community and all relevant stakeholders. (The Czech Republic [119].)
3. Stewardship: Stewardship programs should take into account the interests of both GM and non-GM farmers. Existing product stewardship programs for non-GM crops in farming should be a starting point for developing stewardship schemes for GM crops.
4. Research: The scientific community should be encouraged to fill the knowledge gaps that have been identified. Projects are needed to validate models and guidelines, including long-term studies. Building up mechanistic, probabilistic, and predictive models of gene flow etc. Methods for restricting gene flow by eliminating the fertility of pollen or seeds (apomixis, cytoplasmic male sterility, plastid transformation, Genetic Use Restriction Technology (GURT), etc.).

The World Trade Organization ruled in 2006 that a 6-year European ban on genetically engineered crops violates international trade. The three-person panel issued its decision ruling in favor of the three countries, USA, Canada, and Argentina, on a large majority of the 25 crops under dispute in the case while issuing mixed rulings on a few crops. The panel also ruled in favor of challenging national bans on specific biotech crops issued by Austria, France, Germany, Greece, Italy, and Luxembourg. The EU had argued that it did not have a moratorium but that it just took more time to weigh the possible risks to health and the environment posed by genetically engineered foods. It said it needed to take a "precautionary" approach to regulation, which is different from what it called Washington's "laissez-faire" stance.

The trade organization panel appears not to have challenged Europe's regulatory process for biotech

crops. Rather, it said Europe failed to follow its own procedures, resulting in undue delay of decisions. Interestingly one of the most comprehensive assessments on the technology was conducted by EU scientists. An EU Commission Report [120] that summarized biosafety research of 400 scientific teams from all original 15 EU countries conducted over 15 years stated that research on biotechnology-derived plants and products so far developed and marketed, following usual risk assessment procedures, has not shown any new risks to human health or the environment beyond the usual uncertainties of conventional plant breeding. Indeed, the use of more precise technology and the greater regulatory scrutiny probably make them even safer than conventional plants and foods. If there are unforeseen environmental effects – none have appeared as yet – these should be rapidly detected by existing monitoring systems. This analysis was repeated in a 2008 EU Joint Research Centre (JRC) Report commissioned by Members of the European Parliament (MEPs) conducted by world experts including the European Food Safety Authority (EFSA), the World Health Organization (WHO), and others [120]. Their report concluded that there is no evidence that genetically modified foods have any harmful effects; a declaration signed by over 3,500 scientists including 25 Nobel Laureates reiterates this position [28].

Future Directions

As agriculture must adapt to rapidly changing needs and growing conditions it is important to become more effective at producing more or less with limited resources and only the tools of biotechnology will allow us to bypass physiological and environmental limitations to produce sufficient food, feed, fuels, and fiber on ever diminishing arable land to meet ever increasing demand. The challenges going forward are foremost technical as one strives to modify qualitative as opposed to quantitative traits and intricates metabolic pathways and networks as opposed to single genes; the scientific hurdles to achieve these aims are not trivial. However, with the tools now coming online in the fields of genomics, proteomics, metabolmics, and bioinformatics, there is the potential to make major modifications to introgress desirable traits. For example, tools such as next generation sequencing, RNA interference (RNAi), transcription factors (Tfs), transcription activator-like effector nucleases (TALENs), mini-chromosomes, combinatorial transformation, epigenetic modification, network engineering, and systems biology will allow us to apply both reductive and holistic approaches to identify, modify, introgress, and subsequently and simultaneously study the expression and interaction of transgenes on tens of thousands of endogenous genes in elite germplasm backgrounds. With these newly evolving tools, one is beginning to dissect the global effects of metabolic engineering on metabolites, enzyme activities, and fluxes. With rapidly emerging technologies, the increase in our understanding of and ability to manipulate plant metabolism during the coming decades should place plant researchers in the position of being able to modify crop traits to respond to the diversity of needs from minimizing environmental impact to optimizing productivity and quality output.

Non-technical limitations include intellectual property restrictions which may limit translation of public research if not managed judiciously; secondly, liability concerns over abuse or misuse of constructs; thirdly prohibitive and asymmetric biosafety regimes and finally public acceptance. The latter two in many ways are the most insidious of limitations as they have little basis in rational process and thus are difficult to redress effectively – the last in particular is often predicated on how much of the former is perceived to be of concern, and how positions are presented by the opposing factions. It is often easier to appeal to emotion and self-fear than it is to present reasoned and judicious scientific rational for basing risk analysis. Indeed the actual commercialization of biotech products may have little to do with technical limitations and more to do with these external constraints primarily the process of regulatory approval. The flagship of improved nutritional varieties, namely, beta carotene–enhanced rice commonly referred to as golden rice, despite being under consideration since the late 1990s and subject to a barrage of risk assessments is unlikely to be approved until 2012 at the earliest. Ingo Potrykus, the developer, says an unreasonable amount of testing has been required without scientific justification. In a recent *Nature* article [121], he lays the blame largely on the regulatory process which he considers excessive observing that unjustified and impractical legal requirements are preventing genetically engineered crops from saving millions from starvation and malnutrition.

In the final analysis, resources are finite and true sustainability can come only from an enlightened philosophy that promotes the development of resource-enhancing technologies. Antithetically, those who claim to be the stalwarts of sustainability are, on occasion, the very ones who oppose the development and application of those tools that can help to insure such sustainability. The only sure way to insure food security and protect the planet's resources is not to settle into the complacency of maintaining the status quo but to engage in continual, constructive change based on scientific knowledge. Thus, if one is to be accountable to posterity it is not just the choice but one's duty to promote and apply responsible science and technology in all endeavors.

Bibliography

1. World Bank (2007) Agriculture for development, overview. World Bank, Washington, DC
2. Lavelle M, Garber K (2008) Eight ways to fix the global food crisis US news and world reports. Posted 9 May 2008. http://www.climos.com/news/articles/8waysto.htm
3. F. W. F. a. A. O. o. t. U. N. W. H. (2004) The state of food and agriculture 2003-2004, agricultural biotechnology, meeting the needs of the poor? vol 35. Food and Agriculture Organization of the United Nations, Rome, pp 1–209
4. Economic and Social Council: Commission on Science and Technology for Development (2004) Resolution 2004/68. Science and Technology for Development. Council, E. a. S
5. Agbioworld (2000) Seven scientific academies support GM crops reproduced from The Times (London), 12 July 2000. http://www.agbioworld.org/biotech-info/topics/dev-world/academies.html
6. James C (2011) Global status of commercialized biotech/GM Crops: 2010. ISAAA briefs 42-2010. ISAAA, Ithaca. http://www.isaaa.org/resources/publications/briefs/42/executivesummary/default.asp
7. Ghadar F, Spindler H (2005) Industrial management the growth of biotechnology (Global tectonics: Part 1). http://www.entrepreneur.com/tradejournals/article/135568229.html
8. Brooks G, Barfoot P (2008) Co-existence of GM and Non-GM arable crops: the Non-GM and organic context in the EU. PG Economics, Dorchester
9. Brooks G, Barfoot P (2005) The global economic and environmental impact – The first nine years 1996–2004. AgBioForum 8:187–196
10. Elmegaard N, Pedersen MB (2001) Flora and fauna in Roundup tolerant fodder beet fields. National Environment Research Institute, Technical Report No. 349, p 40
11. Trewavas A (2001) Urban myths of organic farming. Nature 410:409–410
12. Folcher L, Delos M, Marengue E, Jarry M, Weissenberger A, Eychenne N, Regnault-Roger C (2010) Lower mycotoxin levels in Bt maize grain. Agron Sustain Dev 30(4):711–719
13. Green RE, Cornell SJ, Scharlemann JP, Balmford A (2005) Farming and the fate of wild nature. Science 307:550–555
14. Huang J, Rozelle S, Pray C, Wang Q (2002) Plant biotechnology in China. Science 295:674–676
15. Choudhary B, Gaur K (2010) BT cotton in India: a country profile. ISAAA, Ithaca
16. Choudhary B, Gaur K (2008) The development and regulation of BT brinjal in India (Eggplant/Aubergine). ISAAA Brief No. 38, Ithaca
17. Bricknel T (2010) India delays BT brinjal decision. Fruitnet.com
18. Monsanto (2010) Insect protected roundup ready 2 yield soybeans achieve important milestone toward commercialization in Brazil
19. Stern N (2006) Review on the economics of climate change. HM Treasury, London
20. Svoboda E (2008) The future of farming is in nitrogen efficiency fast company. Posted 1 Oct 2008. http://www.fastcompany.com/magazine/129/seed-money.html
21. Sottosanto JB, Saranga Y, Blumwald E (2007) Impact of AtNHX1, a vacuolar Na+/H+ antiporter, upon gene expression during short- and long-term salt stress in Arabidopsis thaliana. BMC Plant Biol 7:18
22. Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25:1263–1274
23. Deng X, Phillips J, Brautigam A, Engstrom P, Johannesson H, Ouwerkerk PB, Ruberti I, Salinas J, Vera P, Iannacone R, Meijer AH, Bartels D (2006) A homeodomain leucine zipper gene from Craterostigma plantagineum regulates abscisic acid responsive gene expression and physiological responses. Plant Mol Biol 61:469–489
24. Rivero RM, Shulaev V, Blumwald E (2009) Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiol 150:1530–1540
25. Foundation AAT (2007) Combining breeding and biotechnology to develop water efficient maize for Africa (WEMA)
26. Jung KH, Seo YS, Walia H, Cao P, Fukao T, Canlas PE, Amonpant F, Bailey-Serres J, Ronald PC (2010) The submergence tolerance regulator Sub1A mediates stress-responsive expression of AP2/ERF transcription factors. Plant Physiol 152:1674–1692
27. Lopez-Bucio J, Nieto-Jacobo MF, Ramirez-Rodriguez VV, Herrera-Estrella L (2000) Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci 160:1–13
28. Newell-McGloughlin M (2006) "Functional foods" and biopharmaceuticals: the next generation of the gm revolution. American Enterprise Institute, Washington, DC, pp 163–178
29. Newell-McGloughlin M (2008) Nutritionally improved agricultural crops. Plant Physiol 147:939–953

30. Luciani G, Wofford DS (2005) Over-expression of a soybean vegetative storage protein gene in Bahiagrass (Paspalumnotatum var. Flugge). http://www.ufgi.ufl.edu/Symposium/FG2005program_rev.pdf
31. Roesler K et al (1997) Targeting of the Arabidopsis homomeric acetyl-coenzymeA carboxylase to plastids of rapeseeds. Plant Physiol 113:75–81
32. Cromwell GL et al (1967) Nutritional value of opaque-2 corn for swine. J Anim Sci 26:1325–1331
33. Yang SH et al (2002) Expression of a synthetic porcine alpha-lactalbumingene in the kernels of transgenic maize. Transgenic Res 11:11–20
34. O'Quinn PR et al (2000) Nutritional value of a genetically improved highlysine, high-oil corn for young pigs. J Anim Sci 78:2144–2149
35. Young TE et al (2004) Senescence-induced expression of cytokinin reverses pistil abortion during maize flower development. Plant J 38:910–922
36. Chakraborty S et al (2000) Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from Amaranthushypochondriacus. Proc Natl Acad Sci USA 97:3724–3729
37. Li LLS et al (2001) Increase of sulphur-containing amino acids in transgenicpotato with 10 kuzein gene from maize. Chin Sci Bull 46:482–484
38. Yu J, Ao G (1997) Expression of 10 kDa sulfur-rich prolamin gene of rice inpotato. Acta Bot Sin 39:329–334
39. Atanassov ABA et al (2004) To reach the poor – results from the ISNAR-IFPRINext Harvest study on genetically modified crops, public research, and policyimplications. EPTD discussion paper – Environment and Production Technology Division, International Food Policy Research Institute, Washington, DC
40. Katsube T et al (1999) Accumulation of soybean glycinin and its assembly withthe glutelins in rice. Plant Physiol 120:1063–1074
41. Rapp W (2002) Development of soybeans with improved amino acid composition. In: 93 rd AOCS annual meeting and expo. American Oil Chemists' Society Press, Montreal, pp 79–86
42. Dinkins RDRM et al (2001) Increased sulfur amino acids in soybean plantsoverexpressing the maize 15 kDazein protein. In Vitro Cell Dev Biol Plant 37:742–747
43. Prakash CS, Jaynes J (2000) Increasing the protein content in sweet potato using a synthetic storage protein gene. Abstr Papers Am Chem Soc 219:U36
44. Uauy C et al (2006) Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314:1298–1301
45. Falco SC et al (1995) Transgenic canola and soybean seeds with increased lysine. Biotechnology 13:577–582, New York
46. White CLTL et al (2001) Increased efficiency of wool growth and live weightgain in Merino sheep fed transgenic lupin seed containing sunflower albumin.J. Sci. Food Agric 81:147–154
47. Lai JS (2002) Increasing maize seed methionine by mRNA stability. Plant J 30:395–402
48. Zeh M et al (2001) Antisense inhibition of threonine synthase leads to highmethionine content in transgenic potato plants. Plant Physiol 127:792–802
49. Galili G et al (2002) Genetic, molecular, and genomic approaches to improvethe value of plant foods and feeds. Crit Rev Plant Sci 21:167–204
50. Del Vecchio A (1996) High-laurate canola. How Calgene's program began, where it's headed. INFORM Int News Fats Oils Relat Mater 7:230–243
51. Froman B (2002) Genetic modification of oils for improved health benefits:production of long chain omega-3 fatty acids in plants. Abstr Papers Am Chem Soc 223:U35
52. James MJ et al (2003) Metabolism of stearidonic acid in human subjects:comparison with the metabolism of other n-3 fatty acids. Am J Clin Nutr 77:1140–1145
53. Ursin VM (2003) Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. J Nutr 133:4271–4274
54. Chapman KDA-BS et al (2001) Transgenic cotton plants with increased seedoleic acid content. J Am Oil Chem Soc 78:941–947
55. Liu Q et al (2002) High-oleic and high-stearic cottonseed oils: nutritionally improved cooking oils developed using gene silencing. J Am Coll Nutr 21(3 suppl):205S–211S
56. Abbadi ADF et al (2004) Biosynthesis of very-long-chain polyunsaturatedfatty acids in transgenic oilseeds: constraints on their accumulation. Plant Cell 16:2734–2748
57. Parveez G (2003) Novel products from transgenic oil palm. AgBiotechNet 5:1–8
58. Jalani BSCS et al (1997) Improvement of palm oil through breeding andbiotechnology. J Am Oil Chem Soc 74:1451–1455
59. Anai T et al (2003) Improvement of rice (Oryza sativa L.) seed oil qualitythrough introduction of a soybean microsomal omega-3 fatty acid desaturasegene. Plant Cell Rep 21:988–992
60. Kinney AJ (1998) Designer oils: the high oleic acid soybean. In: Roller SHS (ed) Genetic modification in the food industry: a strategy for food quality improvement. Blackie Academic and Professional, Oxford
61. Reddy AS, Thomas TL (1996) Expression of a cyanobacterial delta 6-desaturase gene results in gamma-linolenic acid production in transgenicplants. Nat Biotechnol 14:639–642
62. Arcadia Biosciences (2008) Arcadia biosciences and bioriginal food and science corp. Enter strategic alliance to market high GLA safflower oil. *Business Wire*. http://findarticles.com/p/articles/mi_m0EIN/is_2008_Feb_22/ai_n24320185/
63. Smeekens S (1997) Engineering plant metabolism. Trends Plant Sci 2:286–287
64. Sprenger N et al (1997) Fructan synthesis in transgenic tobacco and chicoryplants expressing barley sucrose: fructan 6-fructosyltransferase. FEBS Lett 400:355–358
65. Sevenier R et al (1998) High level fructan accumulation in a transgenic sugarbeet. Nat Biotechnol 16:843–846
66. Caimi PG et al (1996) Fructan accumulation and sucrose metabolism intransgenic maize endosperm expressing a Bacillus amyloliquefaciens SacB gene. Plant Physiol 110:355–363

67. Hellwege EM et al (1997) Transgenic potato tubers accumulate high levels of1-kestose and nystose: functional identification of a sucrose 1-fructosyltransferase of artichoke (Cynarascolymus) blossom discs. Plant J 12:1057–1065
68. Hartwig E et al (1997) Seed protein and its relationship to soluble sugars insoybeans. Crop Sci 37:770–773
69. Hellwege EM et al (2000) Transgenic potato (Solanumtuberosum) tubers synthesize the full spectrum of inulin molecules naturally occurring in globeartichoke (Cynarascolymus) roots. Proc Natl Acad Sci USA 97:8699–8704
70. Chiang CYF et al (2005) Expression of a bi-functional and thermostableamylopullulanase in transgenic rice seeds leads to autohydrolysis and alteredcomposition of starch. Mol Breed 15:125–143
71. Schwall GP et al (2000) Production of very-high-amylose potato starch byinhibition of SBE A and B. Nat Biotechnol 18:551–554
72. Shintani D, DellaPenna D (1998) Elevating the vitamin E content ofplants through metabolic engineering. Science 282:2098–2100
73. Rocheford TR et al (2002) Enhancement of vitamin E levels in corn. J Am Coll Nutr 21(3 suppl):191S–198S
74. Cahoon EB et al (2003) Metabolic redesign of vitamin E biosynthesis in plantsfor tocotrienol production and increased antioxidant content. Nat Biotechnol 21:1082–1087
75. Chen Z et al (2003) Increasing vitamin C content of plants through enhancedascorbate recycling. Proc Natl Acad Sci USA 100:3525–3530
76. Naqvi S et al (2009) Transgenic multivitamin corn through biofortification ofendosperm with three vitamins representing three distinct metabolic pathways. Proc Nat Acad Sci USA 106:7762–7767
77. Shewmaker CK et al (1999) Seed-specific overexpression of phytoenesynthase: increase in carotenoids and other metabolic effects. Plant J 20:401–412
78. Ducreux LJ et al (2005) Metabolic engineering of high carotenoid potatotubers containing enhanced levels of beta-carotene and lutein. J Exp Bot 56:81–89
79. Ye X et al (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305
80. Agius F et al (2003) Engineering increased vitamin C levels in plants byoverexpression of a D-galacturonic acid reductase. Nat Biotechnol 21:177–181
81. Diaz de la Garza R et al (2004) Folatebiofortification in tomatoes byengineering the pteridine branch of folate synthesis. Proc Natl Acad Sci USA 101:13720–13725
82. Enfissi EM et al (2005) Metabolic engineering of the mevalonate and nonmevalonateisopentenyldiphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol J 3:17–27
83. Fraser PDRS et al (2001) Elevation of carotenoids in tomato by geneticmanipulation. J Sci Food Agric 81:822–827
84. Rosati C et al (2000) Metabolic engineering of beta-carotene and lycopenecontent in tomato fruit. Plant J 24:413–419
85. Szankowski I et al (2003) Transformation of apple (MalusdomesticaBorkh.)with the stilbene synthase gene from grapevine (Vitisvinifera L.) and a PGIPgene from kiwi (Actinidiadeliciosa). Plant Cell Rep 22:141–149
86. Hipskind JD, Paiva NL (2000) Constitutive accumulation of aresveratrol-glucoside in transgenic alfalfa increases resistance to Phomamedicaginis. Mol Plant Microbe Interact 13:551–562
87. Kobayashi SDC et al (2000) Kiwifruits (Actinidiadeliciosa) transformed with aVitisstilbene synthase gene produce piceid (resveratrol-glucoside). Plant Cell Rep 19:904–910
88. Yu O et al (2000) Production of the isoflavonesgenistein and daidzein in nonlegumedicot and monocot tissues. Plant Physiol 124:781–794
89. Lukaszewicz M et al (2004) Antioxidant capacity manipulation in transgenicpotato tuber by changes in phenolic compounds content. J Agric Food Chem 52:1526–1533
90. Stark-Lorenzen PNB et al (1997) Transfer of a grapevine stilbene synthasegene to rice (Oryza sativa L.). Plant Cell Rep 16:668–673
91. Yu O et al (2003) Metabolic engineering to increase isoflavone biosynthesis insoybean seed. Phytochemistry 63:753–763
92. Giovinazzo G et al (2005) Antioxidant metabolite profiles in tomato fruitconstitutively expressing the grapevine stilbene synthase gene. Plant Biotechnol J 3:57–69
93. Niggeweg R et al (2004) Engineering plants with increased levels of theantioxidant chlorogenic acid. Nat Biotechnol 22:746–754
94. Muir SR et al (2001) Overexpression of petunia chalconeisomerase in tomatoresults in fruit containing increased levels of flavonols. Nat Biotechnol 19:470–474
95. Gonzali S et al (2009) Purple as a tomato: towards high anthocyanin tomatoes. Trends Plant Sci 14:237–241
96. UPI (2002) Wheat inhibits colon cancer. United Press International, Washington, DC
97. Austin-Phillips SBE et al (1999) Production of industrial and animal feed enzymes in transgenic Alfalfa. http://www.molecularfarming.com/nonmedical.html
98. Goto F et al (2000) Iron accumulation and enhanced growth in transgeniclettuce plants expressing the iron-binding protein ferritin. Theor Appl Genet 100:658–664
99. Lucca P et al (2002) Fighting iron deficiency anemia with iron-rich rice. J Am Coll Nutr 21(3 suppl):184S–190S
100. Drakakaki G et al (2005) Endosperm-specific co-expression of recombinant soybean ferritin and Aspergillusphytase in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 59:869–880
101. Han G (2009) Origin agritech announces final approval of world's first genetically modified phytase corn. http://www.allbusiness.com/science-technology/biology-biotechnology-genetic/13453842-1.html
102. Denbow DM et al (1998) Soybeans transformed with a fungal phytasegene improve phosphorus availability for broilers. Poult Sci 77:878–881

103. Brinch-Pedersen H et al (2000) Generation of transgenic wheat (Triticumaestivum L.) for constitutive accumulation of an Aspergillusphytase. Mol Breed 6:195–206
104. Zhang ZB, Kornegay ET, Radcliffe JS, Wilson JH, Veit HP (2000) Comparison of phytase from genetically engineered Aspergillus and canola in weanling pig diets. J Anim Sci 78:2868–2878
105. Lucca P, Hurrell R, Potrykus I (2002) Fighting iron deficiency anemia with iron-rich rice. J Am Coll Nutr 21:184S
106. Newell-McGloughlin M (2006) A Retrospective prospective perspective on agricultural biotechnology ten years on. J Commer Biotechnol 12(1):20–27
107. Programme UNE (2009) Towards sustainable production and use of resources: assessing biofuels, United Nations Environment
108. Heinzelman P, Snow CD, Wu I, Nguyen C, Villalobos A, Govindarajan S, Minshull J, Arnold FH (2009) A family of thermostable fungal cellulases created by structure-guided recombination. Proc Natl Acad Sci USA A106:5610–5615
109. Ritch E (2008) Microbes drive new Amyris biodiesel plant. Cleantech Group, San Francisco
110. Hartman E (2008) A promising oil alternative: algae energy. The Washington Post
111. Goldenberg S (2010) Algae to solve the Pentagon's jet fuel problem. The Guardian, London
112. Newell-McGloughlin M (2002) Ten reasons why biotechnology will be important to the developing world. AgBioWorld 2(3 and 4)
113. Gonsalves D (1998) Control of papaya ringspot virus in papaya: a case study. Annu Rev Phytopathol 36:415–437
114. Anderson K, Jackson LA (2005) Some implications of GM food technology policies for Sub-Saharan Africa. J Afr Econ 14(3):385–410
115. Wambugu F (1999) Why Africa needs agricultural biotech. Nature 400:15–16
116. Council IFI (2010) Consumer perceptions of food technology. Cogent Research of Cambridge, Massachusetts for the International Food Information Council, Cambridge
117. Brookes G, Barfoot P (2004) Co-existence of GM and non-GM arable crops: the non-GM and organic context in the EU. PG Economics, Dorchester
118. Kershen D (2006) Legal liability issues in agricultural biotechnology. http://www.nationalaglawcenter.org/assets/articles/kershen_biotech.pdf
119. Křístková M (2008) Principles of co-existence. Department of Agriculture, The Czech Republic. http://www.mzp.cz/www/webdav_biosafety.nsf/biosafety/pdf/coexistence.pdf
120. Council EUJR (2008) Scientific and technical contribution to the development of an overall health strategy in the area of GMOs
121. Potrykus I (2010) Regulation must be revolutionized. Nature 466:561
122. Boettiger S, Alvarez S, Getting better technologies to the poor. A public intellectual property resource
123. Potrykus I (1999) Vitamin-A and iron-enriched rices may hold key to combating blindness and malnutrition: a biotechnology advance. Nat Biotechnol 17:37
124. Report EC (2001) GMO research in perspective
125. Agbios (2008) GM crop database. Agbios. http://www.agbios.com/dbase.php?action=ShowForm
126. Zhao ZYGK et al (2002) Nutritionally improved transgenic sorghum. PlantBiotechnology 2002 and beyond. In: Proceedings of the 10th IAPTC&B congress, Orlando, 23–28 June 2002
127. Glei M et al (2006) Both wheat (Triticumaestivum) bran arabinoxylans and gutflora-mediated fermentation products protect human colon cells fromgenotoxic activities of 4-hydroxynonenal and hydrogen peroxide. J Agric Food Chem 54:2088–2095

Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union

Yann Devos[1], Wendy Craig[2], Joachim Schiemann[3]
[1]GMO Unit, European Food Safety Authority (EFSA), Parma, Italy
[2]Biosafety Unit, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy
[3]Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Biosafety of Genetically Modified Plants, Quedlinburg, Germany

Article Outline

Glossary

Biodiversity Biodiversity is the quantity and variability among living organisms within species (genetic diversity), between species and between ecosystems. Biodiversity is not itself an ecosystem service, but underpins the supply of services. The value placed on biodiversity for its own sake is captured under the cultural ecosystem service called "ethical values" (according to the Economics of Ecosystems and Biodiversity report [1]).

Deliberate release Any intentional introduction into the environment of a genetically modified organism (GMO) or a combination of GMOs for which no specific containment measures are used to limit their contact with, and to provide a high level of safety for, the general population and the environment (according to Directive 2001/18/EC on the deliberate release into the environment of GMOs [2]).

Ecosystem An ecosystem is a dynamic complex of plant, animal, and microorganism communities and their non-living environment interacting as a functional unit. Examples of ecosystems include rainforests, grasslands, urban parks, and cultivated farmlands. Ecosystems can be relatively undisturbed by humans, such as virgin rainforests, or can be modified by human activity (according to the Economics of Ecosystems and Biodiversity report [1]).

Ecosystem services Ecosystem services are the benefits that people obtain from ecosystems. Examples include food, freshwater, timber, climate regulation, protection from natural hazards, erosion control, pharmaceutical ingredients, and recreation (according to the Economics of Ecosystems and Biodiversity report [1]).

Environmental harm Environmental harm can be defined as a measurable adverse change in a natural resource or measurable impairment of a natural resource service which may occur directly or indirectly (according to Directive 2004/35/EC on environmental liability [3]).

Genetically modified/transgenic organisms Organisms, such as plants, animals, and microorganisms (with the exception of human beings), in which the genetic material (DNA) has been altered in such a way that does not occur naturally by mating and/or natural recombination (according to Directive 2001/18/EC on the deliberate release into the environment of GMOs [2]).

Living modified organism Any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology (according to the Cartagena Protocol on Biosafety [4]).

Modern biotechnology The application of (1) *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct introduction of nucleic acid into cells or organelles, or (2) fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

selection (according to the Cartagena Protocol on Biosafety [4]).

Organism Any biological entity capable of replication or of transferring genetic material (according to Directive 2001/18/EC on the deliberate release into the environment of GMOs [2]).

Placing on the market Making available to third parties, whether in return for payment or free of charge (according to Directive 2001/18/EC on the deliberate release into the environment of GMOs [2]).

Risk assessment Process of evaluation of risk, including the identification of scientific uncertainties, of the likelihood and severity of an adverse effect(s) or event(s) occurring to human and animal health or the environment following exposure under defined conditions to a risk source(s). A risk assessment comprises problem formulation (or hazard identification), hazard characterization, exposure characterization, and risk characterization (according to [5]).

Definition of the Subject

This contribution describes the risk assessment principles and the regulatory framework for transgenic (genetically modified (GM)) crops in the European Union (EU).

While the global cropping area of GM crops reached 148 million hectares in 2010, the total area cultivated with GM crops in the EU was less than 100,000 ha. Most GM crops are thus cultivated outside the EU, but might subsequently be imported and eventually further processed in the EU, mostly for animal feed purposes.

It is globally accepted that agro-food biotechnology could contribute to achieving the objectives (conservation of biological diversity, sustainable use of its components, fair and equitable sharing of the benefits arising out of the utilization of genetic resources) laid down in the Convention on Biological Diversity, if developed and used with adequate safety measures for both the environment and human health. Generally, the safety measures are embedded in process- or product-based regulatory framework. The EU regulatory framework is process-based, precautionary, and includes mandatory labeling and traceability requirements for GM crops and their derived food and feed products. During its development, the EU regulatory system has become increasingly more stringent.

GM crops and their derived food and feed products are generally subject to a risk analysis before they can be commercialized. In the EU, the risk analysis consists of three components: risk assessment, risk management, and risk communication. In risk assessment, potential adverse impacts associated with a specific activity are scientifically characterized on a case-by-case basis, while in risk management, policy alternatives to accept, minimize, or reduce the characterized risks are weighed and, if needed, appropriate prevention and control options are selected. Risk management is functionally and temporally separate from risk assessment in order to reduce any conflict of interest and to protect the scientific integrity of risk assessment. Risk communication is defined as an interactive exchange of information and opinions on risk throughout risk analysis, between risk assessors, risk managers, and other interested parties.

When analyzing potential risks, it is important to bear in mind that the real choice is not between GM crops that are inherently risky and traditionally bred ones that are completely safe. The cultivation of existing crops and those with novel traits (including GM crops) will have both positive and negative consequences. To fully acknowledge the overall outcome of adopting specific crops, and to assess and manage more effectively the environmental footprint of agriculture as a whole, the conclusion is that broader and more balanced legislative oversight is needed in the EU.

Introduction

The global cropping area of GM crops (including soya bean, maize, cotton, oilseed rape, and sugar beet) has consistently increased each year since they were first commercially cultivated in 1996. While the global cropping area of GM crops reached 148 million hectares in 2010, the total area under GM cultivation in the European Union (EU) was approximately 91,400 ha [6]. Most approved GM crops worldwide are thus currently cultivated outside the EU, but might subsequently be imported and eventually further processed in the EU, mostly for animal feed purposes.

The disparity in adoption rates of GM crops between the EU and the rest of the world is generally attributed both to societal and political opposition toward agro-food biotechnology, and to complex regulatory approval procedures in the EU [7, 8]. In the mid-1990s, the advent of GM crops aroused strong societal concerns [9–12]. Fostered by several highly publicized and successive food safety crises (e.g., bovine spongiform encephalopathy, dioxins, emergence of pathogens such as *Escherichia coli* 0157), public suspicion toward regulatory authorities, scientists and technocratic decision making grew [13]. The media, which was explicitly involved in framing public perception and societal image-building of agro-food biotechnology [14, 15], exacerbated the social amplification of risk [16]. In the late 1990s, increasing societal and political opposition contributed to a *de facto* moratorium on new GM crop market approvals. This was adopted at a meeting of the EU Council of environmental Ministers in June 1999, where five EU Member States indicated that they would oppose any new approvals pending a revision of the legislation [17]. The moratorium did not have a formal status and did not revoke pre-1999 approvals of GM crops or food products, nor did it officially prevent new approvals of GM food products. However, the moratorium was *de facto* effective since a sufficient number of Member States ensured a blocking majority of the legislative process [18]. As a consequence, several GM crop market applications remained blocked in the EU regulatory system for over a decade.

From 1999 onward, policy makers started to continuously revise the legal conditions under which GM crops and derived food and feed products were to be allowed to be used in the EU, in order to slow down further erosion of public and market confidence (reviewed in [10]). These various legal and institutional reforms, although leading to the upheaval of the *de facto* moratorium in 2004 and a regulatory regime that imposes the most stringent criteria for their approval worldwide (cf., WTO dispute between the US and EU), did not dissipate societal concerns. As Gaskell et al. [19] put it "the new regulatory frame appears to have done little to allay the European public's anxieties about agro-food biotechnology," and "the years of controversy have led many people in Europe to believe that anything that has to do with GM food is undesirable". Member States continue to raise safety objections during the approval process for the placing on the market of GM crops. While the new EU regulatory system should guarantee a harmonized and science-based process, none of the GM plant market applications that were positively evaluated by the EU authority responsible for providing advice on the safety of GMOs, the European Food Safety Authority (EFSA), have attained the necessary qualified majority (neither in favor nor against the approval of GMOs) from the relevant regulatory committees or the Council of Ministers, both for exhibiting substantial abstentions in voting [18, 20]. In most cases, the European Commission has adopted favorable decisions that are not endorsed by a qualified majority of Member States, whereas in other cases, decisions are still pending.

In response to the European Commission approvals for the marketing of GM crops, several Member States invoked national safeguard measures to provisionally restrict or prohibit the use and/or sale of approved GMOs in their territory. Even though EFSA concluded that, in terms of risk to human and animal health and the environment, no new scientific evidence had been presented that would invalidate former risk assessments, the Council of Ministers rejected the proposals of the European Commission to repeal invoked safeguard measures. To reduce the recourse of Member States to safeguard measures and to facilitate the decision making process, the European Commission proposed in July 2010 to confer to Member States the freedom to allow, restrict, or ban the cultivation of GMOs on part or all of their territory [21, 22]. In addition, the European Commission issued a new Recommendation on coexistence of GM crops with conventional and/or organic crops [23] that replaces the previous recommendation from 2003. Whether these proposals will help to unlock the European legal gridlock is debatable. The European Parliament and Council are expected to discuss the proposals, with a view to legal implementation, in the autumn of 2010 [24]. However, several Member States have already notified the European Commission of their intention to prohibit the cultivation of the Amflora® starch potato, which was approved for cultivation in March 2010. It was the first EU approval for cultivation of a GM crop in the past decade. This approval has garnered considerable public controversy [25] and is illustrative of the

lasting skeptical and/or ambivalent attitude of the European society toward agro-food biotechnology.

Regulatory Oversight of GM Plants and Their Derived Food and Feed Products

Heightened global awareness and concern over accelerating environmental degradation during the latter quarter of the twentieth century resulted in a desire by the international community to push the protection of the environment higher up the political agenda. These efforts came to fruition in 1992 when the Convention on Biological Diversity (CBD) came into force. Its objectives include "the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources" [26]. During the elaboration of the Convention, negotiators recognized that biotechnology could contribute to achieving these objectives, if developed and used with adequate safety measures for both the environment and human health. Accordingly, procedures were developed to address the safe transfer, handling, and use of any LMO (used interchangeable with GMO in this contribution) resulting from biotechnology that may have an adverse effect on the conservation and sustainable use of biological diversity (Article 19.3, CBD). These procedures formed the Cartagena Protocol on Biosafety (CPB; 4), which came into force in 2003 and has 160 signatory countries to date (October 2010). Parties lacking a cohesive biosafety policy undertook, or are currently undertaking, a number of initiatives to put a national framework in place in order to comply with the CPB.

This period of heightened political activity in environmental protection has coincided with a concomitant rise in GM crop cultivation. The number of countries opting to grow GM crops has increased steadily from 6 in 1996, the first year of commercialization, to 18 in 2003 and 25 in 2009 [27]. Among the top 10 GM crop-growing countries by area, the USA, Argentina, Canada, Uruguay, and Australia are currently not Parties to the CPB. At the same time, many developing countries that have ratified the CPB are still in the process of elaborating a regulatory framework governing the import or cultivation of GM crops. This has led to the current situation where different strategies and standards have been adopted at the national level, caused by the different infrastructures available in developed and developing countries, and has resulted in much confusion and difficulty in harmonizing environment and trade agreements and regulations.

The Cartagena Protocol on Biosafety

The CPB has been the primary driving force behind many countries establishing national biosafety regulatory systems for GM crops and animals. Attempts have been made under the CPB, as an international legally binding treaty, to set forth the scientific and legal boundaries for those systems, and establish a minimum set of rules and procedures to "ensure an adequate level of protection to avoid or minimize potential adverse effects on the conservation and sustainable use of biological diversity, taking into account human health". The CPB also sets minimum standards for regulating certain aspects concerning the safe transfer, handling, and use of LMOs [28].

The necessary set of techniques to produce GMOs has been defined not only by the CPB, but also by other relevant international treaties, guidelines, and standards, including the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology [29], and the International Standards for Phytosanitary Measures [30]. The application of modern biotechnology allows the intentional crossing of natural breeding barriers, the underlying molecular processes of which are qualified as sufficiently "new" so that they and the resulting organisms can be patented as inventions.

In many countries, the terms "genetically modified organism," "genetically engineered organism," and "transgenic organism" are widely used, including in domestic legislation, to describe LMOs covered by the CPB [31]. Different countries therefore have biosafety regulations to guide the development of GM crops. Such regulations are key to ensuring the environmental and human safety of GMOs and give the public confidence in GM products [28].

Although the CPB covers all LMOs, it primarily addresses two particular uses of LMOs: [1] those that will be intentionally introduced into the environment and [2] those used for food, feed, or processing (FFP).

For LMOs used for other purposes, such as LMOs used in the laboratory, the Protocol leaves any regulation to the discretion of the individual country. The CPB also does not cover products derived from LMOs, such as processed foods that have ingredients that came from LMOs.

To ensure the safe transfer, handling, and use of LMOs, the CPB sets up two separate procedures. The first time that an LMO is to be intentionally introduced into the environment, the CPB sets up an Advanced Informed Agreement (AIA) procedure (Article 7). This procedure requires that an exporter of an LMO provides a notice with detailed information about the LMO to the importing country (Article 8). The importing country then reviews the information, conducts a risk assessment, and decides, based on the risk assessment results, whether to approve or reject the LMO (Articles 10 and 15). In deciding whether to accept the LMO, the importing country can invoke risk management measures to address issues that arise from the risk assessment (Article 16). The importing country also can err on the side of precaution (discussed below) and not approve an LMO if there is insufficient information to adequately assess its particular potential risks (Article 10 [6]).

The second procedure set up by the CPB is for LMOs for FFP (such as maize, soya bean, wheat, or other grains that will be fed to humans or animals). For these LMOs, the AIA procedure is not required (Article 11). Instead, the CPB establishes a simpler system which reflects the decreased likelihood that these LMOs will affect the biodiversity of the exporting country. Before the LMO can be exported to another country, the safety decision in the exporting country is communicated to other countries through the Biosafety Clearing-House (BCH; http://bch.cbd.int/). A country may require prior consent, however, under its domestic regulatory framework, as long as that requirement has been posted on the Biosafety Clearing-House (Article 11).

The CPB also contains numerous other provisions that complement the review procedures for LMOs discussed above and address issues important to a uniform and comprehensive biosafety regulatory process. There are provisions on reviewing decisions for new information (Article 12), simplified procedures for certain LMOs that do not present risks (Article 13) and emergency procedures for unintentional releases of LMOs (Article 17). The CPB also addresses issues such as public awareness and participation (Article 23), and what to do about confidential information (Article 21). Thus, the Protocol attempts to establish a complete and comprehensive set of procedures and legal obligations to assess and manage the potential risks of LMOs on biological diversity, also taking into account risks to human health.

While proponents of modern biotechnology state that no new risks are associated with GMOs, others feel that the new methods of producing organisms might be associated with new risks. Premarket procedures have therefore been established by many regulatory authorities around the world, and are applied to assess how the organisms may behave and evolve in the environment, and how they may interact with other species. The CPB sets forth the information about an LMO that is needed before it is released into the environment or used for FFP. Annexes I and II contain detailed lists on the major categories of information needed to assess the potential risks of an LMO. They provide models which a national biosafety regulatory system can use as standard data requirements. Of course, individual countries may add to the list of required information, depending on particular environment issues within their country or if they choose to address other risk areas (such as food safety or socioeconomic concerns).

The CPB also attempts to explain what a scientific risk assessment of an LMO should entail. Annex III sets forth the objective of the risk assessment, what the risk assessment will be used for, the general principles that the risk assessment must follow, the methodology of the risk assessment, and particular points to consider when assessing the potential risks of an LMO. The Annex provides a clear explanation to interested parties about what is expected in the risk assessment, what will guide the risk assessment, and how it will be used. Therefore, the Protocol's Annexes attempts to provide sufficient information and details so that countries which adopt those provisions will establish harmonized and standardized procedures that will be transparent and understandable.

Precaution and the CPB

Based on the reaffirmation of the precautionary approach contained in Principle 15 of the Rio Declaration on Environment and Development (1992), Articles 10 (para. 6) and 11 (para. 8) of the CPB both state that

- "Lack of scientific certainty due to insufficient relevant scientific information and knowledge regarding the extent of the potential adverse effects of a living modified organism on the conservation and sustainable use of biological diversity in the Party of import, taking also into account risks to human health, shall not prevent that Party from taking a decision, as appropriate, with regard to the import of the living modified organism . . .. in order to avoid or minimize such potential adverse effects".

However, ever since the CPB came into force, government regulators and their technical experts, political activists, and GM product developers have debated this inclusion of the precautionary approach. Disagreements have raged over whether such an approach is a useful tool for managing the risks of biotechnology and its products. Resolving these disputes is made difficult by the lack of a formal, established definition, making it unclear exactly what it means in practical terms and what it requires of governments and innovators. Therefore, governments may act at their discretion to restrict or ban products or activities even before obtaining proof that a harm is imminent, although no obligation to do so seems to be implied in the CPB. This had led opponents of such actions to argue that conversely, such decisions can also jeopardize human health and the environment at large, for example, during the severe food shortage of 2002 in Southern Africa when food aid shipments containing transgenic maize were rejected on the basis of potential harm. Until clarity is forthcoming, this will remain fertile ground for dispute and open for possible abuse.

Process-Based Versus Product-Based Approach

In Europe, as in all Parties of the CPB, a *process-based* regulatory system governs the regulation of GMOs, as the techniques used for their production were considered new and raised specific safety concerns. A GMO is thus mainly characterized by the technique used to produce it, and is defined as an organism in which the genetic material has been altered in a way that does not occur naturally by crossing and/or natural recombination [2]. Directive 2001/18/EC, on the deliberate release of GMOs into the environment, provides a list of techniques in Annexes IA and IB that: (1) result in genetic modification; (2) are not considered to result in genetic modification; or that (3) result in genetic modification, but yield organisms that are excluded from the scope of the Directive [2, 32]. Production techniques falling under the EU GMO definition are:

- Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid, or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation
- Techniques involving the direct introduction into an organism of heritable material prepared outside the organism including microinjection, macroinjection, and microencapsulation
- Cell fusion (including protoplast fusion) or hybridization techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally

In vitro fertilization, natural transformation processes (e.g., conjugation, transduction, transformation), and polyploidy induction are not considered to result in genetic modification and are, therefore, currently excluded from the GMO definition. Techniques of genetic modification yielding organisms that currently are excluded from the GMO definition are mutagenesis and cell fusion of plant cells of organisms which can exchange genetic material through "traditional" production techniques [2].

In the USA and Canada, a *product-based* regulatory approach is followed for the regulation of GMOs [33–35]. Legislation focuses on the risks of products, and not the techniques of production, as genetic modification *per se* is not considered inherently risky. Because the focus is on novel traits or attributes introduced into a plant, rather than the method of

production, both plants and their derived food and feed products are regulated under the existing regulatory system.

Whether legal regimes using production techniques instead of the product itself as a trigger for regulatory oversight provide the best framework for an adequate safety assessment of GMOs is at best debatable. On the one hand, it is questionable whether newly developed crop improvement techniques will outgrow the current EU GMO legislation [36, 37]. On the other hand, it is not intuitively obvious why conventionally bred plants and their derived products are not subject to a similar safety assessment as those obtained through genetic modification [38, 39] or, conversely, why GM plants are regulated more strictly than conventionally bred ones in the EU [40, 41]. With techno-scientific advances and innovations, the knowledge about plant genes and their regulation and functions has increased, while new plant production techniques have emerged to induce or select desired plant characteristics, such as RNA interference and oligonucleotide-mediated mutagenesis (see, e.g., [32, 42–44]). These new techniques may challenge the current regulatory definition of a GMO, because it is not always clear whether the products obtained through these techniques would be captured by, or excluded from, the EU GMO definition [36]. The current EU regulation is therefore heavily reliant upon the need to regularly update the list of techniques and their possible uses; otherwise, it runs the risk of quickly becoming obsolete, as the rate of innovation and advances in the field of biotechnology marches on. In addition, the ability to detect products of such emerging technologies will be severely challenged, and as such, there will need to be a greater emphasis placed on traceability mechanisms for this approach to be practically regulated. In recognition of this, EU Member States and the European Commission are considering recent developments in plant breeding and are discussing if the current EU legalization appropriately covers these new techniques and their application, and whether they should be subject to regulatory requirements. Recently, in an answer to a parliamentary question, the European Commission stated that a specific working group of external experts has been put in place to determine which of the newly developed plant production techniques would result in genetic modification and would thus be captured by, or excluded from, the EU GMO definition (cf., Parliamentary question P-6606/07 2008).

Regulatory Framework for GMOs in the EU

The EU has the most stringent and wide-ranging regulations on GM products and commodities in the world. Their development can be traced back to the aforementioned food safety incidences in the mid-1990s that affected European consumer confidence in government regulatory agencies and agribusiness groups. These concerns have developed to include a negative view of GM products and of the companies that develop and market these products. Furthermore, the ongoing maintenance of trade barriers against agricultural imports in general has resulted in strong political pressure to regulate GM products [45].

The EU regulatory approach is precautionary, process-related, and includes mandatory labeling and traceability requirements for food and feed crops, unprocessed or processed. Only non-food GM products (unseeded), such as textile or other industrial products, are not subject to any requirement.

EU legislation is adopted through a system of interactions between the three main EU institutions: the European Parliament; the Council of the European Union (i.e., representatives of all the EU Member States at the ministerial level); and the European Commission. In most cases, the European Commission initiates legislative proposals that are decided jointly by the Council and the European Parliament; the most common legislative measures are Regulations (acts that are binding in their entirety and are immediately applicable throughout the EU) and Directives (acts that require the modification or establishment of national measures, generally for harmonization purposes). Legislation pertaining to GMOs in the EU is further elaborated below.

Contained Use and Deliberate Release Directives

In the early 1990s, two European Directives for the use of GMOs were adopted: to ensure the protection of human and animal health and the environment; to guarantee consumers' freedom of choice without

misleading consumers/users; and to create an internal market that makes the free movement of GMOs possible within the EU without unequal competition and impediments between and within Member States. Directive 90/219/EEC, which has since been amended by Directive 98/81/EEC, regulated the contained use of GM microorganisms, while Directive 90/220/EEC regulated the deliberate release of GMOs into the environment, covering both the release for research purposes (part B) and for commercial use as or in products (part C). This triad reflects the stepwise process GM crops go through, beginning with experiments under contained use (e.g., laboratory, greenhouse), through experimental release, up to the placing on the market. According to the step-by-step principle, the containment of GMOs can be reduced and the scale of release increased gradually, if assessment of earlier steps indicated that the next step can be taken.

Novel Food Regulation

On 15 May 1997, Regulation (EC) No 258/97 – the so-called *Novel Food Regulation* – removed food products derived from GM plants from the scope of Directive 90/220/EEC on the deliberate release of GMOs into the environment. The new regulation covered risk assessment procedures and the marketing and labeling of all types of novel foods, including those produced by new plant production techniques such as genetic engineering, as well as food without a history of safe use in the EU. Under the Novel Food Regulation, the safety assessment of GM food was based on the principle of *substantial equivalence* between the GM food and its traditionally cultivated non-GM counterpart. The non-GM counterpart was generally taken to have a history of safe use, allowing it to serve as baseline in the comparison of its chemical composition and phenotypic characteristics to those of GM food [46–48].

According to the *labeling provisions* of the Novel Food Regulation, labeling was not required when a GM raw material had been treated technically in such a way that neither the new DNA, nor the protein could be detected in the end product. Since May 1997, processed oil from GM oilseed rape, maize, and cotton, and processed food and food ingredients derived from GM maize have been notified as being substantially equivalent and thus approved for human consumption under the simplified procedure of the Novel Food Regulation. Moreover, labeling did not apply to food already used for human consumption in the EU prior to the establishment of the Novel Food Regulation. Food already marketed, such as GTS40-3-2 soya bean and Bt176 maize, were not considered as novel. With the adoption of Regulations (EC) No 1813/97 and 1139/98, the labeling of GTS40-3-2 soya bean and Bt176 maize foodstuffs also became compulsory. From that moment on, the label literally had to contain the words "produced from GM soya bean" or "produced from GM maize" when the new protein or transgene was detectable in the end product intended for consumption. A final product needed no label when a GM raw material had been technically treated in such a way that neither the new protein nor the transgene could be detected (e.g., hydrolyzed soya bean proteins, refined oils). With Regulation (EC) No 50/2000, the labeling provisions were extended to additives and flavorings that have been genetically modified or that have been produced from a GMO.

Revised Deliberate Release Directive

On 17 October 2002, Directive 2001/18/EC replaced (the older) Directive 90/220/EEC. With it (1) the precautionary principle was explicitly adopted as a guide; (2) risk assessment criteria were broadened to include direct, indirect, immediate, delayed, and cumulative long-term adverse effects; (3) post-market environmental monitoring (PMEM) became compulsory; (4) the need for a common methodology for the environmental risk assessment was established; (5) the requirement of reexamination of risk assessment and management conclusions in light of new scientific evidence was strengthened by limiting the duration of market consents to a maximum of ten years; (6) specific considerations related to the use of antibiotic resistance marker genes were introduced; (7) existing labeling provisions applying to GM food were extended to all marketed products containing GMOs; (8) the general concept of traceability at all stages of commercialization was introduced; (9) transparency in the decision making process was increased; (10) consultation of the public became mandatory in the approval procedure; (11) the possibility to consult an ethics committee was confirmed; and (12) the

implementation of national cultivation registers was required, recording the locations where GM plants have been grown for experimental purposes.

General Food Law and Establishment of the European Food Safety Authority

Adding to Directive 2001/18/EC, Regulation (EC) No 178/2002 – the so-called *General Food Law* – laid down general principles of food law and procedures in food and feed safety. It defines food and feed and other agricultural inputs at the level of primary production, as well as hazard, risk, risk analysis, risk assessment, risk management, and risk communication. Furthermore, the General Food Law sets food and feed safety requirements in order to determine whether any food or feed may be injurious to human and animal health. With this Regulation, the application of the precautionary principle was further extended to risk analysis of all food and feed in the EU, whether of GM-origin or not.

In response to a multiple wave of food crises that caused considerable public concern in Europe about food safety and the ability of regulatory authorities to fully protect consumers, the *European Food Safety Authority* (EFSA) was created as a European-wide risk assessment body. EFSA is tasked: (1) to provide science-based advice at the request of the European Commission on any matter within its mission, or in the framework of Community legislation; (2) to issue scientific advice on its own initiative; and (3) to issue advice upon request of the European Parliament or a Member State on matters falling within its mission (Article 29 of the General Food Law). By providing "independent, objective, and transparent" science-based advice, EFSA aims to ensure a high level of consumer protection and to restore and maintain confidence in the EU food supply.

EFSA has ten Scientific Panels addressing food safety issues in the different sectors of food and feed production, and a Scientific Committee. The EFSA Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel) consists of 21 scientific experts from European research institutes, universities, or risk assessment bodies. The EFSA GMO Panel issues: (1) *scientific opinions* on the safety of GMO market approval dossiers and on national safeguard clauses invoked by Member States; and (2) produces *guidelines* for the risk assessment of GMOs upon request of the European Commission in the framework of Directive 2001/18/EC and Regulation (EC) No 1829/2003. These guidelines provide assistance to those preparing and presenting GMO market applications, by describing principles, concepts, data requirements, and issues to be considered in the frame of risk assessment. So far, the following guidances have been issued:

- Guidance document for the risk assessment of GM plants and derived food and feed (issued in 2004; published in 2006; revised in 2008 and 2011)
- Guidance document for the risk assessment of GM microorganisms and their derived products intended for food and feed use (issued in 2006; revised in 2011)
- Guidance document for the renewal of authorizations of existing GMO products (issued in 2006)
- Guidance document for the risk assessment of GM plants containing stacked transformation events (issued in 2007; replaced by the latest revision of the Guidance document for the risk assessment of GM plants and derived food and feed, see above)
- Guidance for the risk assessment of GM plants used for non-food or non-feed purposes (issued in 2009)
- Guidance on the environmental risk assessment of GM plants (issued in 2010)
- Guidance on selection of comparators for the risk assessment of GM plants (issued in 2011)
- Guidance on the PMEM of GM plants (issued in 2011)

Scientific opinions and reports are also issued on specific risk assessment issues regarding food, feed, and environmental safety related to GMOs, such as: the safety of antibiotic resistance marker genes; the use of animal feeding trials for testing of whole GM food and feed; the statistical analysis of results of field trials; the evaluation of GM plants cultivated for non-food/feed purposes; PMEM; statistical considerations for the safety evaluation of GMOs; and the assessment of allergenicity of GM foods. A scientific opinion on the environmental risk assessment of non-target organisms exposed to GM plants was recently adopted, as well as a scientific opinion on the annual PMEM report on the cultivation of maize MON810 in 2009. The EFSA GMO Panel is continuously considering new scientific

information and recent developments in the field of GMO risk assessment, and as such, it has undertaken several initiatives to incorporate them into its operational procedures (see [49, 50] for further details).

GM Food and Feed Regulation

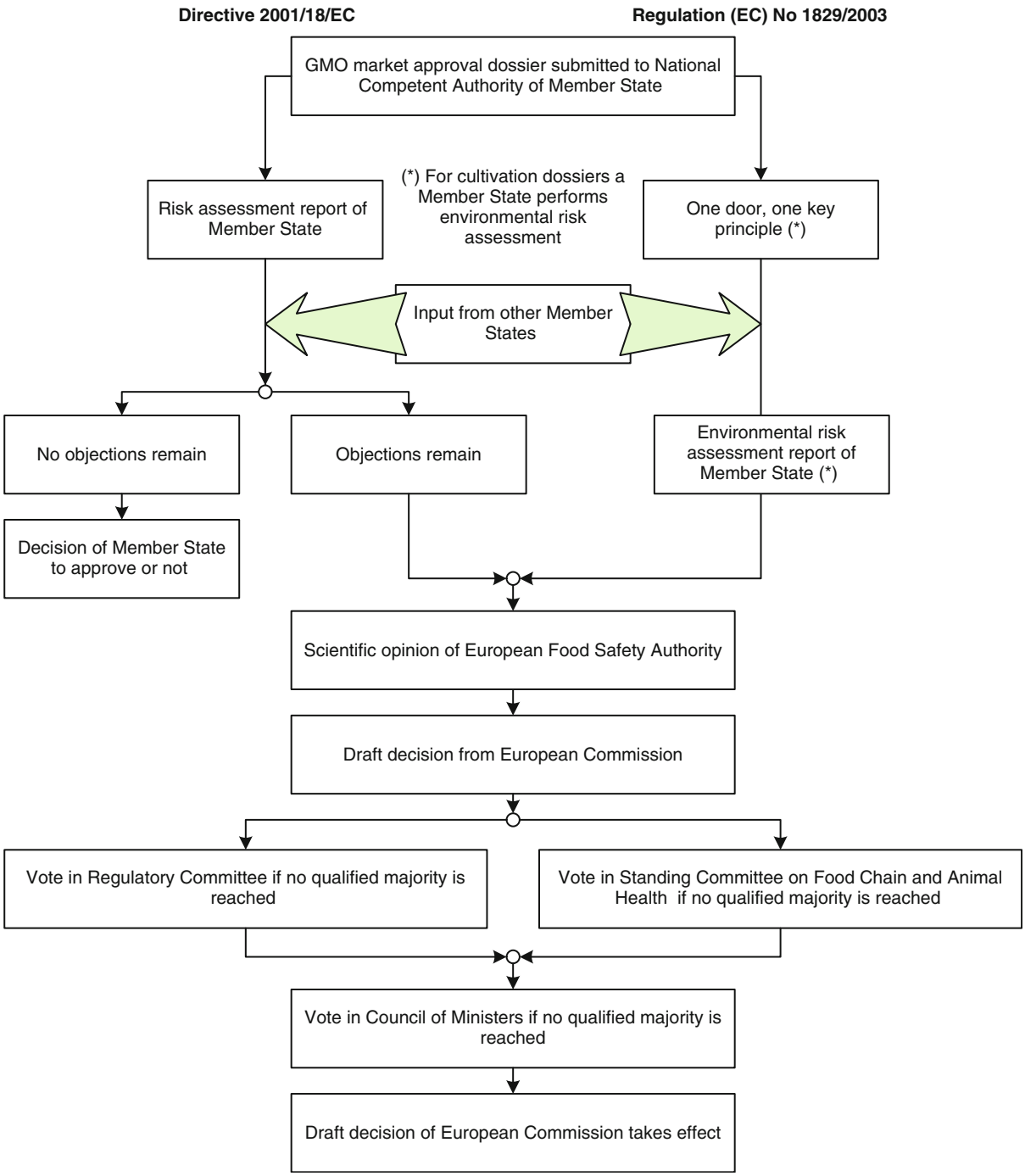
Issued on 18 April 2004, Regulation (EC) No 1829/2003 on GM food and feed covers the commercialization and risk assessment of GM food and feed, such as food/feed containing or consisting of, food/feed produced from, and food/feed containing ingredients produced from GMOs, as well as seed or other plant-propagating material. Prior to this date, approvals for human food use were required under the Novel Food Regulation, whereas feed use was assessed under Directive 2001/18/EC and its predecessor. The amended approval procedure is centralized around EFSA and based on a "*one door – one key*" approach whereby all commercial uses can be covered in the same GM plant market approval dossier. Moreover, it also introduces the need for a GM crop market application to cover both food and feed uses, as it avoids market approval for a single use in case a product is likely to be used for both purposes (e.g., [51]). In response to the heated debates that challenged the "principle of substantial equivalence," the principle was demoted to a "comparative analysis" in the GM Food and Feed Regulation. The safety assessment of GM food and feed remains based on a comparative analysis in which the non-GM counterpart serves as baseline. However, it requires more evidence of safety than before. Since the principle of "substantial equivalence" was intensely criticized by various actors, it was no longer interpreted as the endpoint of risk assessment, but rather as the starting point. Whether GM food is subject to further safety assessment depends on the identified similarities and differences between the GM food and its non-GM counterpart: (1) if substantial equivalence is established, the need for further testing is to be investigated on a case-by-case basis; (2) if substantial equivalence is established, except for a single or few specific traits of GM crops, further tests must be done focusing on these traits in order to assess their potential impact on human and animal health; and (3) if neither partial nor total substantial equivalence is established, the wholesomeness of the food product is to be assessed. In the same line of arguments, the simplified authorization procedure was abandoned under the GM Food and Feed Regulation.

Approval Procedures for placing GM crops on the Market

Procedures for approval of GM crops in the EU follow comitology rules that involve different actors such as the European Commission, EFSA, the Regulatory Committee of Member States representatives under Directive 2001/18/EC, the Standing Committee on the Food Chain and Animal Health (SCFCAH) under Regulation (EC) 1829/2003, and the Councils of Ministers. In principle, the delegation of powers to the European Commission along with the supervision of the European Commission's use of these powers through committees composed of Member States representatives is considered a convenient mechanism. It enables efficient decision making, engenders a close and cooperative working relationship between the European Commission and Member States, and maintains a degree of control over the process by Member States [20]. The voting system is based on the number of votes designated to each of the 27 Member States and is proportional to their percentage of the EU population. A qualified majority should be at least 74% of the total votes (255) *and* 14 Member States *and* 62% of the EU population; a blocking minority is at least 26% of the votes [91] *or* 14 Member States *or* >38% of the EU population [18].

Approved GM products can move freely throughout all EU Member States in conformity with any conditions set out in the approval, and enter into the public register of GM food and feed (Community Register: http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). Authorizations are valid throughout the EU, have a maximum duration of ten years, and can be renewed.

Approval Procedure Under Directive 2001/18/EC
Under Directive 2001/18/EC, the approval procedure for placing a GMO on the market involves all Member States, the European Commission, and possibly EFSA (see Fig. 1). The GM crop market application is first submitted to the National Competent Authority of a Member State. Upon receipt of the application, the National Competent Authority issues a risk



Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union. Figure 1
Approval procedure for placing GM crops on the EU market under Directive 2001/18/EC and Regulation (EC) No 1829/2003 (Figure adapted from EFSA (Parma, Italy) and GHK Consulting Ltd (London, UK))

assessment report that may be favorable or unfavorable regarding market approval of the GMO under consideration. In the event of a favorable opinion, the Member State informs the other Member States via the European Commission. The other Member States and the European Commission examine the risk assessment report and may pose observations and objections.

If there are no objections by other Member States or by the European Commission, the National Competent Authority that carried out the original risk assessment approves the placing on the market of the product, and issues a final approval permitting the marketing of the GMO within the Community.

If objections are raised, the procedure provides for a conciliation phase among the Member States, the European Commission, and the applicant in order to resolve the outstanding questions. In case objections are maintained at the end of the conciliation phase, a Decision is taken at EU level. The European Commission first asks the scientific opinion of the EFSA GMO Panel on the safety of the GMO in question. In the case of EFSA issuing a positive opinion, the European Commission presents a Draft Decision to the Regulatory Committee under Directive 2001/18/EC for an opinion. If the Regulatory Committee gives a favorable opinion by qualified majority, the European Commission adopts the Decision. If not, the Draft Decision is submitted to the Council of Ministers for adoption or rejection by qualified majority. If the Council does not act within three months, the European Commission adopts the draft Decision.

Approval Procedure Under the GM Food and Feed Regulation Under the GM food and feed Regulation, a GM crop market application is submitted to the National Competent Authority of a Member State, which forwards it to EFSA. If the application covers the use of seeds or other plant-propagating material for cultivation, EFSA asks a National Competent Authority under Directive 2001/18/EC to perform the environmental risk assessment that will be considered by EFSA during its final assessment (see e.g., [52]).

From the receipt of a valid GM crop market application, EFSA endeavors to deliver its scientific opinion within a time limit of six months. This time limit can be extended if EFSA or the Commission's Community Reference Laboratory seeks supplementary information from the applicant. Within three months after receiving EFSA's scientific opinion, the European Commission submits a Draft Decision to the SCFCAH. If SCFCAH gives a favorable opinion by qualified majority, the European Commission adopts the Decision. If not, the Draft Decision is submitted to the Council of Ministers for adoption or rejection by qualified majority. If the Council does not act within three months or does not obtain a qualifying majority for adoption or rejection of the Commission Draft Decision, the European Commission adopts the Decision (see Fig. 1).

Labeling and Traceability Regulation

Regulation (EC) No 1830/2003 on the labeling and traceability of GM food and feed complements, clarifies, and makes operational some of the labeling and traceability objectives of previous legislation. This Regulation extended labeling provisions to feed, seed, bulk products, food that are delivered as such to the final consumers or mass caterers, and to products in which GMO-derived DNA or protein (e.g., refined oils) is no longer detectable. These requirements go further than previously, because the use of genetic modification in itself is now sufficient to justify labeling. The label must be shown in a clearly visible, legible, and indelible manner, and must contain the reference "genetically modified," "produced from genetically modified" or "contains genetically modified." When a GM food or feed is different from its conventional counterpart, the label must also provide information about any characteristic or property that renders it different. When there is no conventional counterpart, the label must contain appropriate information about the nature and characteristics of the GM food or feed. Any characteristic or property that gives rise to ethical or even religious concerns also has to be mentioned. Products being produced with the help of GMOs – rather than actually made out of them – do not require labeling. As such, meat, eggs, milk, and dairy products from animals fed GM crops fall outside the remit of labeling provisions. Since substances assisting in food production, carrier substances, or culture media for microorganisms are not considered foods, their labeling is not considered necessary.

Labeling Thresholds

Tolerance thresholds for the unintentional or technically unavoidable presence of approved GM material in non-GM products were established in the EU. A tolerance threshold refers to the maximum admixture level for GMO content under which the comingled product does not have to be labeled as containing a GMO. It is often argued that there is no scientific justification for the established thresholds. Since GM crops and derived food and feed products are declared safe before marketing, thresholds do not relate to safety or health issues. However, thresholds reflect a balance between differently framed societal concerns and requests and technical capabilities. The translation of labeling thresholds in practice still entails enormous technical and scientific challenges.

Regulation (EC) No 49/2000 set the labeling threshold for the adventitious GMO presence in non-GM food at 1% of the food ingredient. The GM Food and Feed Regulation decreased the tolerance threshold to 0.9%, and extended it to feed and products intended for direct processing. There is zero tolerance in the EU concerning unapproved GM plant events, unless they had previously received a favorable scientific risk assessment for marketing from EFSA and a detection method is publicly available. In the latter case, a threshold of 0.5% may be applied transitionally. A threshold has yet to be defined for seeds as discussions have remained at an impasse. Possible thresholds that will be proposed for seeds will be established at levels such that the GMO content of 0.9% can be guaranteed in food, feed, or crops. Proposals made by the Scientific Committee on Plants in 2001 ranged between 0.3% for cross-pollinating crops, and 0.5% for self-pollinating and vegetatively propagated crops [53]. As no such thresholds have been established to date, any seed lot containing authorized GM seed for cultivation in the EU has to be labeled as containing GM material.

Organic growers principally aim at keeping their products free from any GM material. Regulation (EC) No 1804/1999 on organic production of agricultural products states that the use of GMOs is not compatible with the organic production method. The Regulation, however, foresees a *de minimis* tolerance threshold for the unavoidable presence of GM material in organic products. It was thus anticipated that organic producers would opt for a tolerance threshold ranging between the limit of quantification of a DNA analysis (0.1%) and the tolerance threshold for food and feed products (0.9%). In a press release published on 21 December 2005 (IP/05/1679), the European Commission emphasized that an organic product with an adventitious content of GM material below 0.9% could still be labeled as organic. On 12 June 2007, this point of view was confirmed at a meeting of the EU agriculture ministers, where political agreement was reached on a new Regulation on organic production and labeling (IP/07/807). However, in its recent recommendation on guidelines for the development of national coexistence measures to avoid the unintended presence of GMOs in conventional and organic crops [23], the European Commission allows Member States to aim at levels of unintended GMO presence that are *lower* than the 0.9% labeling threshold in certain cases. It stated that

- "The potential loss of income for organic and some conventional producers (e.g., certain food producers) may be due to the presence of GMO traces at levels lower than 0.9%. In those cases, and in the interest of protecting particular types of production, concerned Member States may define measures that aim at reaching levels of presence of GMOs in other crops lower than 0.9%".

In this respect, the European Commission referred to Member States that have developed national standards for different types of "GM-free labeling" [23]. Since the organic sector advocates that GM crops are not compatible with organic farming [54, 55], they will seek to establish the limit of DNA quantification analysis as the basis to determine the tolerance threshold in organic products.

Coexistence

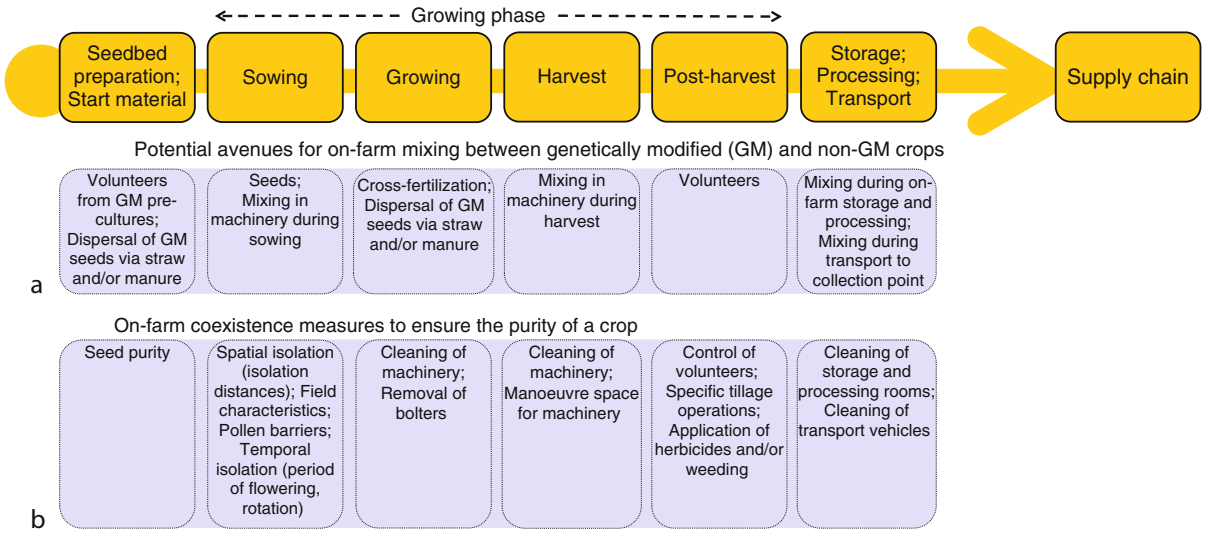
In order to provide a high degree of consumers' choice in the EU, a coexistence policy was adopted to maintain different agricultural production systems. It specifically aimed to enable the side-by-side development of different cropping systems without excluding any agricultural option. In this way, farmers would maintain their ability to make a practical choice between

conventional, organic, and GM crops. Since coexistence only applies to approved GM crops that were judged to be safe before their market entry [56], safety issues fall outside the remit of coexistence [57–59].

The European Commission recognized that completely avoiding the unintentional presence of GM material in non-GM products is difficult in the agricultural context [23, 60]. As agriculture is an open system, a certain amount of adventitious mixing is unavoidable. Various sources have been identified that could contribute to on-farm adventitious mixing between GM and non-GM crops (Fig. 2): (1) the use of impure seed [61, 62]; (2) cross-fertilization due to pollen flow between neighboring fields [63–65]; (3) the occurrence of volunteer plants originating from seeds and/or vegetative plant parts from previous GM crops [66–69]; (4) mixing of plant material in machinery during sowing, harvest, and/or postharvest operations [51]; and – to a lesser extent – (5) cross-fertilization from certain sexually compatible wild relatives and feral plants [70–72]. As completely avoiding admixing is difficult in the agricultural context, tolerance thresholds were established for the unintentional or technically unavoidable presence of approved GM material in non-GM products. If the content of GM material in a non-GM product exceeds the established tolerance threshold of 0.9%, the product has to be labeled as containing GM material, which may affect its market acceptability.

According to Article 43 of the GM Food and Feed Regulation, Member States are empowered to take appropriate measures to avoid the unintentional presence of GM material in other products. There are principally two strategies Member States have established, or are developing, to warrant coexistence of different cropping systems: *ex ante* regulations; and *ex post* liability schemes [23, 73–75]. Regulations are considered *ex ante* if they have to be followed by GM crop adopters while growing GM crops. *Ex ante* regulations prescribe preventive on-farm measures that should ensure that tolerance thresholds are not exceeded in neighboring non-GM agricultural production systems. Contrary to *ex ante* coexistence regulations, *ex post* liability schemes are backward looking: they cover questions of liability and the requirement to redress the incurred economic harm once adventitious mixing in a non-GM product has occurred after the cultivation of GM crops [59].

For decades, seed production regulations have specified statutory segregation measures (so-called identity preservation measures) between seed crops and conventional crop production of the same species to



Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union. Figure 2
(**a**) Potential avenues for on-farm adventitious mixing between GM and non-GM crops and (**b**) on-farm coexistence measures to ensure the purity of a crop during the production process (Figure reprinted from [59])

maximize varietal seed purity. Apart from seed production, experience with identity preservation systems is also available from the cultivation of different crop types grown for different uses [76]. Several of the proposed measures to ensure varietal seed and crop purity can be applied within the context of coexistence to limit the adventitious content of GM material in seeds and plant products. These measures include: (1) the use of certified seed; (2) spatially isolating fields of the same crop; (3) implementing pollen barriers around fields; (4) scheduling different sowing and flowering periods; (5) limiting carryover of GM volunteers into the following crop through the extension of cropping intervals; (6) cleaning agricultural machinery and transport vehicles of seed remnants; (7) controlling volunteers and wild relatives; (8) applying effective postharvest tillage operations; (9) retaining records of field history; and (10) the voluntary clustering of fields (Fig. 2). If Member States can demonstrate that the aforementioned preventive coexistence measures are not sufficient to achieve the desirable levels of purity, the European Commission gives those Member States the possibility "to exclude GMO cultivation from large areas of their territory" [23].

The European Coexistence Bureau (ECoB) recently issued a report on best practices to ensure the coexistence between maize cropping systems. Based on a comprehensive assessment of data generated in field experiments, commercial cultivation of GM maize, as well as modeling exercises under European environmental conditions, the report describes best management practices that can be put in place for each potential source of admixture [77]. It is important to realize that the level of containment needed to ensure coexistence will mainly be driven by the established tolerance thresholds: the lower the tolerance threshold, the stricter the on-farm measures needed to meet labeling requirements. Apart from defining the level of containment needed, tolerance thresholds also determine the level of GM material that initiates the need to redress economic harm due to adventitious mixing. The adventitious presence of GM material above the tolerance threshold set out in EU legislation triggers the need for the product that was intended to be a non-GM product, to be labeled as containing GMOs. In other words, the product has to be labeled as containing GMOs only in cases when the established threshold is exceeded. According to the European Commission,

- "The presence of GM material in food and feed has an economic effect only when it exceeds the 0.9% labelling thresholds", but as mentioned previously, "Member States can aim at levels of unintended GMO presence that are lowerthan the 0.9% labelling threshold in cases where the potential loss of income of organic and some conventional producers may be due to the presence of GMO traces at levels lower than 0.9%" [23].

In its Communication to the Council and the European Parliament from 2003, the European Commission has clearly emphasized that coexistence measures should not go beyond what is necessary to ensure that the unintentional presence of GM material in non-GM products remains below established labeling thresholds, and thus should be proportionate to the objective that is pursued. This point was reiterated in its 2010 coexistence recommendation [23], in which the European Commission stated that

- "Coexistence measures should avoid any necessary burden for farmers, seed producers, cooperatives and other operators associated with any production type". Moreover, it argued that "the choice of measures should take into account the regional and local constraints and characteristics, such as the shape and size of fields in a region, the fragmentation and geographical dispersion of fields belonging to individual farms and regional farm management practices".

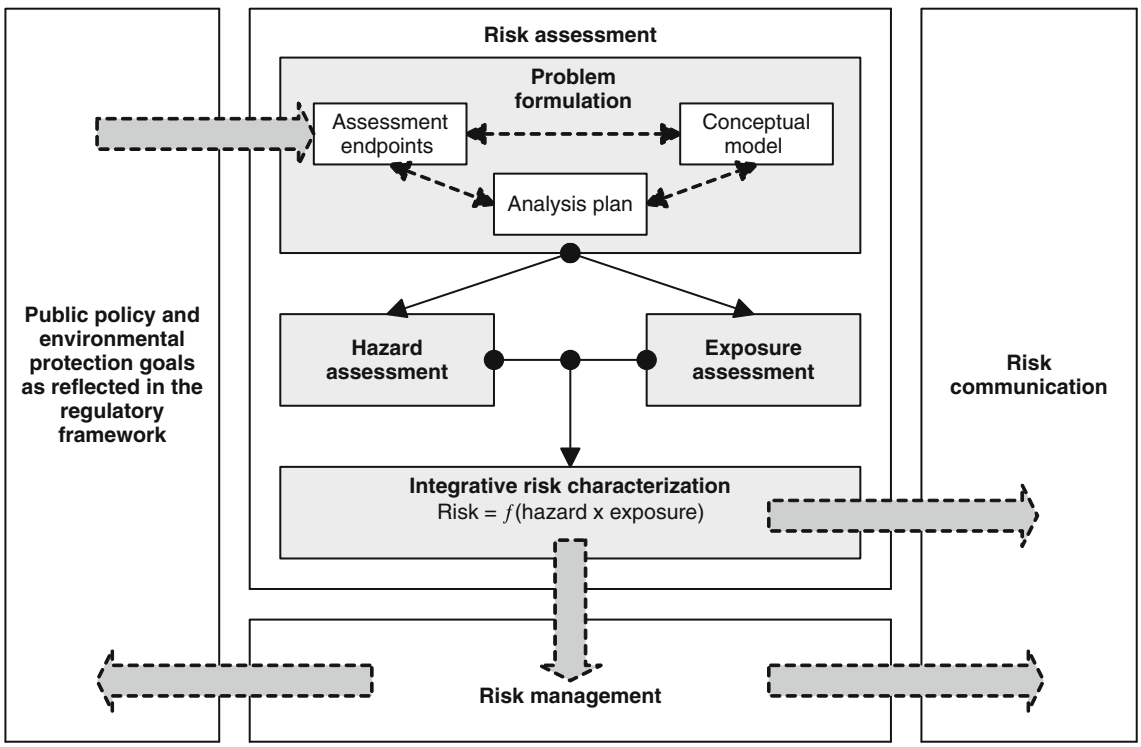
While some Member States have taken this advice into account for most conventional producers, others decided to propose or adopt measures that aim at keeping the amount of GM material present in conventional products as low as possible. In some cases, proposed measures, such as large and fixed isolation distances between fields of GM and conventional crops, appear to entail greater efforts for GM growers than necessary, which raises questions about the proportionality of these measures. Several authors have argued that wide and fixed isolation distances, as currently proposed by several Member States, fail to satisfy several challenges [58, 59, 78–85]. First, they are excessive from a scientific point of view; second, they are difficult to implement in practice; third, they are inconsistent with regional heterogeneity of farming; and fourth, they are not proportional to the economic incentives for coexistence. To enable regionally and

economically proportionate coexistence, policy makers should allow integrating flexibility in *ex ante* coexistence regulations. This might be achieved by allowing plural coexistence measures that are adaptable to local farming and cropping conditions, and that are negotiable amongst farmers. Computer-based decision support tools may thereby play a crucial role in the future case-by-case-based coexistence approach, as they allow the prediction of achievable levels of coexistence between neighboring maize fields under various conditions. One caveat here is that policy makers may be reluctant to adopt such a case-by-case coexistence approach because of the difficulties of implementation.

Risk Assessment Principles

Interplay of Risk Assessment, Risk Management, and Risk Communication

GMOs and their derived food and feed products are generally subject to a risk analysis before they can be commercialized [49, 86]. In the EU, the risk analysis consists of three components: risk assessment, risk management, and risk communication (Fig. 3). In risk assessment, potential adverse impacts associated with a specific activity are scientifically characterized on a case-by-case basis, while in risk management, policy alternatives to accept, minimize, or reduce the characterized risks are weighed and, if needed, appropriate prevention and control options are selected. Because risk managers and regulators rely on risk assessments to make an informed decision on whether or not to approve a certain use of a GM plant, it should explain clearly what assumptions have been made during the risk assessment, and what is the nature and magnitude of uncertainties associated with the characterized risks. The decision whether a certain risk is acceptable and/or tolerable under a particular set of conditions is not part of the risk assessment itself, but part of the wider risk analysis, as this choice is not only based on scientific criteria, but also involves political, social, cultural, and economic



Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union. Figure 3
Risk analysis: The diagram depicts the main components of risk analysis, together with the successive steps comprising the environmental risk assessment of GM plants (Figure reprinted from [50])

considerations. Risk management is also functionally and temporally separate from risk assessment in order to reduce any conflict of interest and to protect the scientific integrity of risk assessment [5]. Risk communication is defined as an interactive exchange of information and opinions on risk throughout risk analysis, between risk assessors, risk managers, and other interested parties. It includes the explanation of risk assessment findings and of the basis upon which risk management decisions are made [87].

Even though there are considerable differences in regulatory requirements for GM crops between countries, environmental priorities (including the preservation of biodiversity) as well as risk terminology, most risk assessments of GM crops follow a science-based assessment process that estimates the level of risk through comparison with a non-GM counterpart [49, 88]. In addition, regulatory requirements involve the consideration of a range of issues relevant to the overall risk assessment in order to determine the impact of the GM crop on human/animal health and the environment relative to the non-GM crop, and thus its relative safety [89, 90]. Some of these elements are discussed in the next sections.

Risk Assessment Methodology and Terminology

Despite the considerable variation among risk assessment frameworks for GM plants, risk assessment generally comprises several sequential steps: (1) problem formulation as a critical first step (including hazard identification); (2) hazard characterization, that examines potential hazards and their magnitude; (3) exposure characterization, that covers levels and likelihood of exposure; and (4) integrative risk characterization, in which the magnitude of consequences and the likelihood of occurrence are integrated (Fig. 3). In the EU, the guidance notes supplementing Annex II of Directive 2001/18/EC on the principles for the environmental risk assessment requires the definition of mitigation measures as a fifth step in the environmental risk assessment in order to manage identified risks. These mitigation measures aim to reduce the identified risks associated with GMO deployment to an acceptable level and should consider defined areas of uncertainty. Accordingly, any proposed mitigation measure is considered for the evaluation of the overall risk of GMO deployment.

Various single risk assessment studies have postulated dire risks when all they have done is characterized either a hazard associated with the use of GM crops, or an exposure to the GM crop without demonstrating whether this exposure is hazardous [5]. These studies confuse hazard or exposure with risk. In this way, they only allow speculative conclusions and contribute to uncertainties in the environmental risk assessment process. Moreover, it is important to distinguish risk from hazard, as the terms have different meanings. The term hazard is associated with the potential of an agent or situation to cause adverse effects or harm. It refers to an inherent property of that agent or situation. Risk is recognized as a function of the probability and severity (magnitude) of an adverse effect occurring to human and animal health or the environment, following exposure to a hazard under defined conditions. Without hazard, there can be no harm and thus no risk.

Problem Formulation (Including Scoping and Planning)

In order to identify the areas of greatest concern or uncertainty related to risks, each risk assessment begins with the identification and formulation of the problem, usually in the context of regulatory decision making [91]. In this respect, the most important questions to be solved and meriting detailed risk characterization are identified [92].

Problem formulation involves: (1) the identification of characteristics of the GM crop capable of causing potential adverse effects to the environment (hazards) together with (2) the identification of pathways of exposure through which the GM crop may adversely affect the environment. This process comprises a qualitative description of the nature of potential adverse effects and its potential triggers, as well as of plausible exposure pathways to the GM crop that might result in environmental harm. Problem formulation also involves: (3) the definition of assessment endpoints, which are explicit and unambiguous targets for protection extracted from legislation and public policy goals; and (4) outlining specific hypotheses to guide the generation and evaluation of data in the subsequent successive risk assessment steps. It involves the development of a methodology – through a conceptual model and analysis plan – that will help

to direct the risk characterization and to produce information that will be relevant for regulatory decision making [92–96]. In this way, problem formulation helps to make the risk assessment process comprehensive and transparent by summarizing existing scientific knowledge and explicitly stating the assumptions and principles underlying the risk assessment. Information considered in problem formulation, comprising existing knowledge of the GM crop and its interactions with the receiving environment, takes many forms. It includes published scientific literature, expert opinions, and research data. Data generated to characterize the GM crop during product development by applicants, and subsequently submitted to regulatory authorities as part of GM crop market application also provide an important source of information considered in the frame of problem formulation. Data to support problem formulation can derive from molecular, compositional, and agronomic/phenotypic analyses performed during GM crop development. Information of this type is frequently available in advance of the environmental risk assessment in order to characterize the GM crop [97–101]. If the level and quality of the available information is high, then the risk assessment can reduce the number of risk hypotheses that need to be tested for risk characterization [94, 95].

Hazard Identification: Characterization of the GM Crop Problem formulation starts with the precise characterization of the GM crop in order to identify potential hazards. This is usually achieved through a comparative safety assessment. A comparison of the characteristics of the GM plant with those of its non-GM counterpart enables the identification of similarities and differences between the two. It enables the characterization of intended differences that were the target of the genetic modification, as well as the identification of unintended differences that may lead to harm [97–101]. The identification of differences in problem formulation is considered of primary importance, because it will direct the subsequent course of actions in the risk assessment [92, 102]. While some differences may be deemed irrelevant to the assessment, others may be meaningful in terms of posing harm to the environment or to human and animal health. Should meaningful differences be identified, the risk that they pose must be evaluated, together with those of the intended modifications, such that an evaluation of their possible biological/ecological relevance can be made (requiring them to be directly linked to those aspects of the environment that are legally protected from harm – see below).

From Protection Goals to Assessment Endpoints A crucial step in problem formulation is to identify aspects of the environment (e.g., valued entity, ecosystem service/function) that need to be protected from harm according to protection goals set out in existing EU legislation, and to translate those into concrete measurable phenomena. Risk assessors need to know *what* to protect, *where* to protect it, and over *what time period*. Usually, aspects of the environment to be protected can be divided into two discrete but interconnected categories: the protection of biodiversity (biodiversity conservation), and the protection of functions/services provided by ecosystems. Ecosystem *services* are distinct from ecosystem *functions* by virtue of the fact that humans, rather than other species, benefit directly from these natural assets and processes. The benefits to humans are many and varied; however, those ecosystem services relevant in an agricultural context include food and feed production, pollination, pest regulation, decomposition of organic matter, soil nutrient cycling, soil structure and formation, water regulation and purification, and cultural services (such as aesthetic value and recreation). The precise relationship between biotic and abiotic processes and the ecosystem functions they drive is an area of considerable scientific debate. Some species contribute to ecosystem functioning in ways that are unique and hence their addition or loss from a community would cause detectable changes in functioning. Most ecosystems, however, exhibit functional redundancy, where several species are able to perform the same critical function. These species are at least partly substitutable and their loss can be compensated for by other species.

The challenge to problem formulation is that, in EU legislation, protection goals are general concepts that are defined in broad terms, which often are too vague to be scientifically useful in the frame of environmental risk assessments of GM crops. Moreover, a broad array and diversity of protection goals are mentioned in EU legislation. The resultant interpretation is that

everything should be protected, everywhere, all of the time. However, to be useful in terms of ecological risks and decision making, it is important that general and broadly defined protection goals are translated into concise and concrete measurable assessment endpoints. If the protection of biodiversity is a public policy goal, then a typical assessment endpoint can be the abundance and species richness of certain groups of organisms at a relevant life stage within a landscape or region over a specific time period [102]. Assessment endpoints represent aspects of the environment to be protected and that can be assessed: they are defined by the valued attribute and/or function (e.g., control of arthropod pest populations) of an ecological entity (e.g., arthropod natural enemies) that could be affected adversely by the GM crop and that requires protection from harm [103]. It is not an abstract goal such as ecosystem health or sustainability, but a real, operationally definable property of an aspect of the environment that reflects management or protection goals laid down in EU legislation. Because arthropod natural enemies fulfill relevant ecological services by contributing to the natural regulation of arthropod pest populations within crop fields, they can be identified as the entity to be preserved; similarly, the biological control functions they perform can be identified as the attribute/function (e.g., [104]). It is important that assessment endpoints are defined as far as possible using measurable criteria relevant to the case under study, such that any change in these endpoints can be readily identified. Moreover, assessment endpoints should ideally be selected based on: relevancy to the protection goal; ecological relevance; susceptibility to the potential stressor; and practicality [105].

Once assessment endpoints have been set, the level of environmental protection to be maintained, or the environmental quality to be preserved, needs to be defined. This process includes establishing the *harmful effect* and both the *spatial* and the *temporal scales* relevant for the ecological entity and its attribute/function to be preserved (Table 1). The harmful effect describes to what extent the environmental quality should be preserved (or above what threshold a difference between the GM crop and its appropriate comparator may lead to harm and would be considered a disturbance in environmental quality). For the conservation of biodiversity, a relevant decrease in abundance of protected or valued species can be seen as a harmful change. Similarly, for ecosystem services, a relevant disturbance in ecological function can be seen as a harmful change [106]. The spatial and temporal scales are the habitats in which, and the period during which, environmental quality should be preserved, respectively [104, 107].

Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union. Table 1 Criteria to operationalise protection goals[a]

Criterion	Measurable variable
Ecological entity	Species of conservation concern; ecosystem service
Attribute	Abundance; ecological function
Unit of protection	Individual; population; community; functional group; assemblage; guild
Harmful effect	Relevant decrease in abundance; relevant disturbance in ecological function
Spatial scale of effect	Field; field margin; other agricultural land; non-agricultural habitats; landscape
Temporal scale of effect	Generations; days; weeks; months; seasons; years

[a]Table adapted from the stakeholders' workshop "Protection goals for environmental risk assessment of pesticides: what and where to protect?" organized by the EFSA Panel on Plant Protection Products and their Residues (PPR) (http://www.efsa.europa.eu/en/scdocs/scdoc/1672.htm); see also [108, 134]

Conceptual Model: Exposure Profiles and Hypotheses The conceptual model describes the consequential exposure scenario of how harm to the assessment endpoint may arise from GM crop deployment and allows for a characterization of risks. Key relationships are described: between the GM crop and the valued entity to be protected from harm; pathways of exposure through which the GM crop may affect the valued entity either directly or indirectly (= exposure profile); and any potential impact of the GM crop to the environment [92, 96]. The conceptual model includes available information on: the nature of the stressor; its intended uses; reasonable exposure profiles; and potential responses of the

assessment endpoint as a result of exposure. It can take an array of forms, from the simplest of statements to complex flowcharts and diagrams.

Environmental pathways and levels of exposure will vary depending upon the intended uses of a GM crop, such as import, processing, food, feed, and/or cultivation. In the case where the use of GM plants does not include cultivation in the EU, problem formulation will consider exposure: (1) via accidental release of propagules, such as seeds of the imported commodity spilled into the environment during transportation and processing, potentially leading to sporadic feral GM plants; (2) indirect exposure through manure and feces from the gastrointestinal tracts of animals mainly fed the GM crop; and/or (3) manure or organic plant matter either imported as a fertilizer or soil amendment or derived from other byproducts of industrial processes. In the case where the GM crop use includes cultivation in the EU, problem formulation will consider exposure resulting from the expected cultivation in the receiving environment.

A well-structured conceptual model in which the components of the system are detailed will allow the identification and formulation of relevant hypotheses that arise from the consideration of potentially significant risks. These hypotheses are necessary to make assumptions and predictions about how a stressor or identified difference in the GM plant could affect and pose harm to an assessment endpoint [94, 95]. Importantly, within the analysis phase, hypotheses amenable to testing and corroboration are defined [92] which further guide the methodological approach taken to evaluate the magnitude of harm [92, 102].

Analysis Plan: Statistical Considerations and Experimental Design The last step of problem formulation comprises an analysis plan in which decisions are made about the most appropriate way to measure the response of each assessment endpoint to GM crop deployment. In this planning phase, approaches are delineated for the generation and evaluation of requisite data, in order to test hypotheses formulated in the conceptual model. Reasonable scenarios are placed in an analysis plan by describing and selecting: (1) the various measures to be used (measurement endpoints) in the assessment and subsequent risk characterization; and (2) the description of methods and criteria for measurement.

The measurement endpoints, derived directly from the assessment endpoints, define the indicator of change that will actually be recorded as part of a comparative risk assessment study [107], and usually constitute estimates of *exposure* or *hazard*. Measures of exposure cover properties of the GM crop and are described in terms of the route, frequency, duration, and intensity of exposure relative to the valued entity [92], while measures of hazard represent the measurable change to the valued entity in response to a changed attribute (e.g., transgenic protein) of the GM crop to which it is exposed [107]. Measures of hazard may be an acute lethal concentration resulting in the death of 50% of the organisms tested (LC_{50}), or a chronic *no observable adverse effect level* (NOAEL) measured for the valued entity [92]. How exposure measurement relates to the hazard measurement is described in the risk formulation.

Once specific measurement endpoints are chosen and given a priority, appropriate methods and criteria of measurement are selected and described in the analysis plan [93]. This includes information on: studies to be conducted; the appropriate tier for analysis; the design of protocols; and statistical power [96, 102, 106, 107, 109, 110]. The selection and prioritizing of measures to be used and testing needed enable the allocation of appropriate human and financial resources [111], so that only essential data for risk characterization are collected [94].

It is important to realize that for practical reasons not all potentially exposed non-target organisms can be tested for regulatory purposes [102]. Therefore, it is necessary to select an appropriate subset of species that can be tested effectively under laboratory conditions [102], or that are available in sufficient numbers in the field to give statistically meaningful results [96, 112–115]. This selection of species is based on several criteria, comprising: ecological relevance of the species; the likely exposure of the species to the GM crop; species susceptibility to known or potential stressors; the anthropocentric value of the species; and species testability [96, 102, 113, 116]. The environmental risk assessment may also consider species with special aesthetic or cultural values, or species of conservation importance and that are classified as threatened or endangered, although these are unlikely to be tested directly but substituted by a surrogate species in tests. The number

and type of species that are to be tested will depend upon the hypotheses generated during the conceptual model.

Once assessment and measurement endpoints have been set and approaches have been delineated for the generation and evaluation of requisite data to test hypotheses formulated during problem formulation, the level of environmental change that constitutes harm is to be set through *limits of concern*. These limits of concern define the minimum relevant ecological effect that is deemed of sufficient magnitude to cause harm, and is therefore biologically significant. Limits of concern are directly related to the type of studies that are to be performed either in the laboratory or in the (semi)-field. For laboratory studies, limits of concern are conservative trigger values which, if exceeded, will indicate potential risks and the need for exposure assessments and determination of field scale effects. For field studies, the lower limit, which corresponds for example to a decrease in the abundance of a particular species in the presence of the GM plant relative to that for its appropriate comparator, will usually be defined by the threshold effect deemed to be of just sufficient magnitude to cause environmental harm [106]. Knowing in advance what effect size is to be determined is crucial, as this information will enable to design the study in a way that it has sufficient statistical power to detect the anticipated effect. Limits of concern are estimated from literature data, modelling, and existing knowledge, and may be based on political, social, cultural and economic considerations. To define the minimum relevant ecological effect that is deemed biologically significant and that is deemed of sufficient magnitude to cause harm, it is important that (sets of) limits of concern for each assessment endpoint are set. Usually, the lower limit, which corresponds for example to a decrease in the abundance of a particular species in the presence of the GM crop relative to that for the non-GM counterpart, will be defined by the threshold effect that was deemed to be of just sufficient magnitude to cause environmental harm [106]. If these limits are exceeded, then detailed quantitative modeling of exposure may be required to scale up effects at the field level, both temporally and spatially. For studies in environment(s) that are controlled, the limits of concern will usually be trigger values which, if exceeded, will either lead to conclusions on risks or the need for further assessment in field [106]. Limits of concern can be defined by, for example, literature data, modeling, existing knowledge and policy goals. Ideally, these limits should be set by risk managers, as they will describe the extent of impact tolerated on a protection goal resulting from GM crop market approvals.

Conclusion Problem formulation is the crucial starting point for risk assessments, as it enables a structured, logical approach to detecting potential risks. Having a properly constructed analysis plan based on a conceptual model that is clearly linked to assessment endpoints helps to guide the collection of data that are relevant to investigating the possible safety of a GM crop. Moreover, it helps the risk assessment process to be comprehensive (by summarizing existing knowledge of the system under study) and transparent (by explicitly stating significant assumptions underlying the risk assessment, and ultimately regulatory decision making). In contrast, poor problem formulation can lead to the collection of data that are either unnecessary, superfluous, or irrelevant, diverting time and efforts from the more serious of the identified risks, thereby slowing down the procedure and increasing associated costs [90, 92, 94, 95, 117].

Risk Assessment Concepts and Approaches

Several concepts and approaches are considered during the risk assessment of GM crops. Risk assessment of GM crops: (1) is science-based, where quantitative information is available, and uses qualitative information in the form of expert judgment; (2) uses a comparative approach; (3) is case specific; (4) is iterative and, in a transparent manner, examines previous conclusions in light of new information; and (5) follows a tiered approach.

Science-Based Assessment

The evaluation of potential adverse effects is based on scientific and technical data, and on common methodology for the identification, gathering, and interpretation of relevant data.

Comparative Safety Assessment and Familiarity Concept

The risk assessment strategy for GM crops seeks to use appropriate methods to compare a GM crop

with its non-GM counterpart. The importance of risks posed by a GM crop is placed in the context of risks posed by its non-GM counterpart. A two-step approach is followed, starting with the identification of possible differences between the GM and non-GM counterpart (= proof of difference); which is then followed by the assessment of the environmental and food/feed consequences of any identified differences (= proof of equivalence) (see [106] for further details). The proof of difference approach verifies whether the GM crop is different from its non-GM counterpart, and may lead to the characterization of a potential risk depending on the type of the identified difference, and the extent and pattern of its exposure. The proof of equivalence approach aims to verify whether the GM crop is equivalent to its non-GM counterpart within bounds defined by so-called limits of concern. The two approaches are complementary: statistically significant differences may point to biological changes caused by the genetic modification, but these may or may not be relevant from the viewpoint of environmental harm [106, 118].

For the *food/feed safety assessment*, the equivalence test involves a comparison of the GM crop with a selection of non-GM counterparts, such as commercial varieties, aiming to identify any values of the GM crop that lie within the range of values from natural variation (i.e., observed among the set of non-GM counterparts included in the trial). The underlying assumption is that traditionally bred plants have a history of safe use for the consumer or animals and the environment, and familiarity for the consumer. For the *environmental risk assessment*, conventional cropping systems are not characterized as having a history of safe use, therefore, a near-isogenic conventional counterpart is used to identify any differences with the GM crop which might result in environmental harm. However, in any agricultural system, environmental impact critically depends upon crop management practices, such as the application of pesticides, environmental stewardship, and any mitigation measures adopted. Hence, the characterization of management regimes is an integral part of the definition of whatever comparator is chosen for the environmental risk assessment of GM crops [106].

The concept of familiarity is based on the fact that most GM crops are developed from more traditionally bred crops, the biology of which is well known. The knowledge about the non-GM crop, gained through experience over time, can therefore be used in a risk assessment to establish differences associated with the genetic modification and the subsequent management of the GM crop. According to the Organisation for Economic Co-operation and Development (OECD), familiarity is derived from the knowledge and experience available from conducting a risk analysis prior to the scale-up of any new crop variety in a particular environment, and from previous GM crop market applications for similar constructs and traits in similar or different crops [119]. However, it is important to bear in mind that familiarity is not an endpoint in risk assessment and does not necessarily equate to safety. If differences between the GM crop and its appropriate comparator have been identified, it needs to be determined whether these differences have any significance for the assessment endpoint under consideration.

The comparative safety assessment is usually based on data from three different sources: molecular, compositional, and agronomic/phenotypic analyses. The molecular characterization includes a precise characterization of the inserted DNA, information on which genes are expressed in the GM crop, and evidence that no detectable unintended effects have occurred because of the insertion [86]. Agronomic, phenotypic, and compositional data collected from field trials carried out in a range of agricultural environments that are typical of the place where the crop is grown will highlight whether the GM crop is equivalent in its functional properties to the non-GM counterpart and, in particular, how stable they are from year to year and across environments.

Molecular Characterization In an environmental risk assessment, molecular characterization data can be used to test the hypothesis that the inserted DNA does not disrupt endogenous plant genes, or trigger the production of new proteins/metabolites (other than the intended ones). If any endogenous plant genes were disrupted, rearranged, or overexpressed, these differences would be considered as unintended effects of the genetic modification, and would be further

assessed to determine the biological significance thereof. A variety of data and information is therefore necessary as no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health or environmental impact. Specifically, detailed information is usually required on: the source of the donor DNA; the transformation method; the organization of the inserted DNA at the insertion site(s); and on the expression and stability of the insert. Information concerning vector construction and the transformation method employed, as well as on all known gene regulatory elements and coding sequences is also relevant. Many authorities require the modification system to be mapped to a degree consistent with available technology, usually to the level of base sequence. An evaluation of whether the transgenes behave similarly to endogenous plant genes in their stability and inheritance between generations is also carried out. Hence, a risk assessment would have to be conducted to determine whether any detected unintended effects could result in potential adverse effects on human or animal health and the environment [100, 101]. Notably, insertional effects (intended or otherwise) are related to the specific transformation event, and not to the function of the inserted transgene; therefore, as with random mutation, most unintended changes caused by deliberate insertions of genetic material will be, at best, neutral to the plant. It is extremely rare for a significant improvement in ecological fitness or substantial compositional changes to result [120].

Compositional Analysis Compositional analyses enable the determination of any differences in key components as a result of the genetic modification, and to test the hypothesis that no difference in key component concentrations exists between the GM crop and its non-GM counterpart. Compositional data are generated in field trials carried out in several locations throughout the intended area of cultivation of the crop. In these field trials, the GM crop is grown alongside its appropriate comparator, and samples are taken. Parameters are selected that are typical for the crop that is assessed, and that are representative of the main metabolic pathways. Significant changes in these parameters are expected to be indicative of more fundamental changes in the crop that requires further evaluation [121]. The lack of statistically significant differences in the concentration of multiple analytes is a strong indication that the genetic modification has not introduced new constituents, nor harmful unintended changes. However, should statistically significant differences be detected, a harmful change is not necessarily indicated: should the different concentration of a specific analyte in the GM crop be within the range known for the crop, then it can be considered biologically insignificant, otherwise they will constitute "unintended identified" differences that require consideration in the risk assessment [100, 101].

Agronomic and Phenotypic Characterization Information on agronomic and phenotypic characteristics is obtained from multi-location agronomic field trials representative of different environments where the GM crop may be grown. To assess the agronomic performance of the GM crop, a sound understanding of the biology of the crop is required, as well as its relationship with the environment in which it is to be released. A variety of plant characteristics, such as plant vigor, growth habit, yield, crop quality, insect and disease susceptibility, fertility, dispersal mechanisms, and endogenous toxins, are recorded and any differences in the GM crop identified, which could potentially cause it to become a weed of agricultural or natural habitats, or otherwise interact differently than the non-GM counterpart in the environment. If there are no significant differences in characteristics associated with survival, growth, and reproduction, then it is likely that the genetic modification did not alter the persistence, invasiveness or gene flow potential of the GM crop. If significant differences are identified, the values obtained in the GM crop will be assessed for their biological relevance by comparing them with the range of values known for (non-GM) commercial varieties [98–101].

Case-by-Case Principle

No two environmental risk assessments are the same, as each is dependent upon a range of variables found when considering: both the source and target environments; the biological and ecological characteristics of the GM crop; the scale and frequency of the proposed deliberate release; and the interactions

amongst them [97, 122]. It is obvious that each range of variables will also differ between risk assessments, and thus a case-by-case approach is taken. In this way, the required information for the environmental risk assessment may vary depending on: the plant species under consideration; its intended use; and potential receiving environments, taking into account specific cultivation requirements and the presence of GM crops already in the environment.

Iterative and Adaptive

It is recognized that an environmental risk assessment is framed within the scientific knowledge available at the time it is conducted, and that regulatory decisions are made in this context. The environmental risk assessment has to take into account uncertainty at various levels, and which may arise from: limitations in the data (e.g., limited exposure data); gaps in the effect database; the limitation of the test systems and measurement endpoints selected; inadequacy of study designs; and the uncertainties in extrapolating between species. Scientific uncertainty may also arise from differing interpretations of existing data or the lack of some relevant data. Uncertainty may relate to qualitative or quantitative elements of the analysis [106]. Although it may be impossible to identify all the uncertainties present, the assessment should include a description of the types of uncertainties encountered and considered during the different risk assessment steps. Their relative importance and influence on the assessment outcome should also be described. Under current EU legislation, a high precision in environmental risk assessments becomes near impossible to achieve, as the identification of any areas of uncertainty which relate to areas outside current knowledge and the limited scope of the assessment is a mandatory requirement, for example, the impact of large-scale exposure of different environments from GM crop cultivation, the impact of exposure over long periods of time, and any cumulative long-term effects.

PMEM, which became mandatory under current EU legislation, aims to identify possible unanticipated adverse effects on human health or the environment which could arise directly or indirectly from GM crops. It also allows for the collection of additional data during the cultivation phase. The scientific knowledge obtained during the monitoring of GM crops, along with experiences gained from their cultivation and any other new knowledge (generated through biosafety research), provides valuable information to risk assessors to update environmental risk assessments and reduce any remaining uncertainties. In the EU, the objectives of a monitoring plan according to Annex VII of Directive 2001/18/EC are: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the environmental risk assessment are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human or animal health or the environment which were not anticipated in the environmental risk assessment. A PMEM plan of GM crops is mandatory in all market approval dossiers submitted under Directive 2001/18/EC and the GM Food and Feed Regulation.

In the EU, PMEM is composed of *case-specific monitoring* and *general surveillance*. Case-specific monitoring is not obligatory, but may be required to verify risk assessment assumptions and conclusions, whereas a general surveillance plan, as part of a GM crop market application, is a legal obligation. Due to different objectives between case-specific monitoring and general surveillance, their underlying concepts differ [123]. Case-specific monitoring enables the determination of whether, and to what extent, anticipated adverse effects occur during GM crop deployment, and thus to relate observed changes to specific causes. It is mainly triggered by scientific uncertainties that were identified in the environmental risk assessment. Therefore, a hypothesis is established that can be tested on the basis of newly collected monitoring data ("bottom-up approach"). In general surveillance, by contrast, the general status of the environment that is associated with the GM crop deployment is monitored without any preconceived hypothesis in order to detect any possible effects that were not anticipated in the environmental risk assessment, or that are long term and cumulative. Should any such effects be observed, they are studied in more detail to determine whether the effect is adverse and whether it is associated with the use of a GM crop [87]. General surveillance data can originate from various sources: (1) farm questionnaires; (2) existing surveillance networks (such as

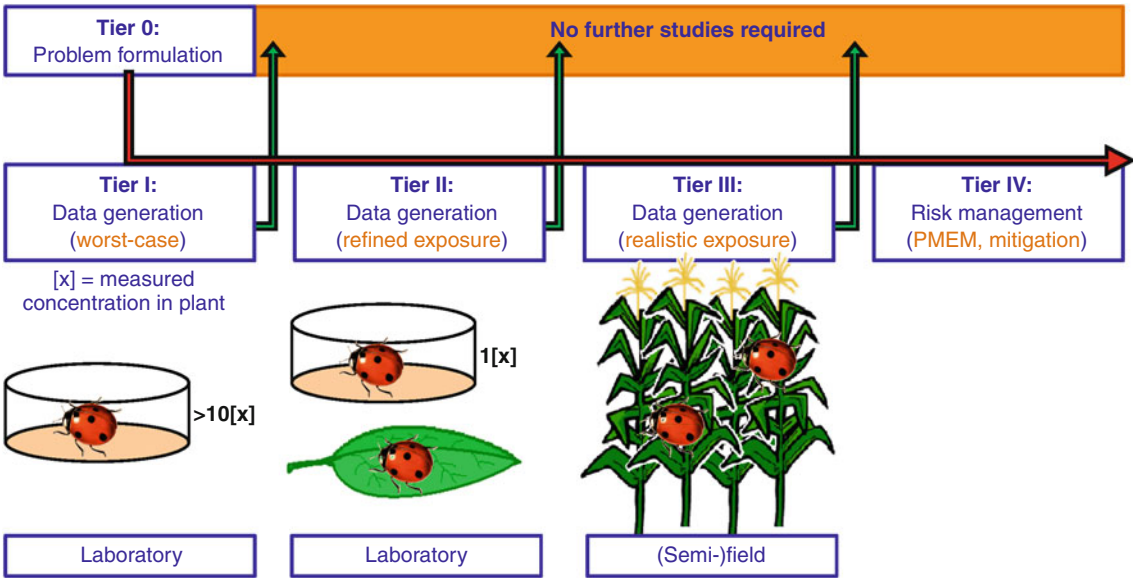
plant health surveys, soil surveys, ecological and environmental observations); (3) scientific literature; (4) industry stewardship programs; and (5) alert issues. Questionnaires for farmers form a useful part of general surveillance, as this tool enables the reporting of any observations of effects linked with GM crop cultivation: farm questionnaires use first-hand observations and rely on farmers' knowledge and experience of their local agricultural environments, comparative crop performance and other factors that may influence events on their land [124]. With this tool, monitoring focuses mainly on the cultivation area of GM crops and its surroundings, which is relevant to protection goals such as sustainable agriculture, soil function, or plant health. Aspects of biodiversity, however, are only addressed indirectly (such as the adoption of conservation/no-till practices, rotation regimes, biological control failures) and may not be sufficiently resolved [125]. Therefore, additional sources of information should be considered in the frame of general surveillance. Moreover, farm questionnaires should include different combinations and mixtures of GM crops that may be grown in sequence or rotation in fields [126]. Directive 2001/18/EC proposes to make use of established routine surveillance networks. EU Member States have various networks in place – some of which have a long history of data collection – that may be helpful in the context of general surveillance of GM crops. The networks involved in routine surveillance offer recognized expertise in a specific domain and have the tools to capture information on important environmental aspects over a large geographical area. However, networks fully meeting all the needs of GM crop monitoring are limited: most do not always provide data of relevance to monitoring the impacts of GM crop cultivation [125, 127]. Concern has been raised, predominantly outside of the EU, however, that in the absence of a valid hypothesis, PMEM for undefined hypothetical adverse effects from a GM crop is not feasible, and adds nothing to the premarket testing results, while potentially undermining confidence in the overall safety assessment process [128].

Risk assessments undertaken in the EU are always iterative, in the sense that regulatory decisions are temporary, reversible, and adaptable in light of new information that becomes available. Under Directive 2001/18/EC, the requirement of reexamination has been strengthened by limiting the duration of any market consent to a maximum period of ten years.

Tiered Approach

An environmental risk assessment is generally conducted in a tiered manner, where information collected in lower tiers directs the extent and nature of any experimentation conducted in higher tiers (Fig. 4). Thereby, hazards are evaluated within different tiers that progress from worst-case scenario conditions, framed in highly controlled laboratory environments, to more realistic conditions in the field [87, 97, 102, 122, 129–131]. Usually, three tiers is the norm, comprising of experimental tests under controlled conditions (tier 1: laboratory tests where test organisms are exposed to a high level of the newly expressed protein as pure protein; tier 2: laboratory tests where test organisms are exposed to refined, more realistic, levels of the newly expressed protein as pure protein or plant material); and (semi-)field tests (tier 3). Within each tier, all relevant data are gathered in order to determine whether there is sufficient information to conclude the risk assessment at that tier. The conclusion can only be made if any residual uncertainty has been defined, otherwise additional investigations to generate further data at a higher tier (s) are designed and undertaken to do so [102]. In the case that no reliable risk conclusions can be drawn, decision making can consider whether risk management measures (tier 4) should be put in place to reduce the overall risk. It is important that throughout the assessment, the problem being addressed (tier 0) remains appropriate and is revised, if necessary.

Lower-tier tests serve to identify and test potential hazards under worst-case scenario conditions and thus involve conservative assumptions, acknowledging that the likelihood of detecting any potential adverse effects of the GM crop or its products on target and nontarget biota increases directly with increasing (usually excessive) levels of exposure. These studies are conducted under controlled laboratory or growth room conditions in order to: quantify effects in relation to known exposure levels; to provide high levels of replication and control; and to increase the statistical power for testing the established hypotheses. Effects of the GM crop on indirectly exposed organisms, that is, those



Transgenic Crops, Risk Assessment and Regulatory Framework in the European Union. Figure 4
Tiered approach to non-target organism testing (Figure adapted from [97])

that are one or two steps removed in the food chain (e.g., predators and parasites of primary phytophagous or plant pathogenic organisms), are generally assessed in the second tier, which is also generally conducted under controlled laboratory, growth room or glasshouse conditions in order to measure effects in relation to known exposure levels [87]. If no hazards are identified and the GM crop is not different from the appropriate comparator, the tested product may be regarded as safe, and no further testing at a higher tier deemed necessary.

However, should potential hazards be detected in early-tier tests or if unacceptable uncertainties concerning possible hazards remain, additional information is required to confirm whether the observed effect might still be detected at more realistic rates and routes of exposure [87, 97, 102, 131]. Progression to larger-scale experiments in higher tiers aims to provide increasingly refined estimates of exposure. Field trials are then established in which the cultivation of the GM crop is conducted with greater environmental realism and thus relevance. As such, actual levels of exposure of different biota can be quantified. In comparison with the appropriate comparator and its management, likely ecological adverse effects due to the GM plant and its management can be determined. While higher-tier studies offer greater environmental relevance, they may have lower statistical power due to the higher variability of environmental conditions (e.g., climate) that can mask effects generated by the GM crop or its product [131]. In exceptional cases, higher-tier studies may be conducted at an initial stage when early-tier tests are not possible or meaningful. As such, many risk assessments are conducted in a tiered manner, meaning that risk assessment studies increase in complexity depending upon the findings at each level of assessment [91]. In cases where uncertainty about the risk remains after higher-tier studies, one can always return to lower tiers to conduct additional studies [102].

The tiered approach is consistent with the iterative or adaptive nature of risk assessment where conclusions are reviewed when new information is obtained. Uncertainty in risk assessment is reduced because each tier is guided by results obtained in the previous tier, and specific, testable, and relevant hypotheses are formulated based on these data [87, 97, 102, 122, 131].

Future Directions

Since one of the main objectives of the EU GMO regulatory framework is to ensure a high level of protection of human and animal health and the

environment, the focus is on the assessment of risks. Whether GM crops fulfill wider socioeconomic and ecological aspirations is not considered explicitly. However, when analyzing potential risks, it is important to bear in mind that the real choice is not between GM crops that are inherently risky and traditionally bred ones that are completely safe. Both existing crops and those with novel traits (including GM crops) will have both positive and negative attributes [56]. To fully acknowledge the overall consequences of adopting specific crops, and to assess and manage more effectively the environmental footprint of agriculture as a whole, it has been suggested that broader and more balanced legislative oversight is needed in the EU [132, 133]. At the EFSA scientific colloquium on challenges and approaches for the environmental risk assessment of GM plants [133], the discussion group on broadening the scope of the environmental risk assessment provided the following recommendations:

- A paradigm shift would be required to change from risk assessment as it is currently practiced, to a more sophisticated assessment which balances risks and benefits: (i) The focus on only GM crops defies scientific evidence. In the longer term, risk assessors could develop an alternative approach on a scientific basis. 'Novelty' is one option. (ii) The status quo, in which risk assessment is interpreted very narrowly in terms of adverse impacts, is not sustainable, and perceptions of the quality of environmental risk assessments suffer as a result. A framework for the future is required. (iii) There is a need to build decision support tools for the risk assessors to better consider impacts of whole farming systems.

The assessment of GM crops has intersected with a wider debate about sustainable agriculture in the EU, blurring any distinctions between environmental, agricultural, and socioeconomic issues. A sustainability assessment may be helpful in recovering public and market confidence, as it integrates larger societal concerns. It enables to define and integrate the underlying values at stake, trade possible risks against benefits, compare technological alternatives, evaluate the usefulness of GM crops, and to assess a whole agricultural system. In this way, it may promote finding a better balance between agricultural production and biodiversity, and evolve toward a socially more robust risk analysis, in which sustainable development is the goal.

While such an integral sustainability assessment certainly will not make things easier and might be an unachievable ideal, it may offer a helpful framing of the debate about multiple controversial aspects. Social, ecological, and economic considerations (such as ensuring social equity and cohesion, a high level of environmental protection, and economic prosperity) that have too often been treated in isolation could then be brought together and explored in a holistic and long-term perspective. Preferences for certain options would no longer be advanced independently of the broader, more complex agricultural and societal context. Likewise, underlying values at stake and normative perspectives fueling the debate might be brought to light.

Disclaimer

Opinions and views expressed in this entry are strictly those of the authors, and may not necessarily represent those of the organizations where the authors are currently employed.

Acknowledgments

The authors wish to thank Salvatore Arpaia, Detlef Bartsch, Adinda De Schrijver, Matty Demont, Achim Gathmann, Rosemary Hails, Jozsef Kiss, Antoine Messéan, Karin Nienstedt, Joe Perry, Dirk Reheul, Olivier Sanvido, and Jeremy Sweet for inspiring discussions that helped to develop this entry.

Bibliography

1. EC (2008) The Economics of Ecosystems and Biodiversity – Interim Report 2008. http://ec.europa.eu/environment/nature/biodiversity/economics
2. EC (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Off J Eur Communities L106:1–39
3. EC (2004) Directive 2004/35/EC of the European Parliament and of the Council of 21 April 2004 on environmental liability with regard to the prevention and remedying of environmental damage. Off J Eur Communities L143:56–75
4. Secretariat of the CBD (2000) Cartagena protocol on biosafety to the convention on biological diversity: text and annexes.

Secretariat of the Convention on Biological Diversity (CBD), Montreal, Canada. http://www.cbd.int/doc/legal/cartagena-protocol-en.pdf
5. Johnson KL, Raybould AF, Hudson MD, Poppy GM (2007) How does scientific risk assessment of GM crops fit within the wider risk analysis? Trends Plant Sci 12:1–5
6. James C (2010) Global status of commercialized biotech/GM crops: 2010. Highlights of ISAAA briefs No 42, Ithaca, New York
7. Chapotin SM, Wolt JD (2007) Genetically modified crops for the bioeconomy: meeting public and regulatory expectations. Transgenic Res 16:675–688
8. Herring RJ (2008) Opposition to transgenic technologies: ideology, interests and collective action frames. Nat Rev 9:458–463
9. Levidow L, Carr S, Wield D (2005) European Union regulation of agribiotechnology: precautionary links between science, expertise and policy. Sci Public Policy 32:261–276
10. Devos Y, Reheul D, De Waele D, Van Speybroeck L (2006) The interplay between societal concerns and the regulatory frame on GM crops in the European Union. Environ Biosaf Res 5:127–149
11. Devos Y, Maeseele P, Reheul D, Van Speybroeck L, De Waele D (2008) Ethics in the societal debate on genetically modified organisms: a (re)quest for *Sense* and *Sensibility*. J Agric Environ Ethics 21:29–61
12. Levidow L, Carr S (2007) GM crops on trial: technological development as a real world experiment. Futures 39:408–431
13. Lofstedt RE (2006) How can we make food risk communication better: where are we and where are we going? J Risk Res 9:869–890
14. Marks LA, Kalaitzandonakes N, Wilkins L, Zakharova L (2007) Mass media framing of biotechnology news. Public Underst Sci 16:183–203
15. Maeseele PA, Schuurman D (2008) Biotechnology and the popular press in Northern Belgium. Sci Commun 29:435–471
16. Kasperson RE, Kasperson JX (1996) The social amplification and attenuation of risk. Ann Am Acad Polit Soc Sci 545:95–105
17. Winickoff D, Jasanoff S, Busch L, Grove-White R, Wynne B (2005) Adjudicating the GM food wars: science, risk, and democracy in world trade law. Yale J Int Law 30:81–123
18. Kuiper HA, Davies HV (2010) The SAFE FOODS risk analysis framework suitable for GMOs? Food Control. doi:10.1016/j.foodcont.2010.02.011
19. Gaskell G, Allansdottir A, Allum N, Corchero C, Fischler C, Hampel J, Jackson J, Kronberger N, Mejlgaard N, Revuelta G, Schreiner C, Stares S, Torgersen H, Wagner W (2006) Europeans and Biotechnology in 2005: Patterns and Trends, Eurobarometer 64.3. http://ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf
20. Christiansen T, Polak J (2009) Comitology between political decision-making and technocratic governance: regulating GMOs in the European Union. Eipascope Bull 1:5–11
21. EC (2010) Proposal for a Regulation of the European Parliament and of the Council amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of GMOs in their territory. http://ec.europa.eu/food/food/biotechnology/docs/proposal_en.pdf
22. EC (2010) Communication from the Commission to the European Parliament, the Council, the Economics and Social Committee and the Committee of the Regions on the freedom for Member States to decide on the cultivation of genetically modified crops. http://ec.europa.eu/food/food/biotechnology/docs/communication_en.pdf
23. EC (2010) Commission Recommendation of 13 July 2010 on guidelines for the development of national co-existence measures to avoid the unintended presence of GMOs in conventional and organic crops. http://ec.europa.eu/food/food/biotechnology/docs/new_recommendation_en.pdf
24. Chipman A (2010) Fears over Europe's GM crop plan. Nature 466:542–543
25. Ryffel GU (2010) Making the most of GM potatoes. Nat Biotechnol 28:318
26. Secretariat of the CBD (1992) Convention on biological diversity. Secretariat of the Convention on Biological Diversity (CBD), Montreal, Canada. http://www.biodiv.org/doc/legal/cbd-en.pdf
27. James C (2009) Global status of commercialized biotech/GM crops: 2009. ISAAA Brief No. 41. ISAAA, Ithaca
28. Jaffe G (2004) Regulating transgenic crops: a comparative analysis of different regulatory processes. Transgenic Res 13:5–19
29. FAO/WHO (2000) Safety aspects of genetically modified foods of plant origin. A joint FAO/WHO consultation on foods derived from biotechnology, Geneva, Switzerland, 29 May–2 June 2000. World Health Organization, Geneva
30. Secretariat of the IPPC (2005) Pest risk analysis for quarantine pests, including analysis of environmental risks and living modified organisms. International Standards for Phytosanitary Measures (ISPM) No. 11. Secretariat of the International Plant Protection Convention (IPPC), Food and Agriculture Organization of the United Nations, Rome, Italy. ftp://ftp.fao.org/docrep/fao/008/y5874e/y5874e00.pdf
31. Mackenzie R, Burhenne-Guilmin F, La Vina AGM, Werksman JD (2003) An explanatory guide to the Cartagena protocol on biosafety. In cooperation with Ascencio A, Kinderlerer J, Kummer K, Tapper R. IUCN, Gland, Switzerland and Cambridge, UK. www.cbd.int/doc/books/2003/B-01669.pdf
32. Breyer D, Herman P, Brandenburger A, Gheysen G, Remaut E, Soumillion P, Van Doorsselaere J, Custers R, Pauwels K, Sneyers M, Reheul D (2009) Commentary: Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge? Environ Biosaf Res 8:57–64
33. Macdonald P, Yarrow S (2003) Regulation of Bt crops in Canada. J Invertebr Pathol 83:93–99
34. McHughen A, Smyth S (2008) US regulatory system for genetically modified [genetically modified organisms (GMO), rDNA or transgenic] crop cultivars. Plant Biotechnol J 6:2–12

35. Smyth S, McHughen A (2008) Regulating innovative crops technologies in Canada: the case of regulation genetically modified crops. Plant Biotechnol J 6:213–225
36. COGEM (2006) New techniques in plant biotechnology. COGEM Report CGM/061024-02. http://www.cogem.net/ContentFiles/CGM061024-02%20new%20techniques%20in%20plantbiotechnology.pdf
37. Morris SH, Spillane C (2008) GM directive deficiencies in the European Union. EMBO Rep 9:500–504
38. Batista R, Saibo N, Lourenço T, Oliveira MM (2008) Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion. Proc Natl Acad Sci USA 105:3640–3645
39. Kok EJ, Keijer J, Kleter GA, Kuiper HA (2008) Comparative safety assessment of plant-derived foods. Regul Toxicol Pharmacol 50:98–113
40. Bradford KJ, Van Deynze A, Gutterson N, Parrott W, Strauss SH (2005) Regulating transgenic crops sensibly: lessons from plant breeding, biotechnology and genomics. Nat Biotechnol 23:439–444
41. Morris SH (2007) EU biotech crop regulations and environmental risk: a case of the emperor's new clothes? Trends Biotechnol 25:2–6
42. Jacobsen E, Schouten HJ (2007) Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends Biotechnol 25:219–223
43. Rommens CM, Haring MA, Swords K, Davies HV, Belknap WR (2007) The intragenic approach as a new extension to traditional plant breeding. Trends Plant Sci 12:397–403
44. COGEM (2009) Novel plant breeding techniques – consequences of new genetic modification-based plant breeding techniques in comparison to conventional plant breeding, report 2009-02. http://www.cogem.net/ContentFiles/Microsoft%20Word%20-%20New%20plant%20breeding%20techniques%20definitieve%20versie.pdf
45. Foster M, Berry P, Hogan J (2003) Market access issues for GM products. Australian Bureau of Agricultural and Resource Economics (ABARE) eReport 03.13, Canberra, Australia. http://abareonlineshop.com/PdfFiles/PC12559.pdf
46. Kuiper HA, Kleter GA, Noteborn HPJM, Kok EJ (2002) Substantial equivalence – an appropriate paradigm for the safety assessment of genetically modified food. Toxicology 181–182:427–431
47. Kok EJ, Kuiper HA (2003) Comparative safety assessment for biotech crops. Trends Biotechnol 21:439–444
48. König A, Cockburn A, Crevel RWR, Debruyne E, Grafstroem R, Hammerling U, Kimber I, Knudsen I, Kuiper HA, Peijnenburg AACM, Penninks AH, Poulsen M, Schauzu M, Wal JM (2004) Assessment of the safety of foods derived from genetically modified (GM) crops. Food Chem Toxicol 42:1047–1088
49. Paoletti C, Flamm E, Yan W, Meek S, Renckens S, Fellous M, Kuiper H (2008) GMO risk assessment around the world: some examples. Trends Food Sci Technol 19:S66–S74
50. Devos Y, Lheureux K, Schiemann J (2010) Regulatory oversight and safety assessment of plants with novel traits. In: Kempken F, Jung C (eds) Genetic modification of plants – agriculture, horticulture & forestry, Series: Biotechnology in Agriculture and Forestry, vol 64. Springer, Berlin, pp 553–574
51. Demeke T, Perry DJ, Scowcroft WR (2006) Adventitious presence of GMOs: scientific overview for Canadian grains. Can J Plant Sci 86:1–23
52. Lheureux K, Mestdagh S, Devos Y (2008) Collaboration between the EFSA GMO Panel and national EU competent authorities for the environmental risk assessment of GM crop cultivation applications. J Consum Prot Food Saf 3(S2):51
53. SCP (2001) Opinion of the Scientific Committee on Plants concerning the adventitious presence of GM seeds in conventional seeds. http://ec.europa.eu/comm/food/fs/sc/scp/out93_gmo_en.pdf
54. Verhoog H, Matze M, Lammerts Van Bueren E, Baars T (2003) The role of the concept of the natural (naturalness) in organic farming. J Agric Environ Ethics 16:29–49
55. Altieri MA (2005) The myth of coexistence: why transgenic crops are not compatible with agroecologically based systems of production. Bull Sci Technol Soc 25:1–11
56. Sanvido O, Romeis J, Bigler F (2007) Ecological impacts of genetically modified crops: ten years of field research and commercial cultivation. Adv Biochem Eng Biotechnol 107:235–278
57. Schiemann J (2003) Co-existence of genetically modified crops with conventional and organic farming. Environ Biosaf Res 2:213–217
58. Devos Y, Demont M, Sanvido O (2008) Coexistence in the EU–return of the moratorium on GM crops? Nat Biotechnol 26:1223–1225
59. Devos Y, Demont M, Dillen K, Reheul D, Kaiser M, Sanvido O (2009) Coexistence of genetically modified (GM) and non-GM crops in the European Union. Agron Sustain Dev 29:11–30
60. EC (2003) Commission Recommendation of 23 July 2003 on guidelines for the development of national strategies and best practices to ensure the coexistence of genetically modified crops with conventional and organic farming. Off J Eur Communities L189:36–47
61. Friesen LF, Nelson AG, Van Acker RC (2003) Evidence of contamination of pedigreed canola (*Brassica napus*) seedlots in western Canada with genetically modified herbicide resistance traits. Agron J 95:1342–1347
62. Jørgensen T, Hauser TP, Jørgensen RB (2007) Adventitious presence of other varieties in oilseed rape (*Brassica napus*) from seed banks and certified seed. Seed Sci Res 17:115–125
63. Devos Y, Reheul D, De Schrijver A (2005) The co-existence between transgenic and non-transgenic maize in the European Union: a focus on pollen flow and cross-fertilization. Environ Biosaf Res 4:71–87
64. Hüsken A, Dietz-Pfeilstetter A (2007) Pollen-mediated intraspecific gene flow from herbicide resistant oilseed rape (*Brassica napus* L.). Transgenic Res 16:557–569

65. Sanvido O, Widmer F, Winzeler M, Streit B, Szerencsits E, Bigler F (2008) Definition and feasibility of isolation distances for transgenic maize. Transgenic Res 17:317–355
66. Devos Y, Reheul D, De Schrijver A, Cors F, Moens W (2004) Management of transgenic herbicide-tolerant oilseed rape in Europe: a case study on minimizing vertical gene flow. Environ Biosaf Res 3:135–148
67. Lutman PJW, Berry K, Payne RW, Simpson E, Sweet JB, Champion GT, May MJ, Wightman P, Walker K, Lainsbury M (2005) Persistence of seeds from crops of conventional and herbicide tolerant oilseed rape (*Brassica napus*). Proc R Soc Lond B 272:1909–1915
68. Messéan A, Sausse C, Gasquez J, Darmency H (2007) Occurrence of genetically modified oilseed rape seeds in the harvests of subsequent conventional oilseed rape over time. Eur J Agron 27:115–122
69. Gruber S, Colbach N, Barbottin A, Pekrun C (2008) Post-harvest gene escape and approaches for minimizing it. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour 3:1–17
70. Jørgensen RB (2007) Oilseed rape: co-existence and gene flow from wild species. Adv Bot Res 45:451–464
71. Knispel AL, McLachlan S, Van Acker R, Friesen LF (2008) Gene flow and multiple herbicide resistance in escaped canola populations. Weed Sci 56:72–80
72. Pivard S, Adamczyk K, Lecomte J, Lavigne C, Bouvier A, Deville A, Gouyon PH, Huet S (2008) Where do the feral oilseed rape populations come from? A large-scale study of their possible origin in a farmland area. J Appl Ecol 45:476–485
73. Beckmann V, Soregaroli C, Wesseler J (2006) Coexistence rules and regulations in the European Union. Am J Agric Econ 88:1193–1199
74. EC (2006) Report on the implementation of national measures on the co-existence of genetically modified crops with conventional and organic farming, European Commission. http://ec.europa.eu/agriculture/coexistence/sec313_en.pdf
75. Koch BA (2007) Liability and compensation schemes for damage resulting from the presence of genetically modified organisms in non-GM crops. http://ec.europa.eu/agriculture/analysis/external/liability_gmo/full_text_en.pdf
76. Sundstrom F, Williams J, Van Deynze A, Bradford KJ (2002) Identity preservation of agricultural commodities. ANR Publication No. 8077, University of California
77. Czarnak-Klos M, Rodríguez-Cerezo E (2010) Best practice documents for coexistence of genetically modified crops with conventional and organic farming. 1. Maize crop production. European Coexistence Bureau (ECoB) report. http://ecob.jrc.ec.europa.eu/documents/Maize.pdf
78. Demont M, Devos Y (2008) Regulating coexistence of GM and non-GM crops without jeopardizing economic incentives. Trends Biotechnol 26:353–358
79. Demont M, Daems W, Dillen K, Mathijs E, Sausse C, Tollens E (2008) Regulating coexistence in Europe: Beware of the domino-effect! Ecol Econ 64:683–689
80. Demont M, Dillen K, Daems W, Sausse C, Tollens E, Mathijs E (2009) On the proportionality of EU spatial *ex ante* coexistence regulations. Food Pol 34:508–518
81. Demont M, Devos Y, Sanvido O (2010) Towards flexible coexistence regulations for GM crops in the EU. EuroChoices 9:18–24
82. Demont M, Dillen K, Daems W, Sausse C, Tollens E, Mathijs E (2010) On the proportionality of EU spatial *ex ante* coexistence regulations: Reply. Food Pol 35:183–184
83. Beckmann V, Soregaroli C, Wesseler J (2010) *Ex-ante* regulation and *ex-post* liability under uncertainty and irreversibility: governing the coexistence of GM crops. Econ 4, http://www.economics-ejournal.org/economics/journalarticles/2010-9
84. Ramessar K, Capell T, Twyman RM, Christou P (2010) Going to ridiculous lengths: European coexistence regulations for GM crops. Nat Biotechnol 28:133–136
85. Riesgo L, Areal FJ, Sanvido O, Rodríguez-Cerezo E (2010) Distances needed to limit cross-fertilization between GM and conventional maize in Europe. Nat Biotechnol 28:780–782
86. Craig W, Tepfer M (2007) Introduction to the safety/risk assessment of GM crops. J Agric Invest 5:36–42
87. EFSA (2006) Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed. EFSA J 99:1–100
88. Hill RA (2005) Conceptualizing risk assessment methodology for genetically modified organisms. Environ Biosaf Res 4:67–70
89. Conner AJ, Glare TR, Nap J-P (2003) The release of genetically modified crops into the environment. Part II: overview of ecological risk assessment. Plant J 33:19–46
90. Craig W, Tepfer M, Degrassi G, Ripandelli D (2008) An overview of general features of risk assessment of genetically modified crops. Euphytica 164:853–880
91. Hill RA, Sendashonga C (2003) General principles for risk assessment of living modified organisms: lessons from chemical risk assessment. Environ Biosaf Res 2:81–88
92. Wolt JD, Keese P, Raybould A, Fitzpatrick JW, Burachik M, Gray A, Olin SS, Schiemann J, Sears M, Wu F (2010) Problem formulation in the environmental risk assessment for genetically modified plants. Transgenic Res 19:425–436
93. EPA (1998) Guidelines for ecological risk assessment. EPA/630/R095/002F, US EPA risk assessment forum. US Environmental Protection Agency, Washington DC. http://cfpub.epa.gov/ncea/raf/recordisplay.cfm?deid=12460
94. Raybould A (2006) Problem formulation and hypothesis testing for environmental risk assessments of genetically modified crops. Environ Biosaf Res 5:119–125
95. Raybould A (2007) Ecological versus ecotoxicological methods for assessing the environmental risks of transgenic crops. Plant Sci 173:589–602
96. Romeis J, Hellmich RL, Candolfi MP, Carstens K, De Schrijver A, Gatehouse AMR, Herman RA, Huesing JE, McLean MA, Raybould A, Shelton AM, Waggoner A (2010) Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. Transgenic Res. doi:10.1007/s11248-010-9446-x

97. Garcia-Alonso M, Jacobs E, Raybould A, Nickson TE, Sowig P, Willekens H, Van der Kouwe P, Layton R, Amijee F, Fuentes AM, Tencalla F (2006) A tiered system for assessing the risk of genetically modified plants to non-target organisms. Environ Biosaf Res 5:57–65
98. Horak MJ, Rosenbaum EW, Woodrum CL, Martens AB, Mery RF, Cothren JT, Burns JA, Nickson TE, Pester TA, Jiang C, Hart JL, Sammons B (2007) Characterization of roundup ready flex cotton, 'MON 88913', for use in ecological risk assessment: Evaluation of seed germination, vegetative and reproductive growth, and ecological interactions. Crop Sci 47:268–277
99. Nickson TE (2008) Planning environmental risk assessment for genetically modified crops: problem formulation for stress-tolerant crops. Plant Physiol 147:494–502
100. Garcia-Alonso M (2010) Current challenges in environmental risk assessment: The assessment of unintended effects of GM crops on non-target organisms. IOBC/wprs Bull 52:57–63
101. Raybould A, Tuttle A, Shore S, Stone T (2010) Environmental risk assessment for transgenic crops producing output trait enzymes. Transgenic Res 19:595–609
102. Romeis J, Bartsch D, Bigler F, Candolfi MP, Gielkens MMC, Hartley SE, Hellmich RL, Huesing JE, Jepson PC, Layton R, Quemada H, Raybould A, Rose RI, Schiemann J, Sears MK, Shelton AM, Sweet J, Vaituzis Z, Wolt JD (2008) Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Nat Biotechnol 26:203–208
103. Suter GW (2000) Generic assessment endpoints are needed for ecological risk assessment. Risk Anal 20:173–178
104. Sanvido O, Romeis J, Bigler F (2009) An approach for post-market monitoring of potential environmental effects of Bt-maize expressing Cry1Ab on natural enemies. J Appl Entomol 133:236–248
105. Suter GW (2007) Ecological risk assessment, 2nd edn. CRC Press, Boca Raton, p 643
106. Perry JN, ter Braak CJF, Dixon PM, Duan JJ, Hails RS, Huesken A, Lavielle A, Marvier M, Scardi M, Schmidt K, Tothmeresz B, Schaarschmidt F, van der Voet H (2009) Statistical aspects of environmental risk assessment of GM plants for effects on non-target organisms. Environ Biosaf Res 8:65–78
107. Storkey J, Bohan DA, Haughton AJ, Champion GT, Perry JN, Poppy GM, Woiwod IP (2008) Providing the evidence base for environmental risk assessments of novel farm management practices. Environ Sci Policy 11:579–587
108. EFSA (2010) EFSA Panel on Plant Protection Products and their Residues (PPR); Scientific Opinion on the development of specific protection goal options for environmental risk assessment of pesticides, in particular in relation to the revision of the Guidance Documents on Aquatic and Terrestrial Ecotoxicology (SANCO/3268/2001 and SANCO/10329/2002). EFSA J 1821, 1-55. http://www.efsa.europa.eu/en/scdocs/doc/1821.pdf
109. Marvier M (2002) Improving risk assessment for nontarget safety of transgenic crops. Ecol Appl 12: 1119–1124
110. Lövei GL, Arpaia S (2005) The impact of transgenic plants on natural enemies: a critical review of laboratory studies. Entomol Exp Appl 114:1–14
111. Qi A, Perry JN, Pidgeon JD, Haylock LA, Brooks DR (2008) Cost-efficacy in measuring farmland biodiversity – lessons from the farm scale evaluations of genetically modified herbicide-tolerant crops. Ann Appl Biol 152: 93–101
112. Gathmann A, Wirooks L, Hothhorn LA, Bartsch D, Schuphan I (2006) Impact of Bt-maize pollen (MON810) on lepidopteran larvae living on accompanying weeds. Mol Ecol 15: 2677–2685
113. Todd JH, Ramankutty P, Barraclough EI, Malone LA (2008) A screening method for prioritizing non-target invertebrates for improved biosafety testing of transgenic crops. Environ Biosaf Res 7:35–56
114. Aviron S, Sanvido O, Romeis J, Herzog F, Bigler F (2009) Case-specific monitoring of butterflies to determine potential effects of transgenic *Bt*-maize in Switzerland. Agric Ecosyst Environ 131:137–144
115. Rauschen R, Schultheis E, Pagel-Wieder S, Schuphan I, Eber S (2009) Impact of Bt-corn MON88017 in comparison to three conventional lines on *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae) field densities. Transgenic Res 18:203–214
116. Andow DA, Lövei GL, Arpaia S (2006) Ecological risk assessment for Bt crops. Nat Biotechnol 24:749–751
117. Raybould A (2010) Reducing uncertainty in regulatory decision-making for transgenic crops. More ecological research or clearer environmental risk assessment? GM Crops 1:1–7
118. EFSA (2010) Scientific opinion of the scientific panel on genetically modified organisms on statistical considerations for the safety evaluation of GMOs. EFSA J 1250:1–59
119. OECD (1993) Safety evaluation of foods derived by modern biotechnology, concepts and principles. Organization for Economic Co-operation and Development, Paris. ISBN 92-64-13859-5
120. Petersen W, Umbeck P, Hokanson K, Halsey M (2005) Biosafety considerations for selectable and scorable markers in cassava (*Manihot esculenta* Crantz) biotechnology. Environ Biosaf Res 4:89–102
121. Aumaitre A, Aulrich K, Chesson A, Flachowsky G, Piva G (2002) New feeds from genetically modified plants: substantial equivalence, nutritional equivalence, digestibility, and safety for animals and the food chain. Livest Prod Sci 74:223–238
122. Andow DA, Zwahlen C (2006) Assessing environmental risks of transgenic plants. Ecol Lett 9:196–214
123. Sanvido O, Widmer F, Winzeler M, Bigler F (2005) A conceptual framework for the design of environmental post-market monitoring of genetically modified plants. Environ Biosaf Res 4:13–27

124. Schmidt K, Wilhelm R, Schmidtke J, Beissner L, Mönkemeyer W, Böttinger P, Sweet J, Schiemann J (2008) Farm questionnaires for monitoring genetically modified crops: a case study using GM maize. Environ Biosaf Res 7:163–179
125. Wilhelm R, Sanvido O, Castanera P, Schmidt K, Schiemann J (2009) Monitoring the commercial cultivation of *Bt* maize in Europe – conclusions and recommendations for future monitoring practice. Environ Biosaf Res 8:219–225
126. Sweet JB (2008) General surveillance of multiple transgenic events. J Verbr Lebensm 3:12
127. Graef F, De Schrijver A, Murray A (2008) GMO monitoring data coordination and harmonisation at EU level – outcomes of the European Commission Working Group on Guidance Notes supplementing Annex VII of Directive 2001/18/EC. J Verbr Lebensm 3:17–20
128. ILSI Task Force (2004) Nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology. Task Force of the International Life Science Institute (ILSI) International Food Biotechnology Committee. Compr Rev Food Sci Food Saf 3:35–104
129. Dutton A, Romeis J, Bigler F (2003) Assessing the risks of insect resistant transgenic plants on entomophagous arthropods: Bt-maize expressing Cry1Ab as a case study. BioControl 48:611–636
130. Wilkinson MJ, Sweet J, Poppy GM (2003) Risk assessment of GM plants: avoiding gridlock? Trends Plant Sci 8:208–212
131. Bartsch D, Gathmann A, Saeglitz C, Sinha A (2008) Field testing of transgenic plants. In: Stewart CN Jr (ed) Plant biotechnology and genetic principles, techniques, and applications. Wiley, London, pp 311–323
132. ACRE (2007) Managing the footprint of agriculture: towards a comparative assessment of risks and benefits for novel agricultural systems. DEFRA, London. http://www.defra.gov.uk/environment/acre/fsewiderissues/pdf/acre-wi-final.pdf
133. EFSA (2008) Environmental risk assessment of genetically modified plants – challenges and approaches. EFSA Scientific Colloquium Series 8, June 2007. European Food Safety Authority, Brussels. http://www.efsa.europa.eu/en/colloquiareports/colloquiagmoera.htm
134. Sanvido O, Romeis J, Gathmann A, Gielkens M, Raybould A, Bigler F (2011) Evaluating environmental risks of genetically modified crops – ecological harm criteria for regulatory decision-making. Environment & Science Policy, doi:10.1016/j.envsci.2011.08.006

Transgenic Fishes: Applications, State of the Art, and Risk Concerns

Eric M. Hallerman
Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Article Outline

Glossary

Accessible ecosystem The aquatic environment immediately accessible to an organism were it to escape from the research or culture facility, and more distant habitats in the contiguous environment into which the organism or its offspring reasonably may be expected to disperse.

Confinement A system of physical, chemical, biological, and operations management measures used to contain a transgenic fish within an experimental or culture facility or to prevent its establishment in the accessible ecosystem upon escape or release.

Direct effects (or impacts) Effects of a transgenic fish on an (other) organism(s) in the accessible ecosystem, which are effected through mechanisms involving biotic factors. Examples would include but not be limited to reproduction with or predation upon or competition with members of the same or other species.

Environmental effects (or impacts) Consequences of an experiment or of aquaculture that might include, but are not limited to, changes in the structure, function, or resiliency of the accessible ecosystem; changes in the gene pool of populations resident in the accessible ecosystem; or decline in the abundance of a population of threatened, endangered, or special concern species in the accessible ecosystem.

Harm A perturbation resulting in negative impact to a population or species.

Hazard An agent or process that has the potential to produce harm.

Indirect effects (or impacts) Effects of a transgenic fish on (an) other organism(s) in the accessible ecosystem that are effected through mechanisms involving abiotic factors or additional species. Examples would include, but not be limited to, modification of the physical environment, affecting its suitability as habitat for other species and cascading effects of altered trophic function upon the aquatic community.

Introgression The incorporation of genes from one species into the gene pool of another; alternatively, breeding of a transgenic individual with wild-type individuals, leading to introduction and persistence of the transgene in the wild gene pool.

Novel trait Expression of a compound not normally found in the species, e.g., an antifreeze polypeptide in Atlantic salmon; alternatively, expression of a compound normally found in the species, but under novel gene regulation; for example, expression of a growth hormone gene under the transcriptional control of a promoter element not normally associated with the gene.

Risk The likelihood of harm resulting from exposure to a hazard.

Transgene An artificial DNA molecule containing a structural gene whose expression would confer a novel trait upon its host. The transgene would contain a regulatory element to control its expression in the host and an element signaling the point at which transcription would terminate and a poly-A tail added to the messenger RNA transcript.

Definition of the Subject

A transgenic fish or shellfish bears within its chromosomal DNA a gene construct – that is, a transgene, a gene whose expression is under novel regulation – that was introduced by human intervention. A variety

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

of methods may be utilized to introduce foreign DNA into the genome of fishes [1]. Microinjection of a DNA vector into the cytoplasm of a fertilized egg was the first [2, 3] and remains the most commonly used method for producing founders of a transgenic line. Electroporation, the application of short electrical pulses to make the cell membrane permeable and thereby permit entry of DNA vectors into fertilized fish eggs, has been used to produce transgenic fishes [4, 5]. Electroporation also has been used to introduce DNA vectors into sperm, which in turn was utilized to fertilize eggs to produce transgenic founders [6]. A variation of the "gene gun" method, which involves bombardment of fertilized eggs with DNA-coated microprojectiles, was demonstrated using medaka, or rice fish, a model species [7]. Retroviruses [8] and transposable elements [9] have been modified to carry genes of interest and, using the ability of these elements to insert themselves into the genome of a host, to insert foreign DNA into zebrafish chromosomes.

Because they have large eggs, high fecundity, and external fertilization and development, fishes provide excellent systems for gene transfer. By introducing a major gene whose expression is under novel regulation, biotechnologists can achieve dramatic impacts upon valued phenotypes. Since the mid-1980s when the first transgenic fish were produced [2, 3], gene transfer studies have been conducted using over 35 species [1]. Following the demonstration of the concept [10], a variety of transgenic zebrafish, medaka, and other fishes have been developed as experimental models for biomedical research [11–13]. Many transgenic lines have been developed for potential use in aquaculture (see below), targeting growth rate enhancement and a variety of other production-related traits. Similarly, several sport fishes (e.g., northern pike [14]) have been transformed with growth hormone genes in order to increase growth rate, aggressiveness, or ultimate size. Transgenic zebrafish expressing fluorescent proteins have been marketed as novel aquarium pets [15]. Transgenic lines have been developed for biomonitoring of chemicals in the aquatic environment [16–18].

Because they express novel traits, transgenic fish may prove attractive for aquaculture, fisheries management, or other applications. While production of transgenic fishes may prove profitable, there are broader and longer-term questions regarding whether such use would prove ecologically, economically, and socially sustainable. Because most transgenic fish lines have been produced for purposes of aquaculture, this contribution focuses upon sustainability of transgenic fishes intended for food production.

Introduction

As world population increases, so also does its demand for fisheries products. However, since 1985, the total harvest from capture fisheries has fluctuated at approximately 90 million metric tons (MMT) [19], suggesting that capture fisheries are at or above maximum sustainable yields [20]. Since 1970, the contribution of aquaculture to world supplies of fisheries products has grown from approximately 5 MMT to over 50 MMT annually. Against this background, it is clear that the projected growth in demand for fisheries products can be met only through growth in production from aquaculture. The historical and projected growth in aquaculture raises the question of whether aquaculture is sustainable as currently practiced. First raised by the environmental community [21], the question then was critically examined by the aquaculture community itself [22]. Academic and commercial aquaculturists now are working to define and achieve sustainability [23–25]. Among key innovations so driven are changes in aquaculture feeds to decrease the content of scarce fish meal and oil [26, 27] and selective breeding to develop stocks of fish adapted to utilizing such feeds [28, 29]. There is increasing emphasis among aquaculturists on producing fishes that are herbivores or omnivores rather than carnivores. Within this broader context, whether transgenic fishes would contribute to the sustainability of aquaculture depends upon the host fish, the transgene, the confinement system in which the fish are raised, and any impacts that might follow should transgenic fish escape or be introduced into the accessible environment.

Traits targeted by transgenesis. A number of different traits have been targeted for genetic improvement via transgenesis. Growth hormone (GH) genes have been introduced into over a dozen aquaculture species to increase growth rate. Noteworthy examples where growth was increased several-fold relative to wild-type include Atlantic salmon [30, 31], coho salmon [32, Fig. 1],

Transgenic Fishes: Applications, State of the Art, and Risk Concerns. Figure 1
A coho salmon expressing a salmon growth hormone transgene (*above*) exhibited growth that was dramatically greater than that of a same-aged, non-transgenic full-sibling (*below*) (Courtesy: R.H. Devlin)

Nile tilapia [33], common carp [34], and rohu carp [35]. The most notable example of growth enhancement is that of mud loach, for which up to 35-fold growth rate enhancement was observed [36]. Antifreeze protein genes from Arctic or Antarctic fishes have been transferred to increase the tolerance of species sensitive to freezing in marine waters chilled to below the freezing point of fish flesh (e.g., Atlantic salmon [37]). Genes for bactericidal compounds have been introduced to heighten nonspecific disease resistance of cultured fishes (e.g., cecropin in channel catfish [38] and human lactoferrin in grass carp [39, 40]). The major storage form for phosphorus in grain is phytate, which is largely indigestible and has antinutritional properties; following demonstration that medaka transgenic for a microbial phytase gene exhibited improved phytate utilization [41], phytase also was transferred into tilapia [42]. Transgenic approaches have been proposed for reproductive confinement of fish [43] (see also below), including expression of an antisense DNA complementary to the gonadotropin-releasing hormone transcript [44] and a "sterile-feral" *zBMP2* construct that blocks a critical developmental gene unless a repressor compound is applied to the animal in captivity [45]. When a key biochemical pathway is disrupted by lack of one enzyme, the function of that pathway can be restored by the expression of a transgene; for example, a rat L-gulono-γ-lactone oxidase transgene was introduced into rainbow trout in an attempt to complete the biosynthetic pathway for L-ascorbic acid [46]. While not exhaustive, these examples illustrate the scope of efforts to genetically improve cultured stocks of fishes using gene transfer methodologies.

Most transgenic lines described above have not been subject to the generations of breeding needed to develop a homozygous line stably expressing the transgene. However, development of some GH-transgenic lines is well advanced and efforts to commercialize them are ongoing. A/F protein, producers of GH-transgenic Atlantic salmon, have petitioned the US Food and Drug Administration for approval to market eggs of transgenic Atlantic salmon to commercial aquaculture producers [31]. The Cuban government is considering a request for production of GH-transgenic tilapia [47]. Marketing of a GH-transgenic common carp line is under consideration by the Chinese government [48]. With the prospect of improved production efficiency, it is not surprising that some aquaculturists want to produce GH-transgenic fish commercially. Commercialization of transgenic fishes, however, poses ecological, food safety, regulatory, and animal welfare concerns [49–52].

Confinement systems. Many different fish culture systems are in commercial use. Closed recirculating systems hold fish in indoor tanks and the water is treated in filtration systems; the associated expenses are such that only high-value species can be produced economically. Raceways are long, narrow outdoor tanks, with water from a spring routed through the system once and then discharged; rainbow trout are commonly reared in raceways. Ponds, which may have discharge to a nearby stream or river, are used to produce many species including tilapias and catfishes. Marine netpens, mesh cages suspended in bays or fjords, are used to produce Atlantic salmon, coho salmon, Atlantic cod, and other species. Ocean ranching involves release of juvenile salmon, trout, or charr into the ocean, to be collected later upon their return to spawn as adults. Clearly, these different types of culture systems vary in terms of their production economics and in their ability to reliably confine fishes.

Entry of cultured fish into the accessible ecosystem. Most commercial aquaculture operations have a routine, often significant escape of fish. Escapes can occur through equipment failures, during handling

or transport operations, through predator intrusion into facilities, as a result of storms, or by other mechanisms. In particular, escapes of salmon and trout from marine net-pen facilities are common, ranging from minor incidents where a few fish escape to massive escapes. In the native range of Atlantic salmon, an estimated two million farmed salmon escape each year into the North Atlantic [53]. Outside the native range, millions of Atlantic salmon have escaped on the west coasts of North [54] and South America [55]. Although salmon farm operators are attempting to prevent escapes by upgrading confinement systems, installing predator deterrent devices, and other actions, it still must be assumed that escapes will occur [56].

Escape of cultured fish into the accessible ecosystem and interaction with local intraspecific and interspecific populations is a well-documented environmental concern [57–61]. The potential ecological and genetic interactions between cultured and wild fish have raised concern about the viability of affected wild stocks. Ecological concerns focus upon competition for space and food resources and direct predation [62]. Genetic concerns include the potential breakdown of locally adapted traits through interbreeding and introgression and range up to replacement of native stocks by cultured stocks [63]. Such concerns are posed by the prospect of producing transgenic fish in aquaculture, with the additional unknown posed by possible effects of the transgene. Because of the prospect of escape of cultured transgenic fish, we must consider not only the benefits of transgenic fish to aquaculture, but also any ecological and genetic hazards that they pose. Hazards posed by transgenic fish were first inferred on the basis of ecological principles [64–67], and then supported by empirical studies.

Ecological Risk Assessment for Transgenic Fish in the Accessible Ecosystem

Ecological risk assessment for transgenic organisms should be based upon case-by-case assessment of the host species, transgene construct, transgene integration site within the genome, and receiving ecosystem [49, 64, 68, 69]. Because most fishes do not have a long history of domestication, many cultured stocks retain the ability to survive and reproduce in the accessible ecosystem. Hence, consideration of ecological risk and genetic hazards posed by transgenic fish that might escape from a culture facility is appropriate.

Consideration of harms posed by transgenic fish must be based on an understanding of key concepts underlying the science and practice of risk analysis [49]. In this context, a *harm* is defined as a perturbation resulting in negative impacts to a species. A *hazard* is defined as an *agent or process* that has the potential to produce harm. A *risk* is defined as the *likelihood* of harm resulting from exposure to the hazard. Risk, R, is estimated as the product of the probability of exposure, $P(E)$, and the conditional probability of harm given that exposure has occurred, $P(H|E)$. That is, $R = P(E) \times P(H|E)$. The steps in risk analysis, then, are to: (1) identify potential harms; (2) identify hazards that might lead to harms; (3) define what exposure means for an aquaculture stock and assess the likelihood of exposure, $P(E)$; (4) quantify the likelihood of harm given that exposure has occurred, $P(H|E)$; and (5) multiply the resulting probabilities to yield a quantitative estimate of risk.

Exact probabilities of risk may prove difficult or impossible to determine for all types of possible ecological or genetic harm. Indeed, it is unlikely that all possible harms would be known *a priori*, particularly with respect to any indirect effects. Hence, it may be necessary – based on current knowledge of population genetics, population dynamics, receiving ecological communities, and experience with cultured stocks – to classify levels of concern regarding likely ecological and genetic impacts posed by cultured stocks into *qualitative* categories ranging from low to high.

Potential harms. Harms potentially posed by transgenic fishes would be the outcome of a chain of events occurring after escape or release from a culture system. Examples of harms that potentially might be realized by transgenic fishes entering or becoming established in an accessible ecosystem [49, 64–66] would include decline in abundance of loss of a native species. A locally adapted natural population could be replaced with a transgenic population or experience introgression of the transgene, decreasing the degree of local adaptation. Changes in ecosystem structure or function could result in decreased fisheries production or in ecosystem resiliency in the face of further ecological stressors.

Ecological hazards. Ecological hazards that could lead to harms becoming realized are posed to a range of species with which a transgenic fish might interact in the accessible ecosystem. Ecological hazards include the possibility of heightened predation or competition, as well as alteration of population or community dynamics due to activities of the transgenic fish. Examples from empirical studies illustrate some of these potential ecological hazards.

Several studies have focused on Atlantic salmon expressing a GH transgene. In order to support their rapid growth, these transgenic salmon have significantly faster routine metabolic rates and growth rates than domesticated and wild individuals of equal mass, and therefore require more energy to sustain body function [70, 71]. GH-transgenic salmon must consume food at a more rapid rate than control salmon. Abrahams and Sutterlin [72] observed behavior of size-matched transgenic and control salmon in experiments where fish could feed in safety or in the presence of the predator. Growth-enhanced transgenic fish were significantly more willing to risk exposure to a predator in order to gain access to food, and exhibited increased feeding rate and average speed of movement. However, transgenic fish reduced their exposure to predators in response to increases in the magnitude of risk, suggesting that their more active behavior may not necessarily lead to increased susceptibility to predation.

Devlin et al. [73] compared the intake of contested food pellets by size-matched pairs of one control (1 year older non-transgenic) and one transgenic coho salmon. The transgenic fish consumed 2.5 times more contested pellets than controls. Overall, transgenic fish consumed 2.9 times more pellets than non-transgenic controls, indicating high feeding motivation of transgenics throughout the feeding trials. In subsequent experiments, at low levels of food availability, dominant, generally transgenic individuals dominated acquisition of food resources [74]. Further, when food availability was low, populations containing transgenics crashed, while in contrast, groups containing only non-transgenic salmon exhibited 72% survival and increase of population biomass. Hence, genotype-by-environment interactions affect ecological risk assessment. Sundstrom et al. [75] exposed GH transgenic and wild coho salmon fry to a live predator in a naturalized experimental stream tank under conditions of high and low food abundance. In both cases, mortality rates of transgenic fry were significantly higher than those of wild fry.

Ecological hazards posed by transgenic fish are not limited to salmonid species. Duan et al. [76] observed food consumption, frequency of movement, and feeding hierarchy in transgenic and size-matched control common carp under conditions of limited food supply. Transgenic fish consumed 1.9 times as many pellets, moved 73% more frequently, and exhibited a higher standing in the feeding hierarchy, but did not realize their higher growth potential. The authors concluded that elevated ability to compete for limited food resources could be advantageous to transgenic carp after escape into the accessible ecosystem.

These and other empirical findings to date tend to support the competitive ability of GH-transgenic fishes with conspecifics, and suggest the likelihood that ecological harms could become realized should large numbers of GH transgenics escape from culture or a transgenic population become established. Clearly, not all possible ecological interactions have been considered experimentally. Ecological hazards posed by transgenic fish expressing other sorts of transgenes, for example, a phytase gene that could confer ability to effectively utilize a broader range of foods, have not yet been examined empirically.

Given the range of interacting species and accessible ecosystems, and given the temporal and spatial variation of ecosystems, it is difficult to predict the ecological outcome should transgenic fish escape from aquaculture operations and enter accessible ecosystems. Devlin et al. [77] review methodologies to assess potential ecosystem effects of transgenic fish before they enter unconfined environments, outlining a strategy for identifying the most important data and discussing methods for obtaining them. Key issues include characterization of the accessible ecosystem, phenotypic characterization of the transgenic fish, experimentation appropriate for relating the transgenic phenotype and ecological impacts, and recognizing and accounting for sources of uncertainty.

Genetic hazards. Although reproductive confinement methodologies such as sterilization by triploidy or transgenesis have been proposed to minimize reproductive interaction between transgenic and wild fish

(see below), to date these technologies have not been demonstrated to be completely effective [78]. Should transgenic fish be reproductively fertile, they potentially could interbreed with natural populations. Genetic or evolutionary impacts that could be realized would depend on the fitness of novel genotypes in the wild. There may be cases where fitness relative to the wild type is high, posing introgression of transgenes into natural populations. There may also be cases where maladaptive traits would be introduced into native populations, posing a hazard to the demographic viability of the receiving population. Empirical studies illustrate possible genetic hazards to wild populations of fish.

Considering the possible impact of introgression of transgenes into wild populations, Devlin et al. [79] examined growth enhancement due to expression of a GH transgene in both wild and selectively bred commercial rainbow trout strains and showed that the effect of expression of the transgene varied among the respective strains. Transgenic wild-strain rainbow trout retained the slender body morphology of the wild-type strain, but their final size at maturity was increased by transgenesis. Both domestic and wild-strain trout had reduced viability, and in the case of the domestic strain, all transgenic individuals died before sexual maturation. The greatest response to expression of the transgene was in hybrids of a wild and domesticated strain.

Devlin et al. [80] compared the reproductive performance of transgenic and non-transgenic coho salmon. GH-transgenic fish showed precocious smoltification and onset of sexual maturation but no increase of adult body size, indicating compression of the normal life history. However, strong genotype-by-environment interactions were shown for the effects of transgenesis and culture environment. Bessey et al. [81] found no differences in gamete quality or in vitro offspring production. The transgenic fish were able to breed successfully in the absence of competition, but at a significantly lower level than hatchery fish (i.e., fish reared artificially as young and then released to complete their life history naturally). When in direct competition with hatchery fish, however, the transgenic coho salmon were competitively and reproductively inferior to the point where they had little or no success.

Models have been developed to predict the outcome of introgression of a transgene into a wild population, notably for cases where expression of the transgene differentially affects various components of fitness. Muir and Howard [82] developed a deterministic model that predicted that under certain conditions, a transgene introduced into a natural population by a small number of transgenic fish would spread as a result of enhanced mating advantage enjoyed by larger individuals, but the reduced viability of offspring would cause eventual local extinction of populations. The predicted time to extinction of a wild population would be a function of the mating advantage of transgenic males relative to that of wild-type males and the relative viability of transgenic offspring. Muir and Howard [83] subsequently considered a transgenic organism's fitness components (juvenile viability, adult viability, age at sexual maturity, female fecundity, male fertility, and mating advantage), using various combinations of fitness component values in addition to empirically derived estimates for medaka, a model species, to parameterize and run the model. For a wide range of parameter values, the model predicted that transgenes could spread through populations despite high juvenile viability costs if there were sufficiently high positive effects on other fitness components. Sensitivity analyses showed that transgene effects on age at sexual maturity would have the greatest effect on transgene frequency, followed by juvenile viability, mating advantage, female fecundity, and male fertility, with the least impact due to changes in adult viability. Extinction hazards also could be posed when the transgene: (1) increases male mating success but reduces adult viability; (2) increases adult viability but reduces male fertility; or (3) increases both male mating success and adult viability, but reduces male fertility [84]. Using a different approach, Hedrick [85] developed a deterministic model for invasion of transgenes into natural populations and showed that if a transgene gives rise to a male mating advantage and a general viability disadvantage, then the conditions for its invasion of a natural population are very broad. More specifically, for two-thirds of the possible combinations of mating and viability parameters, the transgene increased in frequency. In addition, the demographic viability of the natural population was reduced, increasing the probability of extinction of the natural population.

Kapuscinski et al. [86] presented a step-by-step method for assessing risk posed by gene flow and possible introgression of a transgene into a wild population (Fig. 2) considering probabilities of escape of a transgenic fish from confinement, encountering wild-type mates, reproducing, and the young surviving to transit the transgene to future generations. Currently, our empirical knowledge of genetic hazards posed by production of transgenic fish and shellfish and their associated risks is limited. Ecological risk assessments are ongoing for GH-transgenic coho and Atlantic salmon, as well as other transgenic fishes, including both model species and aquaculture species. Many critical experiments aimed at estimating fitness parameters [67, 83] have yet to be conducted.

Risk Management

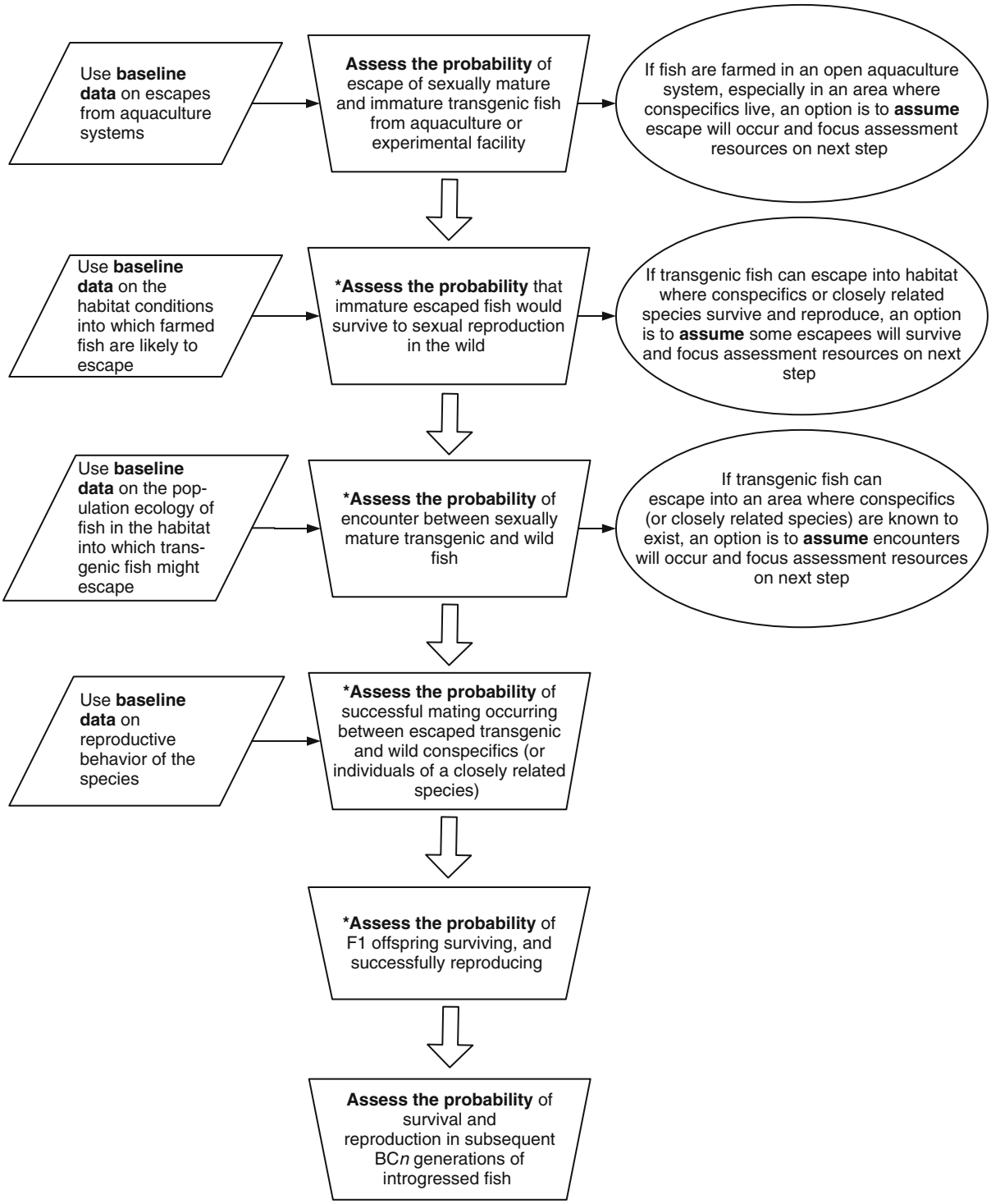
Many key inputs for a quantitative ecological risk assessment currently are unknown. Further, many key aspects of risk assessment are difficult or impossible to address, given the spatial and temporal variability of ecosystems and the adaptive ability of populations. Under at least some circumstances, escaped transgenics could negatively impact accessible ecosystems and populations; hence, should a producer or oversight body determine that production of a transgenic stock poses harm to a population in the accessible ecosystem, the consideration then turns to managing the associated risk. From the viewpoint of ecological sustainability, risk might be managed by producing transgenic fish only under conditions of confinement. In some contexts, production of transgenic fish might go forward only under conditions of strict confinement aimed at ensuring no escape of transgenic fish into the accessible ecosystem.

Risk management is the design, selection, and implementation of a program of actions to minimize risk. In the context of formal risk analysis, it becomes clear that the best approach for minimizing the likelihood of harm being realized is to minimize exposure to the hazard. Three non-mutually exclusive approaches include: (1) physically confining the cultured stock on aquaculture facilities, (2) reproductively confining cultured stocks, and (3) operations management. Management of risks posed by transgenic fishes has been considered for experimental [87] and commercial production systems [88].

Physical confinement. Physical confinement of cultured aquatic organisms will require a combination of measures in order to prove effective [87, 88]. Context is key; the ease or difficulty of managing risk will depend greatly on the geographic location of an aquaculture facility. Sites subject to flooding, violent storms, or wave action are poorly suited for confinement of production stocks. Virtually all physical confinement systems will include barriers to escape of cultured organisms from the culture site, including mechanical or physical/chemical barriers. Mechanical barriers are structures that physically hold back cultured organisms from escaping the project site. Examples include stationary or moving screens, (e.g., floor drains, standpipe screens), tank covers, filters (e.g., gravel traps), grinders or pumps, and French drains. A French drain is a filter for screening effluent from an aquaculture facility that contains gravel and geotextiles through which even small life-stages cannot pass. Physical or chemical barriers use manipulation of physical (e.g., elevation of temperature) or chemical (e.g., administration of chlorine) attributes of effluent water to induce 100% mortality of any escaped organisms before they can reach the accessible ecosystem. The set of barriers must prevent escape of the hardest-to-retain life-stage held at the aquaculture operation, usually the smallest life-stage. Because no barrier is 100% effective at all times, for effective physical confinement, each possible escape path from the aquaculture facility would have redundant barriers to escape of cultured organisms. Barriers also must prevent access of predators that can carry cultured organisms off-site (e.g., avian predators) or damage ponds (e.g., muskrats), allowing escape of cultured organisms.

Reproductive confinement. A key element of many risk management strategies is reproductive confinement, especially for cases where physical confinement alone is unlikely to prove effective. Two approaches, culture of monosex or sterile stocks, might be applied singly or in combination.

Procedures have been developed for producing monosex stocks of fish through direct, hormone-mediated sex reversal and indirectly by sex reversal, followed by progeny testing and selection of broodstock that yield monosex offspring. For example, production of all-female rainbow trout [89] and



Transgenic Fishes: Applications, State of the Art, and Risk Concerns. Figure 2

A pathway for conducting an assessment of the risk of gene flow posed by a transgenic fish (From [86]). *Asterisks* denote assessment steps that require empirical information on traits exhibited by the transgenic fish

all-male Nile tilapia [90] stocks comprise growing segments of the respective aquaculture sectors. Production of monosex stocks may provide an acceptable high level of reproductive confinement in contexts where transgenic escapees have no possibility of encountering prospective mates in the accessible ecosystem. Production of monosex stocks will not provide reliable reproductive confinement if there is a population of the same species or of a closely related species with which escaped transgenics may hybridize in the accessible ecosystem. Further, for monosex production to provide reliable confinement, sex ratios would have to be absolutely monosex, an outcome which has not generally been achieved in commercial production [88].

Reproductive confinement also might be achieved through chromosome set manipulation. Fishes can be produced that have three, instead of the usual two chromosome sets. Such fish are said to be triploid, and are reproductively sterile because they produce gametes with unusual numbers of chromosomes. Should their eggs or sperm give rise to an embryo, the unmatched chromosomes would disrupt normal embryogenesis, leading to death of the embryo. All-triploid stocks can be produced most reliably by the crossing of diploid and tetraploid broodstock, although lack of tetraploid broodstock precludes the approach for many species. Alternatively, triploid stocks can be produced by *de novo* induction. *De novo* triploidy induction is not always 100% effective and, hence, triploid broods will have to be screened to determine whether they are indeed all-triploid [78]. This extra handling and screening add to the cost of seed-stock production.

Other approaches for reproductive confinement may become available in the future, including the possibility of reversible sterility through transgenesis. A transgene that produces antisense gonadotropin-releasing hormone appears to cause sterility in male transgenic rainbow trout [44], but does not seem fully effective in females [91]. Use of a similar approach in tilapia sometimes greatly reduced fertility, but at other times was ineffective in both sexes (N. Maclean, quoted in [45]). Thresher et al. [45] developed a transgene that renders the host sterile, allowing normal development only if a repressor compound is applied during a particular life-history stage. The authors demonstrated its effectiveness in founder-generation transgenic zebrafish, channel catfish, and Pacific oyster. Definitive demonstration of this "sterile-feral" approach would require successful trials in homozygous transgenic lines and demonstration of the effectiveness of the repressor under commercial aquaculture conditions. Wang and Van Eenennaam [43] reviewed developing transgenic approaches to reproductive confinement of transgenic fishes, in addition to the approaches above also considering gonad-specific transgene excision. They suggested that slow progress in development of transgenic reproductive confinement systems is attributable to limited fundamental knowledge of possible gene targets to inactivate in order to induce sterility, preclude gondal development, or disrupt embryogenesis, as well as difficulties in implementing such highly technical approaches in vivo.

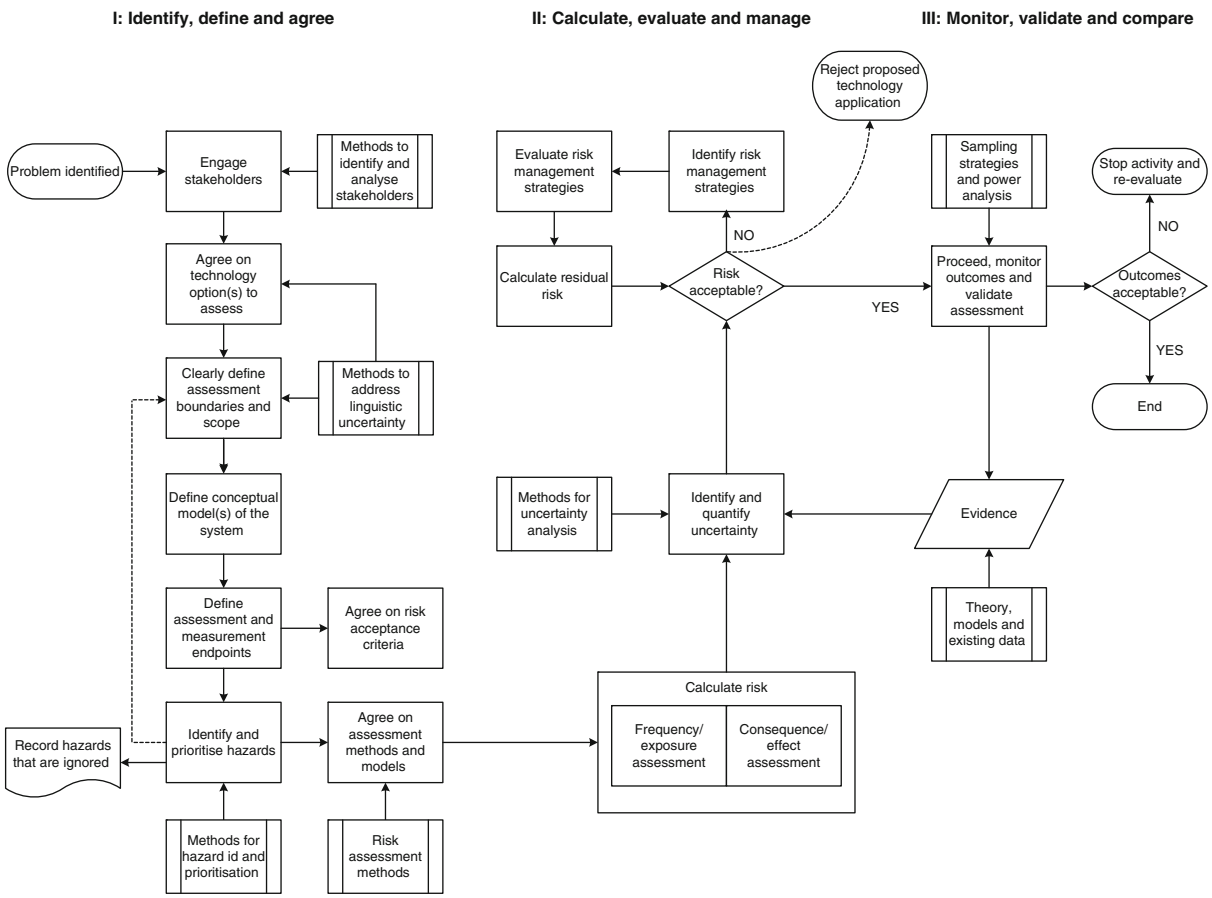
Operations management. Operations management is a key, though often overlooked, aspect of a confinement system [87, 88]. Measures are needed to: (1) ensure that normal activities of workers at the aquaculture operation are consistent with the goal of effective confinement, (2) prevent unauthorized human access to the site, and (3) ensure regular inspection and maintenance of physical confinement systems. Effective supervision of project personnel is critical for operations management.

Operations management also must consider biosecurity after cultured organisms are removed purposefully from the culture site, that is, harvested and transported through the marketing process. For biosecurity purposes, it would be best if only dead fish were sent to market. This is counter to marketing practices in many developing countries, where live sales prevail. Live sale is a known route for introductions of nonindigenous species, and exemplified by recent introduction and establishment of snakeheads in the USA [92].

Effective risk management calls for combinations of confinements. Combinations of risk management measures are advisable so that failure of any one measure will not necessarily lead to escape of confined stocks [87, 88]. It is infeasible to anticipate the best combination of risk management measures for every possible case. Differences in species, production traits, receiving ecosystems, and culture systems will affect the case-by-case determination of appropriate risk management measures.

Future Directions: Transgenic Fish Considered Within the Sustainability Paradigm

Interactive decision-making framework. Recognizing that sustainability has ecological, economic, and social dimensions, what conditions would have to be met for aquaculture production of transgenic fish to prove sustainable? The range of issues posed by a proposed utilization of transgenic fish in aquaculture might best be considered within in a three-stage, interactive framework involving a range of stakeholders [93, 94] (Fig. 3). Involvement of the full range of stakeholders would bring all existing knowledge into the deliberative process, make the process transparent to stakeholders, enhance the understanding and acceptance of the outcome of risk analysis, and promote social acceptance of the technology. Stage I of the process involves identifying the problem at hand, engaging stakeholders, identifying possible technical solutions to the problem at hand, and identifying potential harms, risk pathways, and assessment methods. Stage II of the process is risk



Transgenic Fishes: Applications, State of the Art, and Risk Concerns. Figure 3
Overview of a framework for environmental risk assessment of a transgenic fish embedded within a larger social context (From [94]). The framework has three stages. In the first stage, participants agree of an assessment option, define the scope of the assessment, agree on conceptual models, identify assessment and measurement end points, and agree upon a list of prioritized hazards. In the second stage, the risks and uncertainties associated with these hazards are assessed, risks are compared to predetermined acceptance criteria, and where necessary, risk management strategies are identified and evaluated. In the third stage, predictions of the risk assessment are compared with empirical findings, generating data used to reexamine uncertainty in the risk assessment

assessment, leading to estimating the likelihood that harm will become realized should the proposed production of transgenic fish go forward. Upon estimation of that risk, a decision would be faced as to whether the risk is acceptable. If it proves acceptable, the decision may be made to go forward. If the level of risk proves unacceptably high, risk management measures would be identified and residual risk quantified, and the decision of whether to go forward would again be considered. Should the proposed action be implemented, genetic, ecological, and social outcomes should be monitored. As discussed below, because all potential harms and associated pathways cannot be known and precisely predicted *a priori*, it will be necessary to update the risk analysis as knowledge accumulates using an adaptive management approach [95, 96].

Trade-offs of benefits and risks. The prospect of producing transgenic fishes in aquaculture poses a mix of benefits and risks that would affect the assessment of whether such production would promote ecological, economic, and social sustainability within the decision-making framework. Consideration of several examples will illustrate how trade-offs among benefits and risks will affect the assessment of sustainability.

Benefits and hazards posed by GH transgenics have been considered empirically, and some of the associated risks have been quantified. GH-transgenic fish exhibit rapid growth and perhaps also improved feed conversion ratio, promoting production efficiencies in terms of more efficient utilization of inputs, including facilities, labor, and feedstuffs. However, as noted above, risk assessment research to date suggests a considerable likelihood of ecological or genetic impacts should GH transgenics escape into accessible ecosystems. This risk can be managed by adoption of an appropriate suite of risk management measures (see above). Risk management will have attendant costs, decreasing profitability by some as-yet unquantified amount. Some segments of society are opposed to production of transgenic organisms [97], affecting the social aspect of whether this first wave of transgenic fishes will prove widely marketable and socially sustainable.

Empirical evaluations of both benefits and risks are yet to be conducted for other lines of transgenic fishes that may be considered for commercial production. Trade-offs among benefits and hazards may be anticipated. For example, as noted above, production of fish expressing a phytase transgene would take advantage of their ability to more efficiently utilize seed meals, increasing feed conversion efficiency and reducing the discharge of unutilized phosphorus from feed, decreasing eutrophication of waters in culture facilities and in waters receiving aquaculture discharges [41]. However, ability to efficiently utilize a wider range of natural foods might render escaped transgenic fish more competitive in accessible ecosystems. In particular, phytase-transgenic tilapia [42] might become even more competitive than wild-type tilapia, which are already recognized as invasive in a range of ecosystems in tropical and subtropical regions [98]. While effective confinement might be achievable in well-managed commercial operations, it likely cannot be achieved on subsistence farms, greatly influencing the assessment of whether phytase-transgenic tilapia would promote sustainability in developing countries.

As noted above, several transgenic fish lines have been produced for use as biomonitors [16–18] for detection of waterborne mutagens that would trigger expression of readily detected marker compounds in the fish. Such fishes would be exposed to such compounds by holding them in confinements in the waterbody of interest or by transporting the water to a confined fish-holding system. The benefits of long-term, integrative monitoring of water quality are clear. Any risks that might flow from the possibility of a nonindigenous species being used, escaping, and becoming naturalized in the waterbody at issue could be managed by use of effective confinement or by use of a species that cannot become established in the accessible ecosystem, for example, a tropical species sensitive to winter temperatures in the temperate zone.

These examples indicate how trade-offs of economic benefits and ecological risks, adoption of risk management measures, and ecological and social context interact to affect the sustainability of transgenic fishes for aquaculture and other applications. Explicit consideration of benefit and ecological risk, and hopefully also ecological, economic, and social aspects of sustainability would accompany societal consideration of whether and how to go forward with commercial production of transgenic fishes.

Oversight of production of transgenic fishes. The decision of whether and under what conditions production

of transgenic fish would go forward will largely be made at the national level. As noted above, at least three countries – the USA, Cuba, and the People's Republic of China – are considering applications for commercial production of transgenic fish. The USA, Canada, the European Union, and Norway have regulatory systems in place for oversight of aquaculture biotechnology. Under Article 21 of the United Nations Convention on Environment and Development and the subsequent Cartagena Protocol, signatories commit to developing and implementing policies for oversight of biotechnology. Consequently, many countries – for example, Cuba, Thailand, and the People's Republic of China [99] – are in the process of developing and implementing policy and staffing government offices that would be utilized to consider applications for production of transgenic fish.

Adaptive and proactive management. Many critical unknowns complicate risk assessment and risk management for transgenic aquaculture stocks. The adaptive management approach is based on recognition that knowledge of the environmental and social systems into which the aquaculture stocks would enter is *always* incomplete. Hence, management should evolve as knowledge of these systems increases [96]. Management cannot adapt if realized by a single passage through transgenesis and breeding, decision of whether and how to produce the transgenic stocks, and implementation of the distribution and production process. Instead, adaptive management would include risk assessment for candidate areas for distribution, incorporation of risk management into the production program, and capacity building as appropriate to meet program goals. Once transgenic stocks are distributed, culture operations and receiving ecosystems would be monitored for indicators of ecological and social conditions [96, 100]. Should monitoring indicate that economic and social benefits are being realized without ecological harms occurring, then few if any adjustments to program implementation are required. However, should monitoring indicate that production of cultured stocks is *not* contributing to nutritional and economic well-being or that the stocks are escaping and impacting accessible ecosystems, then it will prove necessary to redefine goals, revise implementation, and continue monitoring.

The issue of whether and under what conditions transgenic fish would promote sustainability of aquaculture has been raised largely after research and development on the first wave of transgenic fish lines was well advanced, after proponents have applied for regulatory approval for commercial production. Kapuscinski et al. [101] proposed a proactive "safety-first" approach in which an early, prospective risk assessment would be conducted in order to guide planning and implementation of measures to prevent or minimize risk as development of a transgenic line progresses. Transgene constructs would be designed for safety. Gene transfer scientists would strive for better control of copy number, genomic insertion site, and control of expression of the trangene, contributing to better-controlled modification of phenotype. Transgenic lines would be evaluated for stability of transgenic expression and transmission. Development of inducible expression of transgenes would reduce risk should transgenic fish escape into the environment. Improvements in confinement also would reduce risk posed by aquaculture production of transgenic fishes.

Applications of transgenic fishes, the science of risk assessment, the practice of risk management, and public policies for oversight of biotechnology are all in development. The degree to which production of transgenic fishes ultimately will prove sustainable will depend upon many societal decisions of whether and under what conditions to utilize transgenic technology in aquaculture.

Bibliography

Primary Literature

1. Zbikowska HM (2003) Fish can be first – advances in fish transgenesis for commercial applications. Transgenic Res 12:379–389
2. Zhu Z, Li G, He L, Chen S (1985) Novel gene transfer into the fertilized eggs of goldfish (*Carassius auratus* L. 1758). Z Angew Ichthyol 1:31–34
3. Chourrout D, Guyomard R, Houdebine L-M (1986) High efficiency gene transfer in rainbow trout (*Salmo gairdneri* Rich.) by microinjection into egg cytoplasm. Aquaculture 51:143–150
4. Inoue K, Yamashita S, Hata J, Kabeno K, Asada S, Nagahisa E, Fujita T (1990) Electroporation as a new technique for producing transgenic fish. Cell Diff Dev 29:123–128
5. Powers DA, Hereford I, Cole T, Chen TT, Lin CM, Kight K, Creech K, Dunham R (1992) Electroporation: a method for transferring

genes into the gamete of zebrafish (*Brachiodanio rerio*), channel catfish (*Ictalurus punctatus*), and common carp (*Cyprinus carpio*). Mol Mar Biol Biotechnol 1:301–308
6. Muller F, Ivics Z, Erdelyi F, Papp T, Varadi I, Horvath L, Maclean N (1992) Introducing foreign genes into fish eggs with electoporated sperm as a carrier. Mol Mar Biol Biotechnol 1:276–281
7. Yamauchi M, Kinoshita M, Sasanuma M, Tsuji S, Terada M, Morimyo M, Ishikawa Y (2000) Introduction of a foreign gene into medakafish using the particle gun method. J Exp Zool 287:285–293
8. Ivics Z, Izsvak Z, Hackett PB (1993) Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. Mol Mar Biol Biotechnol 2:166–173
9. Izsvak Z, Ivics Z, Plasterk RH (2000) Sleeping beauty, a wide host-range transposon vector for genetic transformation in vertebrates. J Mol Biol 302:93–102
10. Stuart GW, McMurray JV, Westerfield M (1988) Replication, integration, and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103:403–412
11. Dodd A, Curtis PM, Williams LC, Love DR (2000) Zebrafish: bridging the gaps between development and disease. Hum Mol Genet 9:2443–2449
12. Dooley K, Zon LI (2000) Zebrafish: a model system for the study of human disease. Curr Opin Genet Dev 10:252–256
13. Ward AC, Lieschke GJ (2002) The zebrafish as a model system for human disease. Front Biosci 7:827–833
14. Gross ML, Schneider JF, Moav N, Alvarez C, Myster SH, Liu Z, Hew C, Hallerman EM, Hackett PB, Guise KS, Faras AJ, Kapuscinski AR (1992) Molecular analysis and growth evaluation of northern pike (*Esox lucius*) microinjected with growth hormone genes. Aquaculture 103:253–273
15. Hallerman EM (2004) GloFish, the first GM animal commercialized, profits amid controversy. ISB (Information Systems for Biotechnology) news report, June 2004. www.isb.vt.edu/news/2004/artspdf/june0405.pdf, accessed November 22, 2010
16. Winn RN, Norris MB, Brayer KJ, Torres C, Muller SL (2000) Detections of mutations in transgenic fish carrying a bacteriophage λ cII transgene target. Proc Nat Acad Sci USA 97:12655–12660
17. Amanuma K, Takeda H, Amanuma H, Aoki Y (2000) Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. Nat Biotechnol 18:62–65
18. Carvan MJ III, Dalton TP, Stuart GW, Nebert DW (2000) Transgenic zebrafish as sentinels for aquatic pollution. Ann NY Acad Sci 919:133–147
19. Food and Agriculture Organization of the United Nations (2009) State of world fisheries and aquaculture. Fisheries and Aquaculture Department, FAO, Rome. http://www.fao.org, accessed October 30, 2009
20. Myers RA, Worm B (2003) Rapid worldwide depletion of predatory fish communities. Nature 423:280–283
21. Goldburg R, Triplett T (1997) Murky waters: environmental effects of aquaculture in the United States. Environmental Defense Fund, New York, p 198. www.edf.documents/490_AQUA.pdf, accessed November 22, 2010
22. Black KD (2001) Environmental impacts of aquaculture. CRC, Boca Raton, p 214
23. Bardach JE (ed) (1997) Sustainable aquaculture. Wiley, New York, p 251
24. Svennevig N, Reinertsen H, New M (eds) (1999) Sustainable aquaculture: food for the future? Balkema Publishers, Rotterdam, p 348
25. Jana BB, Webster CD (eds) (2003) Sustainable aquaculture: global perspectives. Haworth, New York, p 365
26. Gomes EF, Rema P, Kaushik SJ (1995) Replacement of fish meal by plant proteins in the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. Aquaculture 130:177–186
27. Gatlin DM III, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl A, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza EJ, Stone D, Wilson R, Wurtele E (2007) Expanding the utilization of sustainable plant produce in aquafeed: a review. Aquacult Res 38: 551–579
28. Pierce LR, Palti Y, Silverstein JT, Barrows FT, Hallerman EM, Parsons JE (2008) Family growth response to fishmeal and plant-based diets shows genotype x diet interaction in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 278:37–42
29. Dupont-Nivet M, Medale F, Leonard J, LeGuillou S, Tiquet F, Quillet E, Geurden I (2009) Evidence of genotype-diet interactions in the response of rainbow trout (*Oncorhynchus mykiss*) clones to a diet with or without fishmeal at early growth. Aquaculture 295:15–21
30. Du SJ, Gong ZY, Fletcher GL, Shears MA, King MJ, Idler DR, Hew CL (1992) Growth enhancement in transgenic Atlantic salmon by the use of an all-fish chimeric growth hormone gene construct. Biotechnology 10:176–181
31. Fletcher GL, Shears MA, Yaskowiak ES, King MJ, Goddard SV (2004) Gene transfer: potential to enhance the genome of Atlantic salmon for aquaculture. Aust J Exp Agric 44: 1095–1100
32. Devlin RH, Yesaki TY, Biagi CA, Donaldson EM, Swanson P, Chan WK (1994) Extraordinary salmon growth. Nature 371:209–210
33. Rahman MA, Ronyai A, Engidaw BZ, Jauncey K, Hwang GL, Smith A, Roderick E, Penman D, Varadi L, Maclean N (2001) Growth and nutritional trials on transgenic Nile tilapia containing an exogenous fish growth hormone gene. J Fish Biol 59:62–78
34. Hinits Y, Moav B (1999) Growth performance studies in transgenic *Cyprinus carpio*. Aquaculture 173:285–296
35. Venugopal T, Anathy V, Kirankumar S, Pandian TJ (2004) Growth enhancement and food conversion efficiency of transgenic fish, *Labeo rohita*. J Exp Biol 301A:477–490
36. Nam YK, Noh JK, Cho YS, Cho HJ, Cho KN, Kim CG, Kim DS (2001) Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. Transgenic Res 10:353–362

37. Hew CL, Poon R, Xiong F, Gauthier S, Shears M, King M, Davies P, Fletcher G (1999) Liver-specific and seasonal expression of transgenic Atlantic salmon harboring the winter flounder antifreeze protein gene. Transgenic Res 8:405–414
38. Dunham RA, Warr GW, Nichols A, Duncan PL, Argue B, Middleton D, Kucuktas H (2002) Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. Mar Biotechnol 4:338–344
39. Zhong J, Wang Y, Zhu Z (2002) Introduction of the human lactoferrin gene into grass carp (*Ctenopharyngodon idellus*) to increase resistance against GCH virus. Aquaculture 214: 93–101
40. Mao W, Wang Y, Wang W, Wu B, Feng J, Zhu Z (2004) Enhanced resistance to *Aeromonas hydrophila* infection and enhanced phagocytic activities in human lactoferrin-transgenic grass carp (*Ctenopharyngodon idellus*). Aquaculture 242:93–103
41. Hostetler HA, Collodi P, Devlin RH, Muir WM (2005) Improved phytate phosphorus utilization by Japanese medaka transgenic for the *Aspergillus niger* phytase gene. Zebrafish 2:19–31
42. Kemeh H (2004) Development of phytase transgenic Nile tilapia. Ph.D. dissertation, Purdue University, West Lafayette. www.docs.lib.purdue.edu, accessed October 30, 2009
43. Wong AC, Van Eenennaam AL (2008) Transgenic approaches for the reproductive containment of genetically engineered fish. Aquaculture 275:1–12
44. Uzbekova S, Chyb J, Ferrierre F, Bailhache T, Prunet P, Alestrom P, Breton B (2000) Transgenic rainbow trout expressed sGnRH-antisense RNA under the control of sGnRH promoter of Atlantic salmon. J Mol Endocrinol 25:337–350
45. Thresher R, Grewe P, Patil J, Whyhard S, Templeton M, Chaimongkol A, Hardy C, Hinds L, Dunham R (2009) Development of repressible sterility to prevent the establishment of feral populations of exotic and genetically modified animals. Aquaculture 290:104–109
46. Krasnov A, Pitkanen TI, Molsa H (1999) Gene transfer for targeted modification of salmonid fish metabolism. Genet Anal 15:115–119
47. Guillen I, Berlanga J, Valenzuela CM, Morales A, Toledo J, Estrada MP, Puentes P, Hayes O, LaFuente J (1999) Safety evaluation of transgenic tilapia with accelerated growth. Mar Biotechnol 1:2–14
48. Wu G, Sun Y, Zhu Z (2003) Growth hormone gene transfer in common carp. Aquat Living Resour 16:416–420
49. National Research Council (2002) Animal biotechnology: science-based concerns. National Academy, Washington, DC, p 179. http://www.nap.org, accessed November 22, 2010
50. Pew Initiative on Food and Biotechnology (2003) Future fish: issues in science and regulation of transgenic fish. www.pewtrusts.org/our_work_report_detail.aspx?id.33350, accessed November 22, 2010
51. Food and Agriculture Organization of the United Nations and World Health Organization (2004) Safety assessment of foods derived from genetically modified animals, including fish. FAO food and nutrition paper 79, FAO, Rome
52. Hallerman EM, McLean E, Fleming IA (2006) Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: a review identifying research needs. Appl Anim Behav Sci 104:265–294
53. Schiermeier Q (2003) Fish farms' threat to salmon stocks exposed. Nature 425:753
54. Volpe J, Taylor E, Rimmer D, Glickman B (2000) Evidence of natural reproduction of aquaculture-escaped Atlantic salmon in a coastal British Columbia River. Conserv Biol 14:899–903
55. Soto D, Jara F, Moreno C (2001) Escaped salmon in the inner seas, southern Chile: facing ecological and social conflicts. Ecol Appl 11:1750–1762
56. Council on Environmental Quality and Office of Science and Technology Policy (2000) Case study no. 1: growth-enhanced salmon. http://www.ostp.gov/what'snew/casestudies, accessed January 20, 2000
57. Schramm HL Jr, Piper RG (eds) (1995) Uses and effects of cultured fishes in aquatic ecosystems. In: American fisheries society symposium 15, American Fisheries Society, Bethesda
58. McGinnnity P, Prodohl P, Ferguson A, Hynes R, Maoilieidigh NO, Baker N, Cotter D, O'Hea B, Cooke D, Rogan G, Taggart J, Cross T (2003) Fitness reduction and potential extinction of wild populations of Atlantic salmon, *Salmo salar*, as a result of interactions with escaped farmed salmon. Proc R Soc Lond B 270:2443–2450
59. Fleming IA, Hindar K, Mjølnerød IB, Jonsson B, Balstad T, Lamberg A (2000) Lifetime success and interactions of farm salmon invading a native population. Proc R Soc Lond B 267:1517–1523
60. Nickum, MJ, Mazik PM, Nickum JG, MacKinlay DD (eds). (2004) Propagated fish in resource management. In: American fisheries society symposium 44, American Fisheries Society, Bethesda, MD
61. Verspoor E, Stradmeyer L, Nielsen JL (eds) (2007) The Atlantic salmon: genetics, conservation, and management. Blackwell, Oxford
62. Gross M (1998) One species with two biologies: Atlantic salmon (*Salmo salar*) in the wild and in aquaculture. Can J Fish Aquat Sci 55:131–144
63. Saegrov H, Hindar K, Kalas S, Lura H (1997) Escaped farmed Atlantic salmon replace the original salmon stock in the River Vosso, western Norway. ICES J Mar Sci 54:1166–1172
64. Kapuscinski AR, Hallerman EM (1990) Transgenic fish and public policy. Anticipating environmental impacts of transgenic fish. Fisheries 15(1):2–11
65. Kapuscinski AR, Hallerman EM (1991) Implications of introduction of transgenic fish into natural ecosystems. Can J Fish Aquat Sci 48(Suppl 1):99–107
66. Hallerman EM, Kapuscinski AR (1992) Ecological implications of using transgenic fishes in aquaculture. ICES Mar Sci Symp 194:56–66
67. Hallerman EM, Kapuscinski AR (1993) Potential impacts of transgenic and genetically manipulated fish on wild populations: addressing the uncertainties through field testing. In: Cloud JG, Thorgaard GH (eds) Genetic conservation of salmonid fishes. Plenum, New York, pp 93–112

68. Tiedje JM, Colwell RK, Grossman YL, Hodson RE, Lenski RE, Mack RN, Regal PJ (1989) The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology 70:298–315
69. Snow A, Andow D, Gepts P, Hallerman E, Powers A, Tiedje J, Wolfenbarger L (2005) Ecological risks and benefits associated with the environmental release of GMOs. Ecol Appl 15:377–404
70. Stevens ED, Sutterlin A, Cook T (1998) Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. Can J Fish Aquat Sci 55:2028–2035
71. Cook JT, McNiven MA, Sutterlin AM (2000) Metabolic rate of pre-smolt growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture 188:33–45
72. Abrahams MV, Sutterlin A (1999) The foraging and antipredator behavior of growth-enhanced transgenic Atlantic salmon. Anim Behav 58:933–942
73. Devlin RH, Johnsson JI, Smailus DE, Biagi CA, Johnsson E, Bjornsson BT (1999) Increased ability to compete for food by growth hormone transgenic coho salmon (*Oncorhynchus kisutch* Walbaum). Aquacult Res 30:1–4
74. Devlin RH, Biagi CA, Yesaki TY (2004) Growth viability and genetic characteristics of GH transgenic coho salmon strains. Aquaculture 236:607–632
75. Sundstrom LF, Lohmus M, Johnsson JI, Devlin RH (2004) Growth hormone transgenic salmon pay for growth potential with increased predation mortality. Proc R Soc Lond B 271: S350–S352
76. Duan M, Zhang T, Hu W, Sundstrom LF, Wang Y, Li Z, Zhu Z (2009) Elevated ability to compete for limited food resources by "all-fish" growth hormone transgenic common carp (*Cyprinus carpio* L.). J Fish Biol 75:1459–1469
77. Devlin RH, Sundstrom LF, Johnsson JI, Flaming IA, Hayes KR, Ojwang WO, Bamaradeniya C, Zakaria-Ismail M (2007) Assessing ecological effects of transgenic fish prior to entry into nature. In: Kapuscinski AR, Hayes KR, Li S, Dana G, Hallerman EM, Schei PJ (eds) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CAB International, Wallingford, Oxfordshire, pp 151–187
78. National Research Council (2004) Biological confinement of genetically engineered organisms. National Academy, Washington, DC, p 255. http://www.nap.org, accessed November 22, 2010
79. Devlin RH, Biagi CA, Yesaki TY, Smailus DE, Byatt JC (2001) A growth-hormone transgene boosts the size of wild but not domesticated trout. Nature 409:781–782
80. Devlin RH, D'Andrade M, Uh M, Biagi CA (2004) Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. Proc Nat Acad Sci USA 101: 9303–9308
81. Bessey C, Devlin RH, Liley NR, Biagi CA (2004) Reproductive performance of growth-enhanced transgenic coho salmon. Trans Am Fish Soc 133:1205–1220
82. Muir WM, Howard RD (1999) Possible ecological risks of transgenic organism release when transgenes affect mating success: sexual selection and the Trojan gene hypothesis. Proc Nat Acad Sci USA 96:13853–13856
83. Muir WM, Howard RD (2001) Fitness components and ecological risks of transgenic release: a model using Japanese medaka (*Oryzias latipes*). Am Nat 158:1–16
84. Muir WM, Howard RD (2002) Assessment of the possible ecological risks and hazards of transgenic fish with implications for other sexually reproducing organisms. Transgenic Res 11:101–114
85. Hedrick PW (2001) Invasion of transgenes from salmon or other genetically modified organisms into natural populations. Can J Fish Aquat Sci 58:841–844
86. Kapuscinski AR, Hard JJ, Paulson KM, Neira R, Ponniah A, Kamonrat W, Mwanja W, Fleming IA, Gallardo J, Devlin RH, Tresiak J (2007) Approaches to assessing gene flow. In: Kapuscinski AR, Hayes KR, Li S, Dana G, Hallerman EM, Schei PJ (eds) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CAB International, Wallingford, Oxfordshire, pp 112–150
87. Agricultural Biotechnology Research Advisory Committee – US Department of Agriculture (1995) Performance standards for safely conducting research with genetically modified fish and shellfish. http://www.isb.vt.edu/perfstands, accessed October 30, 2009
88. Mair GC, Nam YK, Solar II (2007) Risk management: reducing risk through confinement of transgenic fish. In: Kapuscinski AR, Hayes KR, Li S, Dana G, Hallerman EM, Schei PJ (eds) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CAB International, Wallingford, Oxfordshire, pp 209–238
89. Bye VJ, Lincoln RF (1986) Commercial methods for the control of sexual maturation of rainbow trout (*Salmo gairdneri*). Aquaculture 57:299–309
90. Mair GC, Abucay JS, Skibinski DOF, Abella TA, Beardmore JA (1997) Genetic manipulation of the sex ratio for large scale production of all-male tilapia *Oreochromis niloticus* L. Can J Fish Aquat Sci 54:396–404
91. Dunham RA (2004) Aquaculture and fisheries biotechnology: genetic approaches. CABI, UK, p 372
92. Dolin EJ (2003) Snake head: a fish out of water. Smithsonian Books, Washington, DC
93. Kapuscinski AR, Li S, Hayes K, Dana G, Hallerman E, Schei P (eds) (2007) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CABI Publishers, Wallingford, Oxfordshire
94. Hayes KR, Kapuscinski AR, Dana G, Li S, Devlin RH (2007) Introduction to environmental risk assessment for transgenic fish. In: Kapuscinski AR, Hayes KR, Li S, Dana G, Hallerman EM, Schei PJ (eds) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CAB International, Wallingford, Oxfordshire, pp 1–28
95. National Research Council (1996) Understanding risk: informing decisions in a democratic society. National Academy, Washington, DC

96. Kapuscinski, AR, Nega T, Hallerman EM (1999) Adaptive biosafety assessment and management regimes for aquatic genetically modified organisms in the environment. In: Pullin RSV, Bartley DM, Kooiman J (eds) Towards policies for conservation and sustainable use of aquatic genetic resources. In: ICLARM (International Center for Living Aquatic Resources Management) conference proceedings 59, pp 225–251
97. Rollin BE (1995) The Frankenstein syndrome: ethical and social issues in the genetic engineering of animals. Cambridge University Press, Cambridge, UK
98. Moreau J (1983) A review of introductions of tilapia in open waters of Africa, their influence on ecology and fisheries. In: Fishelson L, Yaron Z (eds) International symposium on tilapia in aquaculture, Tel Aviv University Press, Nazareth, Israel, May 8–13 1983, pp 77–85
99. Nelson KC, Basiao Z, Cooper AM, Dey M, Foniciella D, Lorenzo Hernandez M, Kunawasen S, Leelapatra W, Li S, Ratner BD, Toledo MI (2007) Problem formulation and options assessment: science-guided deliberation in environmental risk assessment of transgenic fish. In: Kapuscinski AR, Hayes KR, Li S, Dana G, Hallerman EM, Schei PJ (eds) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CAB International, Wallingford, Oxfordshire, pp 29–60
100. Senenan W, Hard JJ, Alcivar-Warren A, Trisak J, Zakaria-Ismail M, Lorenzo Hernandez M (2007) Risk management: post-approval monitoring and remediation. In: Kapuscinski AR, Hayes KR, Li S, Dana G, Hallerman EM, Schei PJ (eds) Environmental risk assessment of genetically modified organisms, vol 3, Methodologies for transgenic fish. CAB International, Wallingford, Oxfordshire, pp 239–271
101. Kapuscinski AR, Goodman RM, Hann SD, Jacobs LR, Pullins EE, Johnson CS, Kinsey JD, Krall RL, La Vina A, Mellon M, Ruttan V (2003) Making "safety first" a reality for biotechnology products. Nature Biotech 21:599–601

Books and Reviews

Beaumont AR, Hoare K (2003) Biotechnology and genetics in fisheries and aquaculture. Blackwell, Oxford, p 158

Bondad-Reantaso MG, Arthur JR, Subasinghe RP (eds) (2008) Understanding and applying risk analysis in aquaculture. FAO fisheries technical paper, no 519. FAO, Rome

Devlin RH, Sundstrom LF, Muir WM (2006) Interface of biotechnology and ecology for environmental risk assessments of transgenic fishes. Trends Biotechnol 24:89–97

Ennion SJ, Goldspink G (eds) (1996) Gene expression and manipulation in aquatic organisms. Cambridge University Press, Cambridge, UK, p 214

Hew CL, Fletcher GL (eds) (1992) Transgenic fish. World Scientific Publishing Co., Singapore

Hew CL, Fletcher GL (2001) The role of aquatic biotechnology in aquaculture. Aquaculture 197:191–204

Iyengar A, Muller F, Maclean N (1996) Regulation and expression of transgenes in fish – a review. Transgenic Res 5:147–166

Maclean N, Laight RJ (2000) The application of gene manipulation to aquaculture. Aquaculture 85:1–20

McLean E, Devlin RH (2000) Application of biotechnology to enhance growth of salmonids and other fish. In: Fingerman M, Nagabhushanam R (eds) Recent advances in marine biotechnology, vol 4, Aquaculture, Part B: Fishes. Science Publishers, Enfield, pp 17–55

Mench JA (1999) Ethics, animal welfare and transgenic farm animals. In: Murray JD, Anderson GB, Oberbauer AM, McGloghlin MM (eds) Transgenic animals in agriculture. CABI International, Wallingford, pp 251–268

Millar K, Tomkins S (2007) Ethical analysis of the use of GM fish: emerging issues for aquaculture. J Agr Environ Ethic 20:437–453

Organization for Economic Cooperation and Development (1995) Environmental impacts of aquatic biotechnology. OECD, Paris

Rasmussen RS, Morrisey MT (2007) Biotechnology in aquaculture: transgenics and polyploidy. Comp Rev Food Sci Food Safety 6:2–16

Transgenic Livestock for Food Production, Introduction

C. Bruce A. Whitelaw
The Roslin Institute and Royal (Dick) School of Veterinary Studies, Division of Developmental Biology, University of Edinburgh, Scotland, UK

Our societies currently face many big challenges: how to feed the increasing world population, how to balance the competing demands for land and water, how to mitigate climate change, and how to maintain healthy lifestyle into old age. Among other technologies, the animal genetic revolution offers several strategies to mitigate these challenges and provide benefit for mankind. While achieving this, we need to maintain and where appropriate increase the welfare and existence of animals we use in agriculture and biomedicine. Transgenic technologies, the ability to add or transfer or change the genetic makeup of an animal, can contribute. In the following ten entries, this premise is presented in detail. Why the entries were selected and how they complement each other while giving an overview of the field of transgenic animal biotechnology is described below.

The cornerstone of biological research is the ability to experimentally alter gene activity. In this way, the functional importance of a gene can be studied. This can be achieved indirectly through altering the environment of cells or organisms, for example, adding specific nutrients to a culture condition or infecting with a pathogen. Alternatively, direct manipulation can be achieved through genetic engineering. This section focuses on the later strategy which includes gene addition, gene removal (or inactivation), or gene alteration. To illustrate this, gene addition might be appropriate where an enhanced functionality is required such as food utilization, while gene inactivation would be appropriate if the aim was to remove a food allergen. Both have been successfully applied to animals and this is likely to be so going forward. In addition, as methodology becomes more sophisticated and the ability to directly alter a given gene sequence, this strategy will see more and more application; for example, where gene diversity is identified that could be applied to engineer altered metabolism or reduce disease susceptibility.

Although initially performed in bacteria, researchers quickly developed the tools to genetically modify animals. First in mice at the end of the 1970s, then livestock in the following decade, in parallel strategies for altering the germ line in fish and birds were developed. Researchers have developed strategies which enable precise spatial and temporal changes to target gene activity. Many consider transgenic or genetic modification (GM) methods (► Transgenics: Alternative Gene Transfer Methods) as part of the technical continuum that is available to animal breeders. As such, GM or transgenic animal technology would simply represent new methodology alongside the more established tools as artificial insemination and embryo transfer. These new animal reproductive tools include somatic cloning and in vitro embryo production, and when combined with the emerging molecular genetic tools provide powerful new opportunities for livestock breeding.

The first transgenic livestock were reported in 1985. Since these early days of this technology, there has been a steady increase in the availability of tools available (► Transgenic Livestock Technologies) for this form of assisted reproduction in animals. Some developments have been heralded as breakthroughs only to quickly drop out of favor while others have received limited use. Recently, reagents based on precisely targeted enzymatic cleavage of the genome that in addition to strategies such as gene knockdown provide the animal geneticist with both efficient and sophisticated methods with which to generate transgenic animals.

Perhaps one of the most consequential scientific advances of recent years was the technical breakthrough that produced "Dolly" the sheep at the Roslin Institute. This animal was produced by nuclear transfer (also termed SCNT, somatic cell nuclear transfer), or as it is commonly referred to as cloning. Animal cloning involves the replacement of the genetic material found in an unfertilized egg with that from a donor cell's genetic material. SCNT reflects considerable scientific thinking and offers the experimental biologist considerable insights into the functional reprogramming of cellular function in addition to details of cellular subcomponent roles in normal cell activity (► Livestock Somatic Cell Nuclear Transfer). In addition to being a valuable aspect of research in its own right, SCNT can be combined with genetic engineering tools to precisely

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

modify the genome of mammals (► Nuclear Transfer to Produce Transgenic Mammals). This combination offers exciting commercial opportunities across the fields of agriculture and animal breeding, to the biomedical arena and novel biotechnological processes.

Transgenic technologies are not restricted to mammals. Due to its use in food production and as an experimental organism in scientific research, the chicken is the most widely used bird species for experimental development of avian transgenic technologies (► Avian Specific Transgenesis). Perhaps the most exiting recent advance is that of precise gene modification in special avian cells called primordial stem cells. These new tools offer strategies for the production of transgenic birds other than the chicken. As such, this is an area currently experiencing rapid development and many expect it will be in birds that GM technologies will be first successfully exploited to produce animals that have enhanced resistance to infectious pathogens.

Although it can be argued that technically transgenic technology is most advanced for mammals, and currently developing quickest for birds, it is considered very likely that the first commercial application of transgenic animals that will enter the food chain will be in fish (► Transgenic Fishes: Applications, State of the Art, and Risk Concerns). Fish that grow faster are providing the first test of the regulatory systems while providing the focus for much debate on broader and longer-term questions regarding whether such use would prove ecologically, economically, and socially sustainable. There is also considerable effort being directed at the development of transgenic strategies to combat disease in fish populations, and the technology can be equally applied to food fish as to sport fish.

As the world population increases there is an increasing demand for food including both plant and animal products. The demand for meat and milk are increasing at a faster rate than for plant products because of increased wealth in developing countries. Animal products represent a concentrated protein and vitamin source which complements cereal and other vegetable proteins. Intensification of livestock production to satisfy the demand is exacerbating the deleterious impact of intensive animal agriculture on the environment and new approaches are needed to reduce the impact. Transgenic animals that have a smaller environmental footprint, increased productivity brought about by enhancing aspects of agriculturally important traits can contribute to this societal challenge. For this potential to be realized an active dialogue between all stakeholders is required to achieve ethically accepted sustainable future animal production.

The animals we all recognize in farms today are the result of many centuries of domestication. The driving force for this has largely come from man's desire for meat. This single process has shaped the rural landscape we all recognize today. It is therefore not surprising that animal breeders look to transgenic strategies to enhance the traditional animal breeding traits including food production (► Transgenic Livestock, Enhanced Nutritional Quality in). The focus now is not on quantity but on quality, while reflecting the rapid industrialization of our world and the associate increase in affluence experienced by many (but not all) of our communities. Food choice is now considered alongside sustainability. Animal welfare is paramount while still recognizing that many of the world population live in poverty with starvation a constant threat. Transgenic technologies offer the potential of quick solutions to the production of food products with enhanced specific nutritional characteristics from animals. The goal is healthier and safer food. However, this potential will only be realized with consumer acceptance in GM technology.

Two specific examples of traditional breeding traits that transgenic technology may provide innovative solutions are worthy of specific attention. The first is that of animal fertility (► Transgenic Technologies and Increased Livestock Fertility). Reproductive efficiency of domestic animals is critical to the sustainability of modern livestock industries. With various indicators of reducing fertility now appearing in our farms it is timely to consider innovative approaches to halt this decline. Aspects under investigation include increasing litter size in livestock or egg-laying capacity in poultry. More radically this technology could be applied to reduce the limitations associated with seasonal breeding.

The second topic that is attracting considerable interest in both the scientific research community and in the animal breeding industry is that of animals that are less susceptible to the ravages and distress of disease (► Disease-Resistant Transgenic Animals). Offering both enhanced animal welfare and economic advantages

this field is both fast moving and innovative, building much on the genomic revolution which is in turn constructed from the strong discipline of genetics. With huge impact on global animal health and the associated impact of zoonotic disease in man, this is an area which offers huge benefit to man and animal alike.

The simultaneous need for more food as the world population expands and that of limiting both short-term and longer-term environmental consequences is a huge challenge for man. Increased animal productivity could directly compromising our environment. Transgenic technology through providing animals that have a smaller environmental footprint (► Transgenic Livestock, Decreasing Environmental Impact of) could form an important part of the compromise that is inevitable if we are to provide sufficient food for our increasing human needs. Under development are projects aiming to reduce manure output and decrease greenhouse gas production. It is argued that the food security agenda being promoted by many governments will lead to greater acceptance of transgenic animals, following the slow but every increasing presence and consumption of transgenic plant products.

Transgenic animals present a dilemma for us. This technology offers rapid change with the promise of economic benefit, enhanced food security and better animal welfare. Yet transgenic technologies directly challenge many ethical aspects of thinking (► Transgenic Livestock, Ethical Concerns and Debate). Central to the process of acceptance is a conscious dialogue between all stakeholders – to build up a generally acceptable stance on what is good with respect to the ethical limits of human use of animals.

At the time of writing, there are no transgenic livestock in production – with faster growing fish being the closest and disease resistant birds being the most exciting. By the time of reading, this may well have changed – or be close to such a scenario. Agricultural advances have been enormously successful in providing an inexpensive supply of high-quality and safe foods. The new advance of transgenesis is likely to continue this trend providing some of the solutions to tomorrow's agricultural challenges. Governmental and industrial financial support – ideally in equal measures – is needed to achieve this. Equally important is the public dialogue to expose the benefits this technology can offer all stakeholders in our diverse world society.

Transgenic Livestock Technologies

Cesare Galli[1,2], Andrea Perota[1], Giovanna Lazzari[1], Franco Lucchini[3]
[1]Avantea, Laboratorio di Tecnologie della Riproduzione, Cremona, Italy
[2]Clinical Veterinary Department, University of Bologna, Italy
[3]Centro Ricerche Biotecnologiche, Università Cattolica del Sacro Cuore, Cremona, Italy

Article Outline

Glossary

Genetic engineering Recombinant DNA technology, gene manipulation including experimental manipulation of genetic material for industrial or medical use.

Germ cells Cells able to give rise to a functional gamete in multicellular animals.

Assisted reproduction The medical and laboratory processes used to reproduce the steps necessary to generate a newborn starting from gametes and or embryos.

IVF In Vitro Fertilization, the laboratory process in which a mammalian egg is fertilized outside the body and then put back inside to grow into a new individual.

ICSI Intra Cytoplasmic Sperm Injection, a procedure by which a spermatozoa is injected directly into the cytoplasm thus overcoming artificially the natural barriers of the oocyte.

SCNT Somatic Cell Nuclear Transfer, a technique that generates genomic copies of any given individual animal by replacing inside an oocyte its genome with the genome taken from somatic cells of a donor animal.

iPS cells Induced Pluripotent Stem cells, somatic cells that have been turned into pluripotent stem cells by the overexpression of pluripotency genes like OCT4, Nanog, Sox3, and Klf4.

Preimplantation embryo Following fertilization, the mammalian one-cell embryo (*zygote*) is still enclosed into the transparent proteic shell of the oocytes (*Zona pellucida*). During the journey along the oviduct it repeatedly cleaves to form an aggregate of smaller cells (*Morula Stage)* and finally develops an inner cavity (*Blastocyst stage*). Once in the uterus, the expanded blastocyst hatches breaking the zona pellucida and begins the implantation on the endometrium.

Chimerism The status of an organism made up by cells of different genotypes (like in the mythological monster *Chimera*). Chimeras derive from the aggregation of two different embryos of the same specie or from two different specie closely related (e.g., sheep and goat).

Mosaicism The cells of the organism own the same background genotype but are diverse in carrying or not a single mutation (or transgene). Such different cells may show a "patchy" distribution within tissues (like in a mosaic).

cDNA (complementary DNA) is a sequence of DNA obtained by reverse transcription of the mRNA (messenger RNA). It retains the whole information for coding the protein, but lacks the introns originally present in the chromosomal gene.

Zinc finger nuclease Recombinant enzyme able to bind a specific sequence of DNA and to "cut" it, generating a Double Strand Break (DSB). It is composed by an artificial DNA binding domain (a polypeptide with a repeat of regions each folded around a zinc ion, to evoke the idea of the fingers) linked to a bacterial nuclease domain, capable to hydrolyze the phosphate bond of the DNA.

Transposon A mobile genetic element able to transpose, that is, to excide itself from one site of the genome and to integrate in a different site. It does not move from one cell to another.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Retroviruses A class of viruses with a RNA genome, able to bind specific receptor on the target cell, to enter across the cell membrane and to transcribe its RNA in a DNA copy by means of its own RNA-dependent DNA polymerase (reverse transcriptase). Specific sequences promote the integration of the viral DNA into the genome of the host.

AAV A defective virus, with a small, single-stranded DNA genome, belonging to the family of Parvoviridae. AAV is able to replicate only in case of concomitant presence of an active adenovirus in the same cell. AAV can often integrate a double-stranded form of its DNA into the genome of the host.

Recombinase A family of enzymes able to exchange stretches of DNA among different molecules. Recombinases like CRE, coded by the genome of bacteriophage P1 (a virus parasiting a bacterium), recognize specific sequences as target of their activity. In the presence of such Recombination Recognition Sites, phagic recombinase is able to insert and excide the viral genome from the bacterial chromosome.

siRNA Silencer RNA or short interfering RNA are small synthetic RNA molecules able to trigger the degradation of a target messenger RNA bearing the same sequence. This results in a posttranscriptional inhibition of gene expression. SiRNA complexes derive from the processing of artificial shRNAs (short hairpin RNA) bearing a secondary double-stranded structure that is subsequently processed by the endogenous cell machinery. RNA-mediated inhibition of gene expression, also known as RNA-interference, is naturally observed in most eukaryotes and relies on a specific pathway involving specific endoribonucleases and endogenous micro-RNA (miRNAs) genes.

Definition of the Subject

Recombinant DNA technology and gene transfer were set about 40 years ago and since then have provided powerful tools for the production of biological molecules, the development of new varieties of crops, and for generating key animal models in biomedical research.

Following the synthesis of recombinant insulin, many other pharmacological proteins have been produced by expressing heterologous genes in microorganisms, plants, and animals.

The application of gene transfer or "transgenesis" to animals has been developed mainly in mouse, while its use for generating transgenic livestock has encountered unanticipated resistances originated by social, economical and technical consideration. Apart from public concern and social consideration about the release of Genetically Engineered (GE) products in the market and GE animals into the environment, which will not be discussed in this chapter, the development of transgenic livestock has been slow due to the technical difficulties faced in transferring this technology from mouse to large domestic animals and by the initial discouraging results, outlining the difficulties in obtaining the expected effect upon a genetic modification.

This review will survey the scientific advancement making the generation of GE swine and ruminants more accessible in the last few years, positively influencing the technical and, as a consequence, the economical issues related to their development.

Introduction

Genetic mutations and equally engineering (GE) of living organisms are part of biological processes that are inherited through reproduction across generations. Such events provide the genetic variability that is at the core of evolution of the species and ensure its survival both in plants and animal kingdoms. The understanding of the biology at the basis of such physiological events, the discovery of the mechanisms and the molecules involved has allowed the investigators to exploit and adapt such tools for the artificial modification of the genome of living organisms from virus and bacteria to mammals including primates. These developments that led to definitions and guidelines of what is now called DNA recombinant technology can be traced back to 1975 to the Asilomar Conference where the scientific community met to discuss potential hazard and regulation. DNA recombinant technology had the potential to be taken from bacteria and viruses to plants and animals with, at the time, unknown biological consequences and biosafety concerns. It was not too long in fact that the first GE mammals were produced [1, 2]. Palmiter's results were dramatic in the sense that the mouse engineered to express the rat growth hormone grew to the size double

that of the normal mice, thus opening the door to the application of such technologies in livestock, as stated in the abstract "This approach has implications for studying the biological effects of growth hormone, as a way to accelerate animal growth, as a model for gigantism, as a means of correcting genetic disease, and as a method of farming valuable gene products".

Since then, GE technology in mammals has been developed mainly in the laboratory mouse to study development and disease processes for research and clinical purposes with the ability to manipulate any given gene of the mouse genome. The developments in livestock immediately followed [3]; however it soon became evident that it was only the beginning and to reach such objective it was a difficult task and more knowledge was required. Beside the know-how required to engineer the genome, to make those modifications into the animals inherited through generations, it is necessary to operate during the early stages of embryonic development or on the germ cells to be able to have all the cells originating from the embryo GE. This is the reason why the developments of assisted reproduction techniques have been crucial for the generation of transgenic animals, especially in livestock where the access in vivo to preimplantation embryos is more complex, expensive, and cumbersome than in the mouse. It can be stated that the developments in transgenic livestock have been set by the development of assisted reproduction techniques like oocyte in vitro maturation and fertilization, intracytoplasmic sperm injection, embryo culture, and somatic cell nuclear transfer (SCNT).

The injection of recombinant DNA into the pronucleus of the zygote has been the first technique used to generate GE livestock. This required a large supply of zygotes that can be easily generated in mice in vivo but are very expensive or sometimes impossible to obtain from the donor females of larger animals because of the costs involved and the low efficiency (only 1–5% of the injected embryos result in transgenic offspring).

Nevertheless, first studies on sheep and pigs involved the use of in vivo produced zygotes, adapting the protocols already set for the mouse [1, 3–5]. On the other hand, the generation of transgenic calves was not practically manageable by this route and could only be achieved [6] using in vitro maturation and fertilization of oocytes collected at the slaughterhouse [7] where large numbers can be generated at a low cost without the use of live animals.

Besides the classical microinjection procedure, alternative approaches have been developed to bring exogenous DNA into the nucleus of the embryo. Embryonic stem cells are largely used in the mouse to generate transgenic animals. They can be replicated in culture for a long period without losing their pluripotency, that is, their ability to take part in the formation of a developing embryo. Thanks to this peculiar behavior, they can be GE to obtain cell clones bearing specific modification of the genome. Such cells can be introduced in the early embryo where they participate in the development of a chimeric organism and eventually integrate into the germ line [8]. This approach has opened the way to the development of the so-called gene targeting, allowing the generation of transgenic mice bearing specific mutations, including disruption of specific genes (knock-out) or insertion of new coding sequences in specific loci (knock-in). ES cells are not yet available in livestock, therefore this route is not yet practicable [9].

An alternative approach has been disclosed by the development of somatic cell nuclear transfer (SCNT) technology, currently the preferred route to make transgenic livestock [10]. Somatic cells may be GE, even by gene targeting, and then used for SCNT. Although SCNT is not very efficient, all the animals obtained carry the selected mutation that has been introduced in the somatic cells subsequently used for nuclear transfer.

In addition, alternative approaches have been developed to generate transgenic animals like the infection with an engineered retrovirus of in vitro cultured preimplantation embryos or the injection of a sperm together with the desired recombinant DNA vector during intracytoplasmic sperm injection (ICSI).

In the following paragraphs, the developments in assisted reproduction technologies that have made GE of livestock possible will be described, then the methods currently used, and finally emerging technologies that promise to make GE simpler, more precise, and reliable.

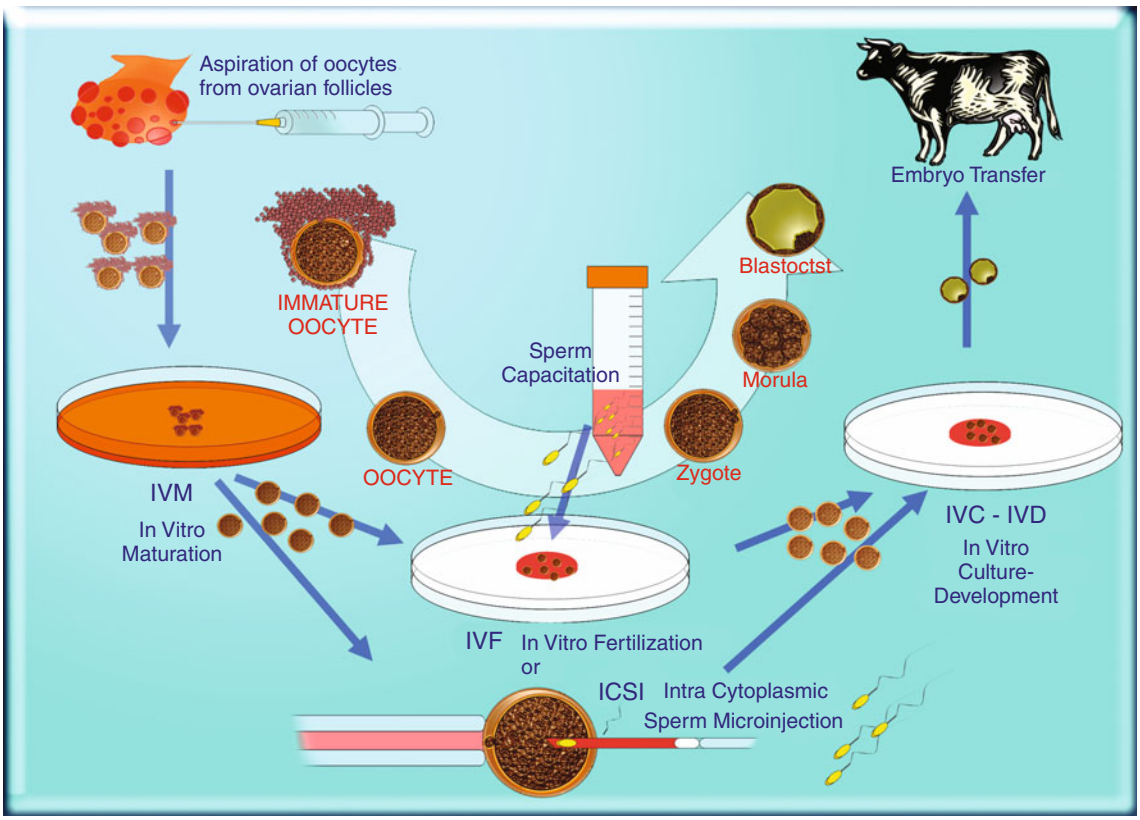
Assisted Reproduction Techniques

Reproduction is the key biological function that is exploited to make a GE animal. Other approaches can

be used, however, if the procedures do not target the germ line or the very early embryo then the genetic modification will not be inherited by the offspring and will be lost in the following generation. This is why the transgene has to be introduced either in the gametes or in an embryo at the very early stage of development, ideally when the embryo it is at the one-cell stage. Therefore, mastering assisted reproduction techniques is the key for the successful generation of GE mammals including livestock (Fig. 1).

Male Germ Line

Male germ cells are located in the testis. In the testis, all stages of development are found from spermatogonial stem cells to mature spermatozoa. Spermatogonia can be collected through a biopsy, cultured in vitro [11] and when are reintroduced in the testis they can give rise to functional spermatozoa [12, 13]. During in vitro culture, spermatogonial stem cells can be GE and later transplanted back to the testis to restore spermatogenesis [14]. More easily from sexually mature animals, spermatozoa are recovered from the ejaculate, extended with appropriate diluents, and stored for fresh insemination or cryopreserved for later use. This is a well-established technique in livestock for use in artificial insemination. Spermatogenesis continues after puberty throughout the life of the animals producing an unlimited amount of spermatozoa. In an alternative route, prior to artificial insemination, spermatozoa can be exposed to the transgene in vitro.



Transgenic Livestock Technologies. Figure 1

Summary of Reproductive Technologies (*in counterclockwise*) Ovarian immature oocytes are recovered from ovaries at slaughterhouse, matured in vitro by culturing in a specific medium, fertilized in vitro by co-incubation with capacitated spermatozoa or microinjected with a spermatozoa with a glass micropipette.The resulting zygotes are cultured in vitro until developed to blastocyst stage and finally transferred to the uterus of a synchronized recipient female by a surgical or a nonsurgical procedure depending on the species

Spermatozoa can bind DNA and then convey it inside the oocyte at the time of fertilization [15]. Although the technique is not always reproducible [16], in this simple way some success in making GE livestock has been reported [94–96].

Female Germ Line and the Embryo

In mammals, primordial follicles, the functional unit that contains the female germ cell, exist on the ovaries but only a tiny proportion of the oocytes are effectively generating a progeny while almost all of the remaining are lost during lifetime through atresia. For many years, several investigators isolated and investigated the biology of intact primordial follicles in rodents [17] and finally grew them successfully in culture [18]. This task proved much more difficult in large animals with bigger ovaries having a lot of connective tissue [19]. Successful development with the birth of live calves, albeit at a low rate, was later obtained by culturing bovine oocytes from more advanced (secondary) follicles [20]. The culture requirements for growing a complex tridimensional structure, such as an ovarian follicle, are so complex that the only progress that has been made is the culture in vivo of thin slices of the ovary following transplantation in humans where this now has a potential application in patients undergoing certain therapies [21].

From the late 1970s, it became clear that also in farm animals it was possible to exploit the oocyte reservoir present in antral follicles from the ovaries of slaughtered animals. Oocytes present in antral follicles have completed the growth phase and they are ready for maturation and fertilization [22, 23]. Initially, successful development was obtained by maturing the oocytes inside the follicles [24]. The subsequent understanding of the crucial role of the surrounding follicular/cumulus cells during the initial phases of maturation [25] lead to the development of a coculture system for in vitro maturation of extrafollicular oocytes obtaining a high proportion of such oocytes developing into live lambs after in vivo transfer [26]. After the successful achievement of in vitro maturation in sheep, the work was expanded investigating in the sheep the effects of gonadotropins [27], and to the cow [28–31] but also to the pig [32] and to the horse [33] and other species as well. Immature oocytes can also be recovered with the ovum pick-up technique in live donor females of different livestock species like cattle [34–36], buffaloes [37], and horses [38]. The development of in vitro maturation is today an irreplaceable, cost-effective and reliable source of mature oocytes for the production of embryos in vitro following in vitro fertilization and the various biotechnological applications connected to it.

Following the seminal work done in laboratory animals [39] in vitro fertilization (IVF) is well developed in farm animal species. Following the birth of the first IVF calf derived from in vivo matured oocytes [40] significant advancements were obtained in IVF when heparin was used as capacitating agent for bull sperm [41]. At about the same time, IVF became a reality in other large species except the horse where the success has been only exceptional and only one foal has been reported as the result of IVF. The horse eventually benefited of the development of intracytoplasmic sperm injection (ICSI) in humans [42]. The use of ICSI has been very efficient as a way to circumvent failure of in vitro fertilization with a high degree of efficiency [23], not matched in other farm animals like cattle [43, 44], sheep [45], or pigs [46] where the efficiency of ICSI for embryo production is lower as compared to IVF and, therefore, this technique is not used for routine practical application. But this is in fact the way to introduce together with the sperm also the DNA vector required to make the genetic modification [47]. In pigs on the other hand, IVF is characterized by a high incidence of polispermy that compromises embryonic development. This problem has been overcome in part by improving the quality of in vitro matured oocytes rather than effectively changing IVF conditions.

The immediate goal of increasing the efficiency of in vitro embryo production (IVP) especially in cattle has been the initial driving force of applied research in this field. However, it became soon obvious that merely counting the number of blastocysts was not an accurate measure of the quality of the overall procedure and of the viability of the embryos [48]. For many years, these constraints on the culture of viable cattle embryos in vitro were overcome by using in vivo culture in the oviduct of sheep [7]. In particular, a major concern after in vitro culture of IVF embryos was caused by the description of the so-called Large Offspring Syndrome (LOS), first in sheep and then in cattle [49]. The use of

serum supplementation and coculture were later recognized as the primary cause of LOS. Later on, an extensive field study [50] demonstrated that the incidence of LOS was greatly reduced using a SOF (Synthetic Oviductal Fluid) [51]-based formulation or similar formulations of the embryo culture media. These findings clearly indicated that in vitro culture can alter development at very early stage and that the Large Offspring Syndrome is correlated to abnormally advanced embryonic growth and gene expression pattern already at very early stages [52]. Nevertheless the in vitro fertilization and preimplantation embryo culture in vitro allow to generate the number of zygotes required either for DNA microinjection or for virus infection, procedures that would otherwise not be possible or be very difficult for livestock using in vivo produced zygotes from superovulated donors as it is done in laboratory mice.

Nuclear Transfer and Cloning

Somatic cell nuclear transfer, better known as cloning, is a technique that allows the generation of individuals with the same genome; thus technically speaking, cloned animals carry the same genome of the donor cell used for the SCNT process, thus they are like twins between themselves and twins of the animal who donated the somatic cell. The genome comes from the nucleus of a cell that can be taken from an embryo, a fetus, or an animal. The nucleus has to be placed into a mature oocyte, as outlined above whose chromosomes are removed before hand. The nuclear transfer step reconstructs an embryo, with its entire genome originating from the cell's nucleus that will subsequently develop like any other embryo conceived by fertilization albeit at a lower efficiency. Cloning by nuclear transfer that brought to the birth of Dolly the sheep [10] is a milestone achievement that has attracted the attention of the general public toward farm animal embryo technologies. This is one of the best examples where the search of new reproductive techniques in farm animals has also provided basic knowledge not previously obtained in laboratory animals. The fundamental work of cloning by nuclear transfer was done in amphybia [53] in the 1960s, however, significant advancements of the technology were achieved with embryo cloning of farm animals [54] when matured oocytes, instead of zygotes, became the recipients of the donor nuclei. Ten years later, it was again farm animals that, with somatic cell nuclear transfer, gave a major contribution to the basic understanding of cell reprogramming and epigenetic control of mammalian development, opening a new era in cell biology. The success with which different mammals were cloned was directly correlated to the availability of good quality mature oocytes and good quality embryos either after in vivo or in vitro culture. Then, it was merely a combination of already available technologies of gamete and embryo manipulation that lead to success and, for example, in the horse it was not until such technologies were developed that cloning was achieved in a consistent and reproducible way [55, 56]. In the last 10 years, the efficiency of somatic cell nuclear transfer as a whole has not improved much and its practical application is limited to the production of animals that have high added value like breeding stock [56–58] or for generating transgenic founder animals when nuclear transfer is combined with genetic engineering of somatic cells [59–62].

Understanding the constraints of nuclear transfer and developing the concept of reprogramming of fully differentiated somatic cells and the derivation of stem cells from cloned embryos [63, 64] for potential biotechnology and therapeutic applications, has fostered the idea of reprogramming differentiated cells in vitro directly without the need of the oocyte. A set of four genes has been demonstrated to be at the core of pluripotency and when transfected to somatic cells gave rise to induced pluripotent stem cells (iPS cells) [65]. These cells, of somatic origin, are extraordinary because they carry most of the properties of embryonic stem cells derived from the early embryo and represent a major breakthrough to overcome the limitation of embryonic stem cells derivation in humans and livestock.

Once the molecular basis of pluripotency will be fully understood, it will be a major step forward that is expected to lead to advances in nuclear transfer technology and cloning of farm animals for agricultural and biomedical applications on a larger, solid basis.

Embryonic Stem Cells

Embryonic stem cells (ES cells) are pluripotent cells that originate from the early embryo either from the inner cell

mass or from the early epiblast. ES cells are cultured and expanded in vitro in undifferentiated conditions, can be GE if necessary, and when reintroduced in the embryos can give rise to any cell type including the germ line, except trophoblast. This is why they are defined as pluripotent and not totipotent cells which is a feature of gametes, zygotes, and early cleavage stages blastomeres only. Today, mouse ES cells technology is the principal route to make GE offspring [8].

The derivation of farm animal ES cells, equivalent to those described for the mouse, has not been reported yet (therefore this route for GE is not available at present in livestock species but it might be so in the near future). However, over the years, from 1981 when mouse ES cells were first discovered [66, 67], many laboratories have attempted ES cell derivation mainly from cattle, pig, and sheep embryos [9, 68]. The original mouse approach has been extensively used and finally proven, with no doubt, unsuccessful although a few reports have been published on the derivation of so-called ES-like cells [69]. However, the stemness (pluripotency) of these cells appeared to be very limited and most likely they represent trophoblastic cells given their epithelial nature, loss of OCT4 expression, and limited differentiation potential [68–70]. The prominent tendency for neural differentiation often observed when attempting ES cell derivation from large animals is another challenge, but it represents an interesting and robust in vitro model for the study of early neurulation events in mammals [64].

In light of all the published reports and observations, it appears that the search for ES cells in farm animals should abandon the original mouse procedure and pay more attention to the recent findings for ES derivation in mouse and humans based on different signaling pathways and/or specific small molecules inhibitors. Advances in mouse and, more recently, in human ES cells culture have demonstrated that a number of different culture conditions can support pluripotency of embryo-derived stem cells. Mouse ES cells can be grown not only in serum-supplemented cultures with LIF and feeders but also in serum-free and feeder-free culture by the addition of BMP molecules. This second method has been developed following the finding that mouse ES cells grown in serum-supplemented medium and LIF can be differentiated in neuroectodermal cells by serum and LIF withdrawal [71]. The role of BMP molecules is to counteract and block the induction of neural differentiation and fix the undifferentiated state in a clever balance between conflicting inductive signaling pathways [71]. Along the same lines, a novel approach for ES derivation in the mouse has been developed on the rationale that the undifferentiated state can be maintained in culture by simply shielding the pluripotent cells of the embryos from endogenous pro-differentiation FGF4 signaling. This method is based on the use of specific inhibitors of the FGF signaling cascade in association with GSK3 inhibitors, the latter acting by improving cell viability. Both mouse and, remarkably for the first time, also rat ES cells have been obtained by inhibition of differentiation signaling introducing the concept that ES cells represent a ground-state pluripotency capable of self-renewing providing that differentiation signals are blocked [72, 73].

Other protocols, based on the stimulation of the nodal-activin signaling pathways, have been shown to maintain the undifferentiated proliferation of human ES cells and mouse epiblast stem cells (EpiSCs). The latter are a type of pluripotent embryo-derived stem cells derived from the egg cylinder as compared to ES cells that derive from inner cell mass or very early epiblast cells. Interestingly, mouse EpiSCs have been shown to be very similar to human ES cells in morphology, growth factors requirement, and gene expression [74, 75] while mouse ES cells differ considerably from human ES in culture requirements for the maintenance of the undifferentiated state, growth rate, and response to inductive signals. Another important difference between mouse ES cells and EpiSCs is the fact that only the former are capable of giving rise to chimeric offspring following blastocyst injection. A recent publication has reported the first success in the derivation of pig EpiSCs [76] and represents a significant step forward toward understanding the requirements for maintaining pluripotency of cultured epiblast cells from farm animal embryos.

In an applied perspective, embryonic stem cells in farm animals are important for several reasons but the most relevant is to provide a method to introduce precise genetic modification into animals by homologous recombination in ES cells [60] followed by blastocyst injection for chimera derivation and breeding, or by somatic cell nuclear transfer. A second important objective is to provide large animal models in which the ES cell technology can be tested for tissue-specific differentiation [77] and cell therapy of various tissues and organs.

Techniques for Genetic Engineering

The meaning commonly given to the term "transgenic" or "GE-" animal, indicate an individual bearing an exogenous fragment of DNA integrated in its genome and transmitting such modification to its descendant through a Mendelian fashion. Therefore, situations like "somatic transgenesis" will not be taken into consideration, where the genetic modification is limited to somatic tissues e.g., in case of gene-therapy protocols applied to adult individuals) or "episomal transgenesis", where the exogenous DNA is present inside the cell nucleus but as an autonomous replicating entity, whose stability and replication are separated from those of host's chromosomes.

The natural processes driving the integration of a transgene into the chomosomes of the host cell are mediated by a set of enzymatic function related to the DNA-repair pathways. The enzymes involved in these pathways are responsible of repairing the host's DNA molecule in case of double strand break (DSB). The resolution of a DSB inside the cell nucleus may follow two alternative pathways, catalyzed by different enzymes. The first and more common process involves the Non-Homologous End Joining (NHEJ) pathway, where the free ends of DNA generated by the DSB are relinked together. The exogenous DNA introduced in the nucleus may be recruited during this process and integrated between the endogenous arms of a broken chromosome. The integration of a transgene through the NHEJ pathway causes its random positioning into the genome of the host and the frequent integration of multiple copies of the transgene in the same locus, end by end. Both the random integration site and the generation of a multiple-copy array can influence the expression of the transgene in an unpredictable way. The alternative enzymatic machinery available in the cell for repairing DSBs is the Homologous Recombination (HR) pathway. It relies on the availability of a full copy of the broken molecule to provide a one-strand template to restore the original DNA sequence. This enzymatic activity is principally evident during gametogenesis (when it is involved in crossing over between sister chromosomes) and maintains higher level during early embryonic development compared to somatic adult cells.

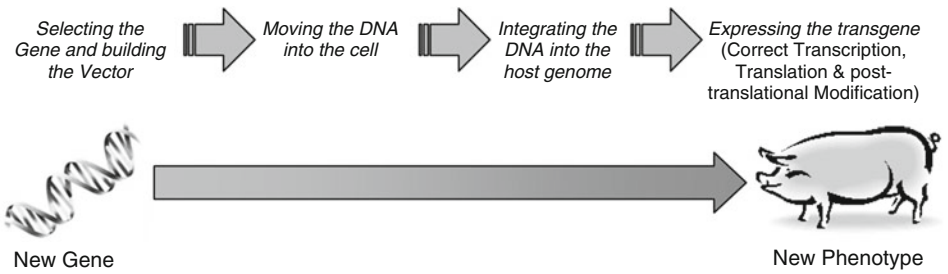
A transgene may be integrated by HR if it carries a region of homology with the target region in the host genome with the advantage of driving the insertion of the transgene in a specific locus and as a single copy. Nevertheless, this event is still rare compared to random integration by NHEJ. Even in ES cells, that retains a significant HR activity, the event of a targeted insertion of a transgene by HR is two to four orders of magnitude less frequent compared to its random integration.

For this reason, the process of gene targeting through natural HR can be performed only on cell cultures, where a large number of integration events can be generated and the cells carrying the desired event can be selected and isolated. The obvious next step to generate a transgenic animal is the capability of turning these cells into new individuals. In mouse, this has be accomplished by performing gene targeting in ES cells, that are able upon being introduced in a host embryo, to take part to the generation of a chimeric animal including part of its germ line and, as a consequence to pass the acquired mutation to its offspring [78]. As functional pluripotent ES cells have never been obtained from species different than the mouse, the possibility of performing gene targeting has been limited to this species until in 1996 Ian Wilmut and colleagues disclosed the way toward mammalian cloning by SCNT [79].

By then, even though somatic cells retain lower HR activity compared to ES cells, the option of performing gene targeting in this cells and to derive a whole animal from their genome has been demonstrated in swine, ovine, and bovine.

Since the generation of the first transgenic large animals by pronuclear microinjection, including transgenic pigs [3], major advances have been made, mainly by use of assisted reproductive technologies [80] as described earlier. Depending on the purpose of the genetic modification, the approach to transgenesis may aim to the random or the targeted integration of the transgene. In both cases, the obvious but sometime underscored consideration is that the final aim of the process is not only to insert a transgene into the genome, but also its proper *expression*. The hard track toward this is the result of intermediate steps, including (Fig. 2):

1. *Accurate design of the transgene*, involving the selection of the desired gene, its regulatory



Transgenic Livestock Technologies. Figure 2

Steps in obtaining a functional transgenic animal. The definition of transgenic is commonly associated with an organism bearing a new piece of DNA, but the true goal of the procedure is obtaining an animal expressing a new gene according to a desired pattern, that is, showing a new phenotype. Anticipating the final expression pattern of a transgene is still the weaker step in this technology (From [141])

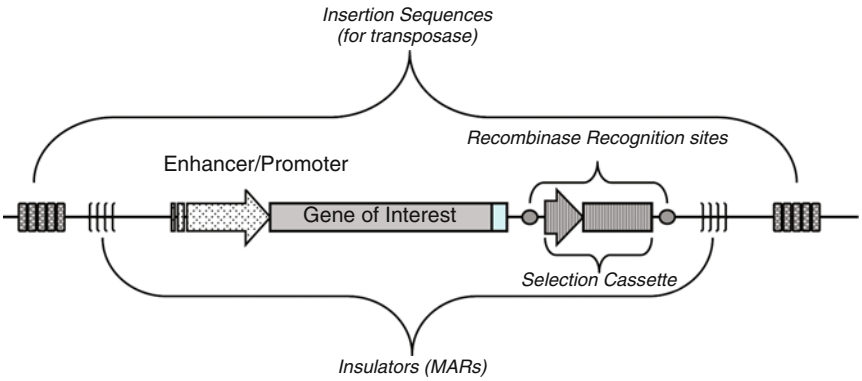
sequences, and all the additional sequences able to drive its integration and the expression of the desired phenotype in the right tissue at the right time

2. *Introduction of the DNA across the cell membrane* of the embryo or of the cell to be used in nuclear transfer
3. *Integration of the new DNA sequence into the genome*. This step may be mediated only by the endogenous NHEJ or HR pathways but, in some cases, a customized integration of the transgene may be artificially induced by the activity of exogenous recombinases or endonucleases specifically introduced into the cell
4. *Correct expression of the transgene* is dependent on the good implementation of all of the previously described phases and on a bit of luck. It is usually necessary to producing a large number of different integration events (i.e., embryos or cell clones) to allow the identification and the selection of the best performing, that will be used to generate and expand the desired transgenic line.

During the last years, the toolbox of the genetic engineer has expanded with new instruments allowing a better control on each of the described steps and an overall improvement on the whole procedure. Moreover, some genetic manipulation procedures initially available only for mice, have become concrete even for livestock.

Choosing the Gene and Assembling the Vector

Once the gene to be transferred has been identified, several alternative options are available to generate a transgenic vector (Fig. 3). The coding sequence of the gene itself may be used as cDNA, as genomic sequence (including introns) or as a minigene (a fusion of cDNA and genomic sequences of the same gene). The presence of one or more introns into the transgene, triggering the splicing process, is a major factor improving the transport of mRNA through the nuclear membrane and the effective expression of the exogenous gene [81, 82]. The regulatory sequences to be included into the transgene are chosen according to the need. The original promoter and enhancers may be conserved to drive the gene according to its original expression pattern, alternatively, heterologous promoters may be used to trigger a different tissue-specific or an ubiquitous expression. In case the gene has to be driven by its original regulatory sequences, the selected genomic fragment should be as large as possible to maximize the chance of a correct expression [83]. Indeed, if a gene has to be transferred from one species to another where the same regulatory signals are recognized, increasing the dimension of the fragment to be transferred allows to including not only the original promoter, but also distal enhancers and chromosomal modifiers [84, 85]. On the other hand, the large size of such DNA molecules influences the choice of the technique required for their introduction into the cell, due to the care required to purify and handle large and fragile DNA molecules. Although microinjection allows the introduction of such big molecules, larger than 100 Kbp, the efficiency of the procedure greatly decreases with the increase of DNA size and a significant part of the resulting transgenic animals



Transgenic Livestock Technologies. Figure 3

Generic structure of a transgenic vector. Beside the expression cassette for the Gene of interest (promoter, CDS and polyA site), additional sequences may be useful to increase the integration frequency of the vector (transposon-derived IS) or to support its regular expression (Insulators). Some features, like drug-resistance genes, are required for isolating cell clones but their persistence in the resulting transgenic organism is undesirable. They can be ablated by means of exogenous recombinases (From [141])

integrates only fragmented transgenes, due to shearing of DNA during injection and/or its enzymatic degradation before going into the chromosome. To improve the transfer of large DNA fragment into the genome, specific procedures have been developed, like the ICSI-mediated Gene Transfer (see below). Besides the selection of the gene and its regulatory sequences, additional features may be inserted into the transgenic vector. In case the transgene has to be introduced in cultured cells, it is often required the insertion of a selection cassette, providing resistance to a specific xenobiotic. As the integration of the vector in the genome of the cell is quite a rare event, the addition of a xenobiotic to the culture medium allows to grow only the transformed cell, that become resistant to it, and to eliminate the rest of the culture.

On the other hand, as the residual presence of this cassette in the transgenic animal may be deleterious or undesired, such cassette may be "floxed" (flanked by lox sites) for a subsequent removal through transient expression of Cre recombinase [86] (see further below).

Random integration of the transgene into the genome is recognized as the main source of variability in the expression of the exogenous genes, due to the influence of flanking genomic regions that may affect the expression pattern of the transgene or "switch it off" by epigenetic silencing. To avoid this behavior, insulator sequences may be inserted to shield the transgene from the influence of the chromosomal location [87–90]. Such insulators, called Matrix Attachment Regions (MARs), bend the transgene in a single transcriptional domain bound by its ends to the nuclear matrix. Additional features of the DNA construct can confer to the vector the behavior of a transposon, being recognized by a transposase that can be cotransferred or transiently co-expressed with the gene during gene transfer. This strategy will be described further below.

Transferring the DNA into the Cell

Pronuclear Microinjection

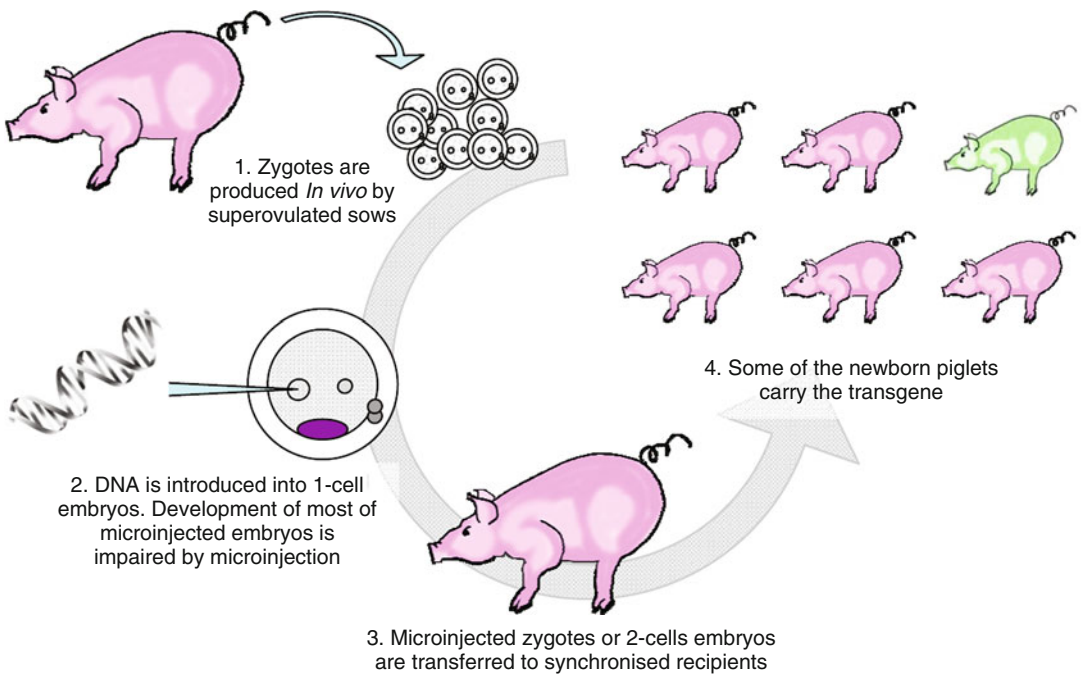
Pronuclear microinjection is now less commonly used in livestock, having been largely replaced by more efficient and less expensive techniques, after the demonstration of the possibility of cloning mammalians by nuclear transfer [10, 91]. Previously, microinjection was considered the most reliable technique to generate transgenic large animals, albeit inefficient and quite expensive in large animals. In vivo production of zygotes provides very viable embryos, but is an expensive approach. The data reported by Krimperfort concerning the generation of the first transgenic calf in 1991, starting from in vitro

produced embryos, evidenced the huge effort made in generating this animal.

Two thousand four hundred and seventy (2,470) oocytes were matured and fertilized in vitro to give 1154 zygotes that underwent microinjection; among them, only 129 developed and were transferred in 99 recipient cows. Twenty one (21) calves were born, only two of which were bearing the transgene. One of them died at birth. The other, a male called Herman, grew to adulthood and was mated, because its transgene, coding for human lactoferrin, was designed to be active into the mammary gland of transgenic cows. Sadly, the expression of this gene in the milk of Herman's daughters was extremely low, making the whole project useless. At the time, the cost of the whole procedure for producing such transgenic bull was estimated around 500,000 dollars.

Different factors may affect the expression of microinjected transgenes, considering that they integrate in random sites of the host genome. The disadvantage compared to what happens for gene transfer in cultured cells, in detecting the transgene integration ahead of embryo transfer, although possible, is not practically convenient following pronuclear microinjection, let alone the assessment of its expression. For this reason, most of the animals resulting from microinjection protocols are non-transgenic (Fig. 4).

Last but not least, obtaining embryos bearing targeted transgene integration, until recently, was considered nearly impossible by this approach. DNA integration upon standard microinjection is achieved mostly via NHEJ, resulting in a random positioning of the transgene into the genome and a consequent



Transgenic Livestock Technologies. Figure 4

Transgenic production by pronuclear microinjection One-cell embryos are recovered from superovulated females. Such zygotes shows high viability but their production in vivo is somewhat expensive. Embryos are centrifuged to reveal pronuclei and DNA is microinjected into one of them. Microinjection of DNA affects embryo viability. Surviving embryos are transferred in synchronized sows. The proportion of transferred microinjected embryos developing to viable offspring is low and variable, moreover only small fraction of them carries the transgene. The pattern of expression is unpredictable. The overall efficiency of this procedure in terms of living transgenic/microinjected embryos usually ranges from 1% to 4% (From [141])

unpredictable effect of the flanking regulatory elements on the expression of the transgene itself. Recent advances in enzymatic engineering has demonstrated the possibility to overcome this limit obtaining the targeted integration of a transgene by means of Zinc Finger Nucleases [92] as discussed below.

Sperm-Mediated Gene Transfer (SMGT)

In 1989, Lavitrano and colleagues reported a new approach to generate transgenic mice based on pre-incubation of spermatozoa with exogenous DNA followed by in vitro fertilization [15]. This unconventional approach was accepted with skepticism by many scientists working in the field of transgenesis [16, 93]. In spite of that, other authors reported success in obtaining transgenic animals by variations of SMGT protocol [94, 95]. After few years, the same technique was adapted to the production of transgenic pigs bearing a hDAF transgene [94–96]. The benefits of this technique, compared to pronuclear microinjection, are low cost and ease of use. Nevertheless, the insertion is still random and the transgene can be rearranged, thus affecting the expression levels. The long-term expression of the transgene remains controversial [97].

ICSI-Mediated Gene Transfer (ICSI-MGT)

ICSI-mediated gene transfer is a technique sharing some analogies with SMGT and microinjection, where both the transgene and the sperm head are introduced with a micropipette into the cytoplasm of the oocyte [47]. In mice, ICSI-MGT is more efficient than standard microinjection and can be particularly effective when a large construct (ranging from 100 kb to more than 0.5–1 Mb, i.e., YAC, BAC, microchromosome) has to be transferred [98]. Together with the use of polyamines to condense DNA, the large size of the micropipette used for injecting the sperm head preserves DNA integrity minimizing mechanical shearing.

Although assisted reproductive technologies (Fig. 1) in pig still suffer some limits, possibly due to specific requirements in embryo culture conditions yet to be defined, some researchers succeeded in producing transgenic pigs by co-incubating sperm with a DNA vector and microinjecting the spermatozoa directly into the ooplasm. After the transfer of 702 embryos into 5 gilts, 2 out of 35 fetuses recovered were transgenic. In vivo production of zygotes provides very viable embryos, but is an expensive approach and requires the use of animals. Nevertheless, this is the preferred source of embryos for this procedure, because microinjection by itself heavily impairs the subsequent embryonic development and decreases the number of newborns following embryo transfer [99].

Viral-Mediated Transgenesis

During their life cycle, retroviruses are able to enter the cytoplasmic membrane of a target cell by binding specific receptor, to reach the nucleus and to retrotranscribe a DNA molecule using their RNA genome as a template. This DNA molecule will often integrate into the host genome.

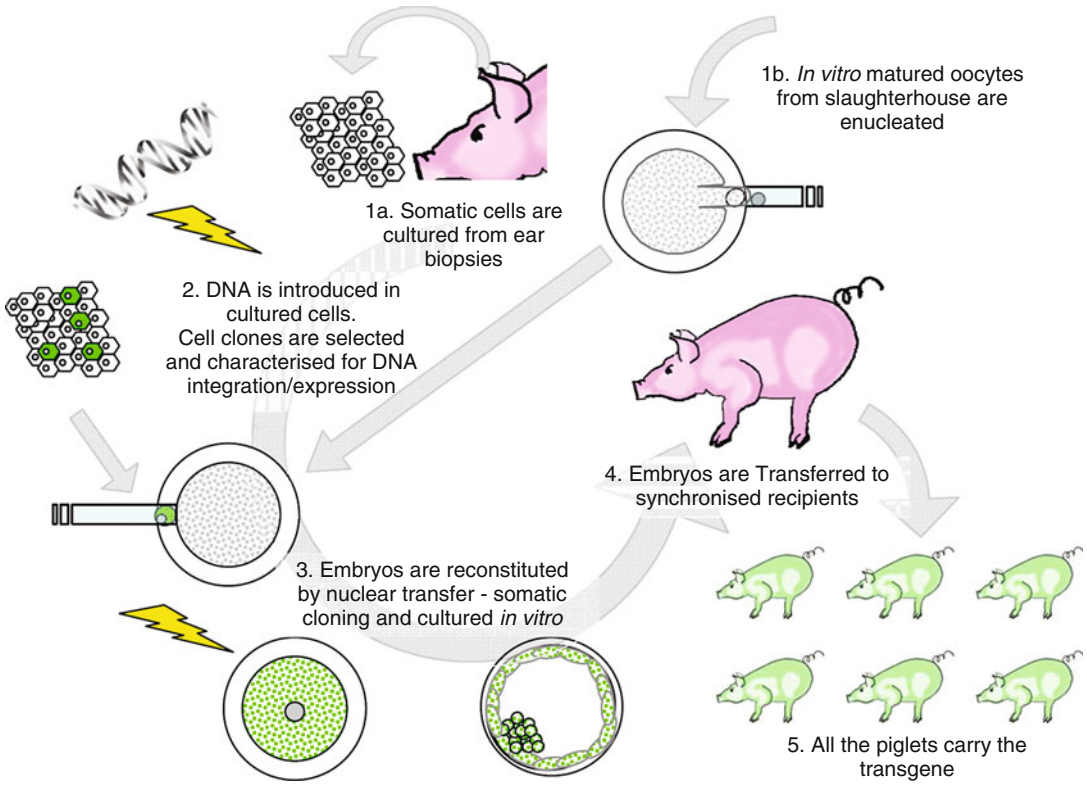
Mimicking such behavior, retroviral-derived vectors have been engineered to carry a specific transgene to be integrated into the target genome, but lack the genes required for completing their replicative cycle. Such retroviral vectors, initially developed to target somatic cells for human gene therapy, can also infect preimplantation embryos, providing that the zona pellucida is removed or at least breached, since it is a barrier to the viruses. The retrovirus integrates its complementary DNA into the genome of the embryo. The cell cycle of the infected cell may be susceptible or not to integration depending on the origin of the selected viral vector. For this reason, early trials with retroviral vectors resulted in mosaic animals, due to the failure of these vectors to enter the genome during one-cell stage but only during subsequent embryo divisions. A more recent generation of viral vectors is based on the structure of lentiviruses like HIV. Lentiviral gene transfer is extremely efficient, with 80–100% of the animals being born transgenic after oocyte or embryo, or somatic cell culture infection@ [100]. Lentiviruses have been used in a variety of experiments to transduce cells with various transgenes. These experiments include siRNA knock-down for stem cells and for somatic cells, and nuclear transfer (see below) for generating desired modifications. Lentiviral transgenesis is one of the main techniques currently proposed for somatic gene therapy. Such approach is associated with known risks and observed limits. During clinical trials, retroviral transgenesis has been associated with oncogene

activation by insertional mutagenesis [101, 102]. To reduce this risk, the interest of the researchers is moving toward replication-deficient vectors [103]. An additional concern about the use of lentiviral vectors is their possible recombination with latent wild retrovirus to generate unpredictable infectious or mobile particles. Among virus-derived vectors, lentiviral (LV) have the property of infecting cells both during replication and in quiescent phase. During lentiviral-mediated transgenesis, the use of some drugs like cytokines or proteasome inhibitors can increase LV gene transfer [60, 104]. Santoni de Sio and colleagues [104] have shown that human hematopoietic stem cells (HSCs) can be transduced to high efficiency by a short exposure to LVs in the presence of SCF, TPO, IL-6, and Flt3L. Moreover, it was shown that the proteasome restricts LV transduction in HSCs and that using the reversible peptide–aldehyde proteasome inhibitor MG132 and the peptide-boronate inhibitor PS-341 during the LV-GFP transduction period, there is a substantial drug-dose-dependent increase in the frequency of transgene expressing cells and in their mean fluorescence intensity [104].

The use of lentiviral vectors for transgenesis results in a significant degree of mosaicism among transgenic newborns, due to the possible multiplicity of integration events during the first cleavages of the embryo [105]. Even though lentiviral vectors enter as a single copy into their insertion site, their integration mechanism is very efficient, frequently resulting in multiple copies integrated in different part of the genome and in a complex pattern of transmission to the offspring [105]. Due to their random positioning into the chromosomes, lentiviral vectors may still undergo epigenetic silencing by methylation [106]. Another class of effective vectors for transgenesis was derived from the adeno-associated virus (AAV). AAV-derived vectors have the advantage to cross the cell membrane delivering single stranded DNA molecules straight to the nucleus. Some adeno-associated viruses are peculiar in their behavior of targeting specific integration site in the host genome [107]. Adenoviral ssDNA shows a high effectiveness in integrating into the host genome through homologous recombination, even in somatic cells [108, 109]. Recently, Rogers et al. produced a CFTR-null pig using AAV-mediated gene targeting and SCNT [110, 111]. These authors generated a pig with both null (knock-out) and ΔF508 (knock-in) modifications. Gene targeting using the AAV approach has resulted in a very efficient strategy for obtaining knock-out of the CFTR gene that is not expressed in fibroblasts.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) has become the leading tool for generating animals from GE somatic cells (Fig. 5). SCNT works better in pigs than in other large animals [112, 113]. A recent innovation to make the technique user-friendly is the zona-free system. After zona removal, enucleation can be performed with a micropipette, for subsequent zona-free fusion, activation, and culture [113, 114], or by cutting the cytoplasts for handmade cloning [115, 116]. Major limitations are represented by the lack of embryonic stem cell (ESC) technologies. Somatic cells are currently being used in the procedures, but they have a limited life span, thus restricting the time the cells can be cultured in vitro for genetic engineering. Fibroblasts (both from fetal and adult origin) have a fine life span and can undergo a maximum of 40–50 population doubling in vitro while maintaining a normal kariotype. Such number of population doublings allow only for a round of cell transfection followed by drug selection to identify a clonal cell population with the desired modification. In fact by the time this has been achieved, the cells have spent most of their proliferative capacity and have to be used for SCNT before they reach complete senescence. So, the use of fibroblasts limits this approach to one modification in vitro at the time since they would not survive a second round of transfection. After SCNT, the fibroblasts are rejuvenated and have fully restored their proliferative capacity. At this point, cloned fetuses are recovered to establish a new fibroblast cell line that can be then subjected to a new round of genetic modification. This process can be repeated several times with apparently no adverse effects [59]; however it requires time and animals. Relating to this topic, recent reports describe the derivation of induced pluripotent stem cells (iPSC) from pig fibroblasts [117–119], however the objective is still far since culture conditions to maintain livestock embryonic stem cells are not known (further discussed below). The opportunity of performing the whole genetic engineering on cell cultured in vitro and finally transferring the manipulated genome to the enucleated oocytes allows selection for gene targeting events, both



Transgenic Livestock Technologies. Figure 5

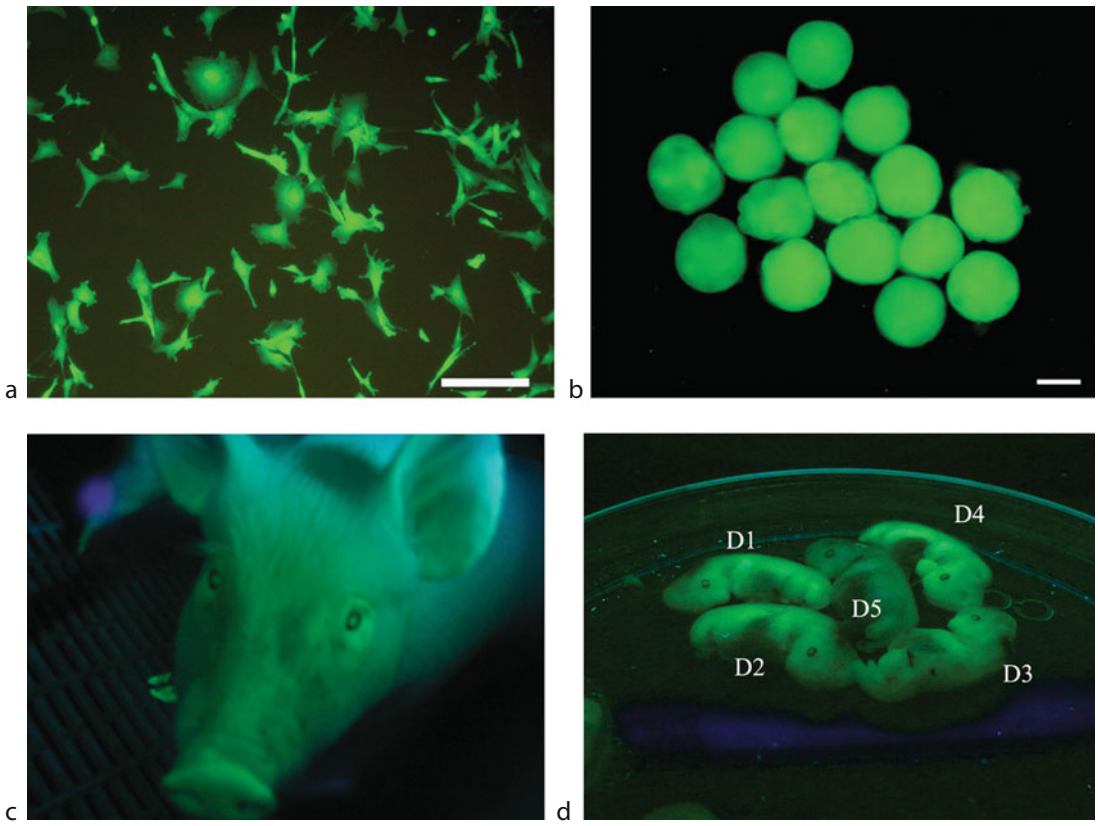
Transgenic production by SCNT. The transgene is transferred into the genome of cultured fibroblasts. Transgenic cell clones are isolated and characterized. This first step is relatively inexpensive. A more accurate prediction of the transgene expression is possible. Next, embryos are reconstituted by SCNT, cultured and transferred in synchronized sows. Although the viability of cloned embryos is variable but usually poor, all of the resulting newborns are transgenic (From [141])

for *knocking out* an unwanted gene or for *knocking in* a transgene in a predetermined reliable position, guaranteeing its best expression.

A similar model, implementing the 1,3 galactosiltransferase knockout in pigs was developed by Takahagi and colleagues that knocked out the first allele of this gene in porcine fetal fibroblast already expressing hDAF and N-AcetylglucosaminylTransferase -III (GNT-III) [120]. In a further step, the authors selected cultures for spontaneous null mutations of the second allele occurring in vitro, and used these cells for nuclear transfer. In this way, the resulting cloned pigs were transgenic for hDAF and GNT-III on a 1,3 galactosiltransferase null background [121].

SCNT technology overcomes many limitations of previous procedures, making possible the gene targeting approach to livestock genome and providing a less expensive way to perform genetic engineering. Indeed, most of the cost and the efforts related to transgenic technology applied to large animal are derived from housing animals, preparation for embryo transfer up to the birth and weaning of the newborn animals.

The opportunity of using cell clones fully characterized in term of transgene integration and expression, guaranteeing 100% of transgenics among born animals, greatly increases the efficiency of the protocol and drops the costs when an ubiquitous or inducible promoter is used and the expression is maintained in subsequent generations through germ line development (Fig. 6) [112]. On the other hand, SCNT is not the final solution for every problem, as most of the matter concerning the proper activity of the transgenes, with regard to its temporal and tissue-specific expression, still needs to be completed on the whole animal.



Transgenic Livestock Technologies. Figure 6

Successful selection of GFP transgenic cells and transmission through the germline of the transgene to the next generation. Clonal fibroblasts selected for high expression of GFP (**a**), GFP expression in embryos after SCNT (**b**) and in the resulting animal (**c**). Fetuses obtained after breeding the GFP boar to a wild type sow (**d**). Fetus D1, D2, D3, D4 are transgenic and express the same high level of GFP of the original boar, D5 is a wild type fetus negative for GFP (From [141])

Possible improvements to overcome these problems are offered by recent advances in genetic engineering.

Emerging Technologies

Several new technologies becoming available may be of great benefit in the future to make GE large animals.

Induced Pluripotent Stem Cells (iPS Cells)

In 2006, a breakthrough study [65] demonstrated that viral transduction of a handful of genes (Oct4, Sox2, Klf4, and c-Myc) can reprogram mouse embryonic fibroblasts into ES cell-like cells which carry all the molecular features of true embryo-derived ES cells including the ability to give rise to germ line chimeras. The following year the generation of human iPS cells was achieved [122, 123] using slightly modified transduction methods and set of genes. Since then, several groups have contributed to the field with new combinations of reprogramming genes, cell types, and viral and nonviral delivery systems. More recently, the addition of small molecules acting on chromatin structure, such as valproic acid, a histone deacetylase inhibitor has allowed further progress by showing that only Oct4 and Sox2 are required for reprogramming of human fibroblasts [124]. Several other small molecules acting on chromatin or on specific signaling pathways have been investigated to improve the efficiency and safety of iPS cells technology (see as review [125]). In large animals, attempts to derive iPS cells have been made resulting in a few reports about induced

reprogramming of pig fibroblasts [117–119]. Although promising data have been presented, the evidence of full reprogramming, measured by sustained activation of endogenous genes and silencing of reprogramming genes, has not been achieved. Most likely, the lack of robust procedures for the establishment embryo-derived ES cells in large animals represents a major limit also for the development of the iPS cells technology. More detailed knowledge on the role of pluripotency genes in the early embryo is needed to advance the field of induced pluripotency not only in the mouse [126] but also in large animal species.

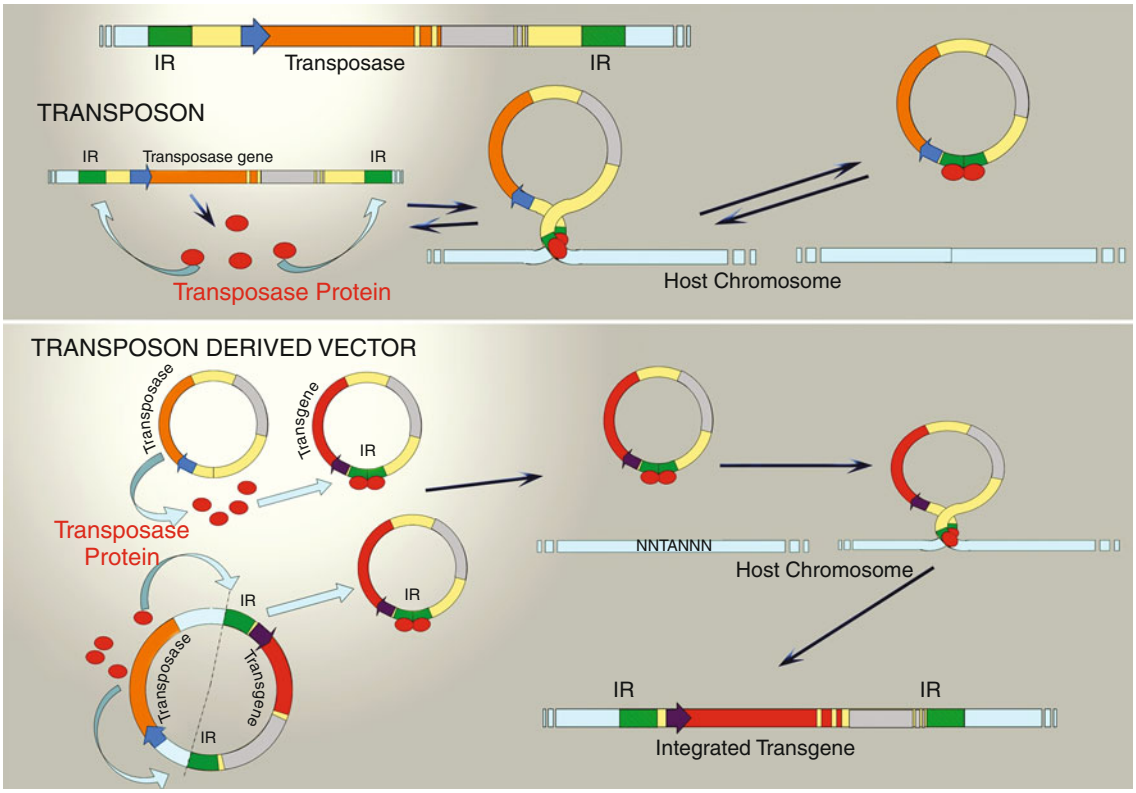
Enzymatic Engineering

Transposons Transposons, called also "jumping genes" are mobile genetic elements; class II transposons are small segments of DNA able to move across the genome of a cell from one region to another, by means of the action of enzymes (transposase) encoded within the transposon itself or supplied in trans by another source (Fig. 7). Transposons have been found in many living organism, from bacteria to plants and animals. The simplest autonomous replicating transposons in vertebrates, like those belonging to TC1/Mariner class, are composed by two inverted terminal repeat flanking a sequence coding for a specific transposase. Although the movement of transposons across different organism does not seem to be a common capability, philogenetic studies strongly suggest that "jumps" of transposons across species have happened during evolution [127]. Modified transposons, like "Sleeping Beauty" and "PiggyBac" have been largely used for precise and efficient delivery of DNA expression cassettes in vertebrate cells. Sleeping Beauty (SB) belongs to the TC1/Mariner class of transposons and these transposases require a TA dinucleotide base pair as integration site, a sequence that is duplicated during the integration process. The SB transposon system consists of two components: (1) a defective transposon, made up by gene of interest flanked by inverted repeats (IRs) but lacking the transposase gene, and (2) a source of transposase. During transposition, the SB transposase recognizes the ends of the IRs and excises the transposon from the delivered plasmid DNA inserting it in to another DNA site.

In a recent study, it was shown that co-transfection of PEGE cells with Sleeping Beauty (SB), Passport (PP) Tol2 and PiggyBac (PB), with their corresponding transposase expression constructs, resulted respectively in 13.5-, 5-, 21-, and 28-fold increases over transfection without transposase [128]. In addition to increasing the efficiency of integration, transposase-mediated transgenesis precisely integrates a single copy of the transposon into one or more locations in the genome, avoiding transgene concatemerization that can cause shutdown of gene expression.

Cre/LoxP Recombinases The integration site of a transgene strongly influences its expression pattern. To overcome the gamble of a random integration, it is possible to target the insertion of the transgene to a transcriptionally active location of the genome, avoiding the risks of both silencing the transgene and disrupting an endogenous gene (insertional mutagenesis).

One possible strategy to obtain this goal is the use of phage recombinases like Cre or FLP that catalyze a conservative DNA recombination event between two short recombinase recognition sites (RRS), loxP and FRT (Fig. 8), respectively. Such enzymes, used by bacteriophages during their infection cycle, allows the excision or inversion of the DNA between two RRSs, depending on their orientation [128]. The artificial modification of the sequences of the parental RRSs has allowed to develop the so-called Recombinase-Mediated Cassette exchange (RMCE), a protocol to modify a specific locus in the genome after an initial "tagging" by the introduction of a pair of incompatible RRSs [129]. In this way, (1) a cell line is modified by inserting a RRS-flanked reporter gene in different random position, (2) the deriving clones are screened to select the one presenting the best integration site, and (3) the gene of interest can be exchanged with the reporter to assure its proper expression. A predictable modification of the genome may be realized by driving the process of DNA repair through homologous recombination (gene targeting). This process, largely used in mouse ES cells, allows the interruption of endogenous sequences (knock-out) or the insertion of new genes (knock-in) in a specific locus [130, 131]. Unfortunately, the HR pathway is much less efficient in somatic cells like

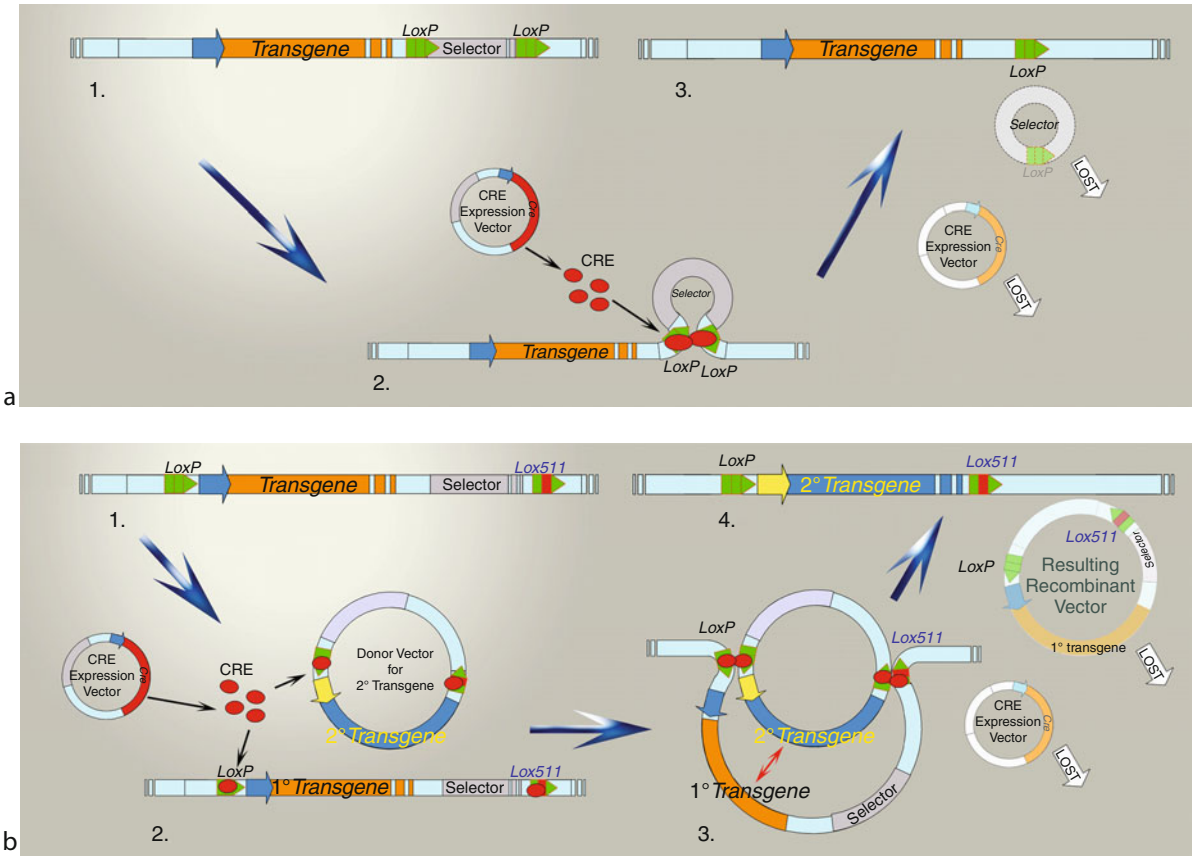


Transgenic Livestock Technologies. Figure 7

Integrative vectors based on Transposon signals *Top*: Natural occurring DNA-transposons consists of a common minimal structure represented by a gene coding for a specific transposase (TP) and two terminal inverted repeats (IRs). Following the expression of the gene, its product is able to bind to the inverted repeats, inducing the circularization and the excision of the complete DNA segment surrounded by IRs. Depending on the type of transposon, excision can leave small footprint in the chromosome or restore the exact original sequence. Following excision, the circular transposon, still bound to transposase, can integrate in a new site of the genome. In presence of transposase, both the excision and the integration of the transposon are catalyzed. Some transposons, like PiggyBac, prefers transcriptionally active genomic region for their integration. *Bottom*: A Transposon-derived vector is represented by a transgene flanked by a pair of IRs, but lacking the transposase CDS between them.The integration of this vector can be obtained with high efficiency by expressing the specific transposase *in trans* – on a different vector (**a**) – or *in cis* – on the same vector but by a cassette located outside the IRs-delimited portion (**b**). The transiently expressed exogenous transposase promotes the integrative cycle of the vector but, due to the absence of a stable source of protein, the integration of the vector is irreversible (From [141])

fibroblasts, making this procedure much harder, in particular for genes that are inactive in the target cell type not allowing the use of gene-trap (promoterless) approaches.

Zinc Finger Nucleases (ZFNs) Zinc finger nucleases (ZFNs) show promise in improving the efficiency of gene targeting by introducing DNA double-strand breaks in target genes, which then stimulate the cell's endogenous HR machinery. Zing finger nucleases are hybrid proteins containing an array of zinc-finger DNA-binding domain and a FokI endonuclease domain (Fig. 9). The DNA zinc-finger domains are designed to recognize a specific sequence, inducing a double stranded break in the target site. Such break promotes a local DNA repair activity that is efficiently accomplished by HR if a template with homologous sequence is provided. Many studies have been



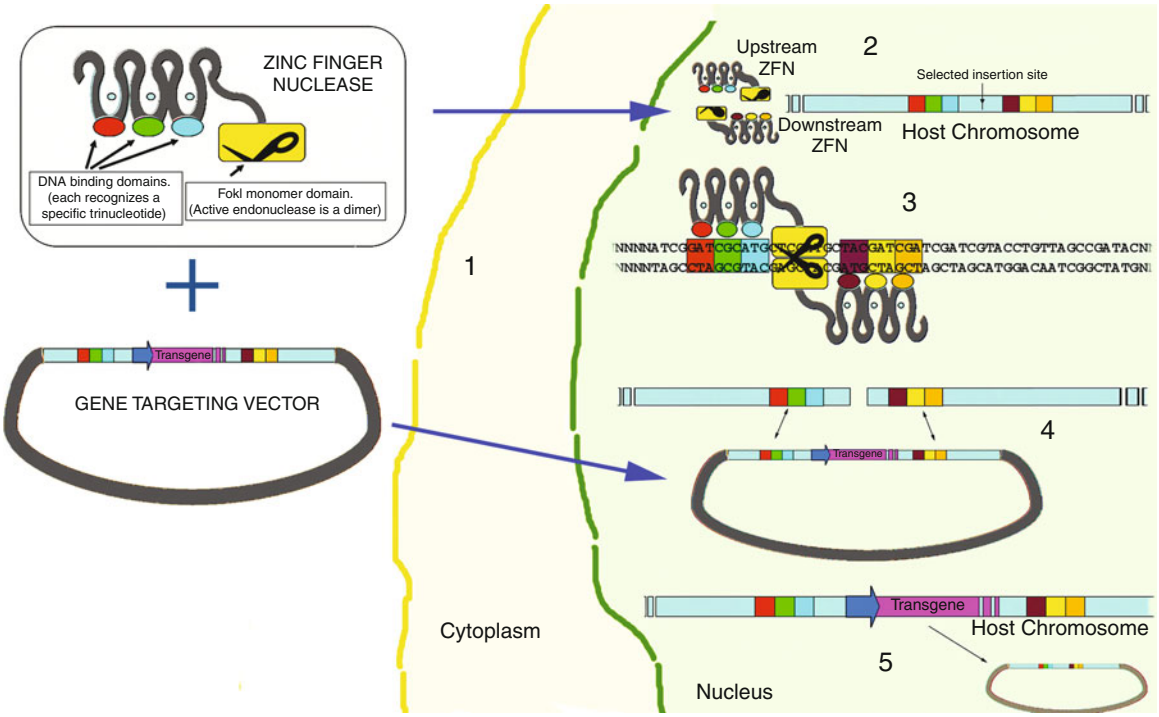
Transgenic Livestock Technologies. Figure 8

Targeted rearrangement of an integrated transgene by Cre recombinase. (**a**) Homologous Lox sites in the same orientation allows the excision of the enclosed region when Cre is expressed (**b**) Heterologous Lox sites do not allow excision but promote RMCE (Recombinase Mediated Cassette Exchange) if a vector bearing a Lox-flanked transgene is introduced together with CRE

developed in human and mouse cells [60, 132]. A recent paper demonstrates the possibility of generating transgenic rats by pronuclear microinjection of specific ZFN expression vectors together with a gene targeting construct [92].

An additional requirement in developing transgenic livestock is the need of expressing multiple transgenes in a coordinate pattern in the same animal. As previously noted, protocol bringing to random integration of multiple transgenes, often result in similarly random level of expression, with some transgenes working and some other not. A strategy to speed multiple transgene integration is represented by recent adaptation of the 2A system from foot and mouth disease virus (FMDV) to mammalian transgenic technology [133, 134]. In this system, the open reading frame (ORF) consists of multiple individual cDNAs separated by sequences encoding 2A and furin cleavage sites. A single complex mRNA is produced and translated into a single polypeptide that is cleaved into individual exogenous proteins at the 2A sites.

siRNA In cells transfected with siRNA vectors, targeted mRNAs are degraded by endonuclease activity and the amount of protein translated may be reduced by over 95%, thus resulting in a significant knockdown and is an alternative approach to achieving more complex and difficult knock-outs (KO). This technique is particularly useful when more than one



Transgenic Livestock Technologies. Figure 9

Homologous Recombination via Zinc Finger Nucleases (ZFNs) 1. A gene targeting DNA vector is prepared, where the transgene is flanked by DNA sequences homologous to the target locus. In different vectors, two ZFNs are designed, each coding for a specific DNA binding region and a FokI endonuclease monomeric domain. The two sequences recognized by the ZFNs are positioned upstream and downstream the selected insertion site in the target locus. The ZFN is introduced into the cell by microinjection, electroporation or transfection of a DNA expression vector or of a mRNA transcript. 2. The mRNAs are translated in two Zinc Finger Proteins. Each "finger" motif of the ZFN recognizes a sequence of three nucleotide (in this example, three "fingers" recognize a 9 bp target). A single ZFN binding to the DNA does not produce any effect. 3. When both the ZFN bind the DNA at the right distance, FokI domains can dimerize and produce a Double Strand Break in the target site. 4. DNA repair on the DSB can proceed through the NHEJ pathway, but, due to the availability of homologous sequences provided by the targeting vector, can also follow the route of Homologous Recombination 5. By Homologous Recombination, the transgene is introduced into the target site. The endogenous allele and the vector backbone sequences are lost (From [141])

copy of the endogenous gene is present and the usual KO approach is not feasible. This is indeed the case of pathogens like porcine endogenous retrovirus (PERV) [135–137]; for example, "knock-down" of PERV expression has been shown in transgenic pigs expressing siRNA corresponding to the viral pol2 sequence [138]. This approach could even be of interest in generating transgenic animals resistant to specific viral pathogens, by steady expression of virus-inhibiting siRNA molecules in their cells.

Future Directions

The GE of livestock is slowly making progress toward possible applications both is science and industry. SCNT has been the major advancement in this field for the last 25 years. Nevertheless, many are the issues that need to be addressed to make GE of livestock robust, reproducible, and affordable. Assisted reproduction techniques are continuously improved and refined, however, the availability of true embryonic stem cells is still the one of the

limiting factors for precise GE and the recent report of alternative sources like iPS cells have yet to prove their value for this purpose and it might take a long time before they become a reality. The designing of more effective DNA delivery vectors relies on the sequence of the genome. In livestock, this is lagging behind that of the human or mouse, therefore, it is not always possible to translate discoveries made in those species to livestock unless genomic information are generated by homology and partial sequencing. The ability to target the insertion of the transgene into a locus on a chromosome to assure its expression also through generations would be a significant advancement since today few of the GE livestock expresses the inserted transgene at the level desired and the expression is often not stable.

The ability to target specific genes either by knockout through HR or knockdown by siRNA, will make it possible to develop functional genomics (i.e., understanding the function of a particular gene) in mammalian species closer to the human other than the mouse and to generate large animal models of human diseases, especially for those diseases that the mouse model has failed to reproduce the human phenotype [110, 139]. Animal models are required both to understand the pathogenesis of a disease, to develop and test possible new therapeutic approaches to it. Biotechnological application of GE animals again for biomedical application include the field of xenotransplantation, i.e., the possibility to transplant tissues and organs from one species (usually the pig) to humans [140, 141]. This requires multiple GE to make pig organs accepted by the human immune system. Another biotech application is the GE of livestock to express antibodies or proteins that have a therapeutic use in human cancer or other diseases [59] in sufficient quantities and affordable costs that would not otherwise be possible by other means.

Although the biomedical field is the more advanced and more receptive for this new technology, there are also potential applications in agriculture for breeding and selection of livestock. Proof of principle have been already obtained for increasing yield in cheese production [62] or better quality pork meat [142] as well as for disease resistance [61, 138].

GE of livestock is an expanding area of research with great potential for applications in the biomedical, biotechnology, and agricultural fields. This technology provides a tremendous potential to select and breed animals with specific genetic makeup much faster than by conventional breeding schemes; however, this will be possible when full understanding of the underlying biological mechanisms will be known.

Acknowledgments

Grant support was received from EU Xenome Project (LSHB-CT-2006-037377), EU PluriSys (n°223485), EU ESNATS (n° 201619), Regione Lombardia and Cariplo Foundation (NOBEL).

Bibliography

1. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 77:7380–7384
2. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611–615
3. Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
4. Simons PJ, Wilmut IA, Clark J, Archibald AL, Bishop JO, Lathe R (1988) Gene transfer into sheep. Biotechnology 6:179–183
5. Wall RJ, Pursel VG, Shamay A, McKnight RA, Pittius CW, Hennighausen L (1991) High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. Proc Natl Acad Sci USA 88:1696–1700
6. Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van den Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R (1991) Generation of transgenic dairy cattle using 'in vitro' embryo production. Bio/technology (Nature Publishing Company) 9:844–847
7. Galli C, Lazzari G (1996) Practical aspects of IVM/IVF n cattle. Anim Reprod Sci 42:371–379
8. Capecchi MR (1989) Altering the genome by homologous recombination. Science 244:1288–1292
9. Galli C, Lazzari G, Flechon JE, Moor RM (1994) Embryonic stem cells in farm animals. Zygote 2:385–389
10. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813
11. Brinster RL (2007) Male germline stem cells: from mice to men. Science 316:404–405
12. Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA 91:11298–11302
13. Dobrinski I (2008) Male germ cell transplantation. Reprod Domest Anim 43(Suppl 2):288–294
14. Brinster RL (2002) Germline stem cell transplantation and transgenesis. Science 296:2174–2176

15. Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C (1989) Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57:717–723
16. Brinster RL, Sandgren EP, Behringer RR, Palmiter RD (1989) No simple solution for making transgenic mice. Cell 59:239–241
17. Roy SK, Greenwald GS (1985) An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. Biol Reprod 32:203–215
18. Eppig JJ, O'Brien MJ (1996) Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 54:197–207
19. Lazzari G, Galli C, Moor RM (1994) Functional changes in the somatic and germinal compartments during follicle growth in pigs. Anim Reprod Sci 35:119–130
20. Miyano T, Manabe N (2007) Oocyte growth and acquisition of meiotic competence. Soc Reprod Fertil Suppl 63:531–538
21. Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Yemini Z, Dor J (2007) Monitoring the ovaries after autotransplantation of cryopreserved ovarian tissue: endocrine studies, in vitro fertilization cycles, and live birth. Fertil Steril 87:418 e417–418 e415
22. Galli C, Moor RM (1991) Development of immature bovine oocytes into viable embryos in vitro. Bull 'Association Anat 228:67–71
23. Galli C, Colleoni S, Duchi R, Lagutina I, Lazzari G (2007) Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. Anim Reprod Sci 98:39–55
24. Moor RM, Trounson AO (1977) Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J Reprod Fertil 49:101–109
25. Moor RM, Osborn JC, Cran DG, Walters DE (1981) Selective effect of gonadotrophins on cell coupling, nuclear maturation and protein synthesis in mammalian oocytes. J Embryol Exp Morphol 61:347–365
26. Staigmiller R, Moor R (1984) Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. Gamete Res 9:221–229
27. Galli C, Moor RM (1991) Gonadotrophin requirements for the in vitro maturation of sheep oocytes and their subsequent embryonic development. Theriogenology 35:1083–1093
28. Carolan C, Monaghan P, Gallagher M, Gordon I (1994) Effect of recovery method on yield of bovine oocytes per ovary and their developmental competence after maturation, fertilization and culture in vitro. Theriogenology 41:1061–1068
29. Lu K, Polge C. A summary of two-year's results in large scale in vitro bovine embryo production. Proc 12th Inter. Cong. Animal Reproduction, The Hague 1992:1315–1317
30. Goto K, Kajihara Y, Kosaka S, Koba M, Nakanishi Y, Ogawa K (1988) Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in-vitro matured follicular oocytes. J Reprod Fertil 83:753–758
31. Xu KP, Greve T, Callesen H, Hyttel P (1987) Pregnancy resulting from cattle oocytes matured and fertilized in vitro. J Reprod Fertil 81:501–504
32. Moor RM, Mattioli M, Ding J, Nagai T (1990) Maturation of pig oocytes in vivo and in vitro. J Reprod Fertil Suppl 40:197–210
33. Zhang JJ, Boyle MS, Allen WR, Galli C (1989) Recent studies on in vivo fertilization of in vitro matured horse oocytes. Equine Vet J 8:101–104
34. Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G (2001) Embryo production by ovum pick up from live donors. Theriogenology 55:1341–1357
35. Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, Willemse AH, Taverne MA (1991) Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 35:857–862
36. Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 43:141–152
37. Galli C, Duchi R, Crotti G, Lazzari G (1998) Embryo production by Ovum Pick Up in Water Buffalo. Theriogenology 49:400
38. Galli C, Crotti G, Turini P, Duchi R, Mari G, Zavaglia G, Duchamp G, Daels P, Lazzari G (2002) Frozen- thawed embryos produced by Ovum Pick Up of immature oocytes and ICSI are capable to establish pregnancies in the horse. Theriogenology 58:705–708
39. Yanagimachi R, Chang MC (1964) In Vitro Fertilization of Golden Hamster Ova. J Exp Zool 156:361–375
40. Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA (1982) Normal development following in vitro fertilization in the cow. Biol Reprod 27:147–158
41. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL (1986) Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25:591–600
42. Palermo G, Joris H, Devroey P, Van Steirteghem AC (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340:17–18
43. Goto K, Yanagita K (1995) Normality of calves obtained by intracytoplasmic sperm injection. Hum Reprod 10:1554
44. Galli C, Vassiliev I, Lagutina I, Galli A, Lazzari G (2003) Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol. Theriogenology 60:1467–1480
45. Catt SL, Catt JW, Gomez MC, Maxwell WM, Evans G (1996) Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm. Vet Rec 139:494–495
46. Kolbe T, Holtz W (2000) Birth of a piglet derived from an oocyte fertilized by intracytoplasmic sperm injection (ICSI). Anim Reprod Sci 64:97–101
47. Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R (1999) Mammalian transgenesis by intracytoplasmic sperm injection. Science 284:1180–1183
48. Gandolfi F, Moor RM (1987) Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 81:23–28
49. Young LE, Sinclair KD, Wilmut I (1998) Large offspring syndrome in cattle and sheep. Rev Reprod 3:155–163

50. van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B, de Ruigh L (2000) Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. Theriogenology 53:575–597
51. Tervit HR, Whittingham DG, Rowson LE (1972) Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 30:493–497
52. Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C (2002) Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 67:767–775
53. Gurdon JB (2006) From nuclear transfer to nuclear reprogramming: the reversal of cell differentiation. Annu Rev Cell Dev Biol 22:1–22
54. Willadsen SM (1986) Nuclear transplantation in sheep embryos. Nature 320:63–65
55. Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G (2003) Pregnancy: a cloned horse born to its dam twin. Nature 424:635
56. Lagutina I, Lazzari G, Duchi R, Colleoni S, Ponderato N, Turini P, Crotti G, Galli C (2005) Somatic cell nuclear transfer in horses: effect of oocyte morphology, embryo reconstruction method and donor cell type. Reproduction 130:559–567
57. Galli C, Duchi R, Moor RM, Lazzari G (1999) Mammalian leukocytes contain all the genetic information necessary for the development of a new individual. Cloning 1:161–170
58. Galli C, Duchi R, Crotti G, Turini P, Ponderato N, Colleoni S, Lagutina I, Lazzari G (2003) Bovine embryo technologies. Theriogenology 59:599–616
59. Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, Tomizuka K, Ishida I, Robl JM (2004) Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet 36:775–780
60. Lombardo A, Genovese P, Beausejour CM, Colleoni S, Lee YL, Kim KA, Ando D, Urnov FD, Galli C, Gregory PD, Holmes MC, Naldini L (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25:1298–1306
61. Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N, Hawk HW (2005) Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat Biotechnol 23:445–451
62. Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G (2003) Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. Nat Biotechnol 21:157–162
63. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292:740–743
64. Lazzari G, Colleoni S, Giannelli SG, Brunetti D, Colombo E, Lagutina I, Galli C, Broccoli V (2006) Direct derivation of neural rosettes from cloned bovine blastocysts: a model of early neurulation events and neural crest specification in vitro. Stem Cells 24:2514–2521
65. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
66. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156
67. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78:7634–7638
68. Keefer CL, Pant D, Blomberg L, Talbot NC (2007) Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. Anim Reprod Sci 98:147–168
69. Notarianni E, Galli C, Laurie S, Moor RM, Evans MJ (1991) Derivation of pluripotent, embryonic cell lines from the pig and sheep. J Reprod Fertil Suppl 43:255–260
70. Iwasaki S, Campbell KH, Galli C, Akiyama K (2000) Production of live calves derived from embryonic stem-like cells aggregated with tetraploid embryos. Biol Reprod 62:470–475
71. Ying QL, Stavridis M, Griffiths D, Li M, Smith A (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 21:183–186
72. Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A (2008) The ground state of embryonic stem cell self-renewal. Nature 453:519–523
73. Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A (2008) Capture of authentic embryonic stem cells from rat blastocysts. Cell 135:1287–1298
74. Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RD (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196–199
75. Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, de Chuva Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448:191–195
76. Alberio R, Croxall N, Allegrucci C. Pig Epiblast Stem Cells Depend on Activin/Nodal Signaling for Pluripotency and Self Renewal. Stem Cells Dev 2010.
77. Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, Baccarini A, Lazzari G, Galli C, Naldini L (2007) Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol 25:1457–1467
78. Mansour SL, Thomas KR, Capecchi MR (1988) Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336:348–352
79. Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380:64–66
80. Galli C, Lazzari G (2008) The manipulation of gametes and embryos in farm animals. Reprod Domest Anim 43(Suppl 2):1–7
81. Brinster RL, Allen JM, Behringer RR, Gelinas RE, Palmiter RD (1988) Introns increase transcriptional efficiency in transgenic mice. Proc Natl Acad Sci USA 85:836–840

82. Palmiter RD, Sandgren EP, Avarbock MR, Allen DD, Brinster RL (1991) Heterologous introns can enhance expression of transgenes in mice. Proc Natl Acad Sci USA 88:478–482
83. Schedl A, Montoliu L, Kelsey G, Schutz G (1993) A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. Nature 362:258–261
84. Giraldo P, Montoliu L (2001) Size matters: use of YACs, BACs and PACs in transgenic animals. Transgenic Res 10:83–103
85. Van Keuren ML, Gavrilina GB, Filipiak WE, Zeidler MG, Saunders TL (2009) Generating transgenic mice from bacterial artificial chromosomes: transgenesis efficiency, integration and expression outcomes. Transgenic Res 18:769–785
86. Sauer B, Henderson N (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. New Biol 2:441–449
87. Allen GC, Spiker S, Thompson WF (2005) Transgene integration: use of matrix attachment regions. Meth Molecular Biology Clifton N J 286:313–326
88. Harraghy N, Gaussin A, Mermod N (2008) Sustained transgene expression using MAR elements. Curr Gene Ther 8:353–366
89. McKnight RA, Shamay A, Sankaran L, Wall RJ, Hennighausen L (1992) Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. Proc Natl Acad Sci USA 89:6943–6947
90. McKnight RA, Spencer M, Wall RJ, Hennighausen L (1996) Severe position effects imposed on a 1 kb mouse whey acidic protein gene promoter are overcome by heterologous matrix attachment regions. Mol Reprod Dev 44:179–184
91. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407:86–90
92. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Menoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325:433
93. Dickson D (1989) "Dangerous" liaisons in cell biology. Science 244:1539–1540
94. Chang K, Qian J, Jiang M, Liu YH, Wu MC, Chen CD, Lai CK, Lo HL, Hsiao CT, Brown L, Bolen J Jr, Huang HI, Ho PY, Shih PY, Yao CW, Lin WJ, Chen CH, Wu FY, Lin YJ, Xu J, Wang K (2002) Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. BMC Biotechnol 2:5
95. Shen W, Li L, Pan Q, Min L, Dong H, Deng J (2006) Efficient and simple production of transgenic mice and rabbits using the new DMSO-sperm mediated exogenous DNA transfer method. Mol Reprod Dev 73:589–594
96. Lavitrano M, Bacci ML, Forni M, Lazzereschi D, Di Stefano C, Fioretti D, Giancotti P, Marfe G, Pucci L, Renzi L, Wang H, Stoppacciaro A, Stassi G, Sargiacomo M, Sinibaldi P, Turchi V, Giovannoni R, Della Casa G, Seren E, Rossi G (2002) Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proc Natl Acad Sci USA 99:14230–14235
97. Wu Z, Li Z, Yang J (2008) Transient transgene transmission to piglets by intrauterine insemination of spermatozoa incubated with DNA fragments. Mol Reprod Dev 75:26–32
98. Moreira PN, Perez-Crespo M, Ramirez MA, Pozueta J, Montoliu L, Gutierrez-Adan A (2007) Effect of transgene concentration, flanking matrix attachment regions, and RecA-coating on the efficiency of mouse transgenesis mediated by intracytoplasmic sperm injection. Biol Reprod 76:336–343
99. Kurome M, Ueda H, Tomii R, Naruse K, Nagashima H (2006) Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. Transgenic Res 15:229–240
100. Whitelaw CB, Radcliffe PA, Ritchie WA, Carlisle A, Ellard FM, Pena RN, Rowe J, Clark AJ, King TJ, Mitrophanous KA (2004) Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Lett 571:233–236
101. Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, MacIntyre E, Dal Cortivo L, Radford I, Brousse N, Sigaux F, Moshous D, Hauer J, Borkhardt A, Belohradsky BH, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N, Blanche S, Bushman FD, Fischer A, Cavazzana-Calvo M (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of\ SCID-X1. J Clin Invest 118: 3132–3142
102. Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H, Brugman MH, Pike-Overzet K, Chatters SJ, de Ridder D, Gilmour KC, Adams S, Thornhill SI, Parsley KL, Staal FJ, Gale RE, Linch DC, Bayford J, Brown L, Quaye M, Kinnon C, Ancliff P, Webb DK, Schmidt M, von Kalle C, Gaspar HB, Thrasher AJ (2008) Insertional mutagenesis combined with acquired somatic mutations causes\ leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 118:3143–3150
103. Romano G (2009) An update on gene therapy programs. Drug News Perspect 22:435–440
104. de Sio FR Santoni, Gritti A, Cascio P, Neri M, Sampaolesi M, Galli C, Luban J, Naldini L (2008) Lentiviral vector gene transfer is limited by the proteasome at postentry steps in various types of stem cells. Stem Cells 26:2142–2152
105. Chan AW, Chong KY, Martinovich C, Simerly C, Schatten G (2001) Transgenic monkeys produced by retroviral gene transfer into mature oocytes. Science 291:309–312
106. Hofmann A, Kessler B, Ewerling S, Kabermann A, Brem G, Wolf E, Pfeifer A (2006) Epigenetic regulation of lentiviral

transgene vectors in a large animal model. Mol Ther 13:59–66

107. Henckaerts E, Dutheil N, Zeltner N, Kattman S, Kohlbrenner E, Ward P, Clement N, Rebollo P, Kennedy M, Keller GM, Linden RM (2009) Site-specific integration of adeno-associated virus involves partial duplication of the target locus. Proc Natl Acad Sci USA 106:7571–7576
108. Miller DG, Wang PR, Petek LM, Hirata RK, Sands MS, Russell DW (2006) Gene targeting in vivo by adeno-associated virus vectors. Nat Biotechnol 24:1022–1026
109. Russell DW, Hirata RK (1998) Human gene targeting by viral vectors. Nat Genet 18:325–330
110. Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ (2008) Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 321:1837–1841
111. Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS (2008) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest 118:1571–1577
112. Brunetti D, Perota A, Lagutina I, Colleoni S, Duchi R, Calabrese F, Seveso M, Cozzi E, Lazzari G, Lucchini F, Galli C (2008) Transgene expression of green fluorescent protein and germ line transmission in cloned pigs derived from in vitro transfected adult fibroblasts. Cloning Stem Cells 10(4):409–420
113. Lagutina I, Lazzari G, Duchi R, Turini P, Tessaro I, Brunetti D, Colleoni S, Crotti G, Galli C (2007) Comparative aspects of somatic cell nuclear transfer with conventional and zona-free method in cattle, horse, pig and sheep. Theriogenology 67:90–98
114. Lagutina I, Lazzari G, Galli C (2006) Birth of cloned pigs from zona-free nuclear transfer blastocysts developed in vitro before transfer. Cloning Stem Cells 8:283–293
115. Kragh PM, Du Y, Corydon TJ, Purup S, Bolund L, Vajta G (2005) Efficient in vitro production of porcine blastocysts by hand-made cloning with a combined electrical and chemical activation. Theriogenology 64:1536–1545
116. Vajta G, Kragh PM, Mtango NR, Callesen H (2005) Hand-made cloning approach: potentials and limitations. Reprod Fertil Dev 17:97–112
117. Esteban MA, Xu J, Yang J, Peng M, Qin D, Li W, Jiang Z, Chen J, Deng K, Zhong M, Cai J, Lai L, Pei D (2009) Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J Biol Chem 284:17634–17640
118. Ezashi T, Telugu BP, Alexenko AP, Sachdev S, Sinha S, Roberts RM (2009) Derivation of induced pluripotent stem cells from pig somatic cells. Proc Natl Acad Sci USA 106:10993–10998
119. Wu Z, Chen J, Ren J, Bao L, Liao J, Cui C, Rao L, Li H, Gu Y, Dai H, Zhu H, Teng X, Cheng L, Xiao L (2009) Generation of pig induced pluripotent stem cells with a drug-inducible system. J Mol Cell Biol 1:46–54
120. Takahagi Y, Fujimura T, Miyagawa S, Nagashima H, Shigehisa T, Shirakura R, Murakami H (2005) Production of alpha 1, 3-galactosyltransferase gene knockout pigs expressing both human decay-accelerating factor and N-acetylglucosaminyltransferase III. Mol Reprod Dev 71: 331–338
121. Fujimura T, Takahagi Y, Shigehisa T, Nagashima H, Miyagawa S, Shirakura R, Murakami H (2008) Production of alpha 1, 3-galactosyltransferase gene-deficient pigs by somatic cell nuclear transfer: a novel selection method for gal alpha 1, 3-Gal antigen-deficient cells. Mol Reprod Dev 75:1372–1378
122. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
123. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
124. Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W, Melton DA (2008) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26:1269–1275
125. Feng B, Ng JH, Heng JC, Ng HH (2009) Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. Cell Stem Cell 4:301–312
126. Ralston A, Rossant J (2010) The genetics of induced pluripotency. Reproduction 139:35–44
127. Bartolome C, Bello X, Maside X (2009) Widespread evidence for horizontal transfer of transposable elements across Drosophila genomes. Genome Biol 10:R22
128. Clark KJ, Carlson DF, Foster LK, Kong BW, Foster DN, Fahrenkrug SC (2007) Enzymatic engineering of the porcine genome with transposons and recombinases. BMC Biotechnol 7:42
129. Araki K, Araki M, Yamamura K (2002) Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. Nucleic Acids Res 30:e103
130. Irion S, Luche H, Gadue P, Fehling HJ, Kennedy M, Keller G (2007) Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat Biotechnol 25:1477–1482
131. Nyabi O, Naessens M, Haigh K, Gembarska A, Goossens S, Maetens M, De Clercq S, Drogat B, Haenebalcke L, Bartunkova S, De Vos I, De Craene B, Karimi M, Berx G, Nagy A, Hilson P, Marine JC, Haigh JJ (2009) Efficient mouse transgenesis using Gateway-compatible ROSA26

locus targeting\ vectors and F1 hybrid ES cells. Nucleic Acids Res 37:e55
132. Porteus MH (2006) Mammalian gene targeting with designed zinc finger nucleases. Mol Ther 13:438–446
133. d'Apice AJ, Cowan PJ (2008) Gene-modified pigs. Xenotransplantation 15:87–90
134. Fang J, Qian JJ, Yi S, Harding TC, Tu GH, VanRoey M, Jooss K (2005) Stable antibody expression at therapeutic levels using the 2A peptide. Nat Biotechnol 23: 584–590
135. Bartosch B, Stefanidis D, Myers R, Weiss R, Patience C, Takeuchi Y (2004) Evidence and consequence of porcine endogenous retrovirus recombination. J Virol 78:13880–13890
136. Moalic Y, Felix H, Takeuchi Y, Jestin A, Blanchard Y (2009) Genome areas with high gene density and CpG island neighborhood strongly attract porcine endogenous retrovirus for integration and favor the formation of hot spots. J Virol 83:1920–1929
137. Takeuchi Y, Patience C, Magre S, Weiss RA, Banerjee PT, Le Tissier P, Stoye JP (1998) Host range and interference studies of three classes of pig endogenous retrovirus. J Virol 72:9986–9991
138. Dieckhoff B, Petersen B, Kues WA, Kurth R, Niemann H, Denner J (2008) Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs. Xenotransplantation 15:36–45
139. Kragh PM, Nielsen AL, Li J, Du Y, Lin L, Schmidt M, Bogh IB, Holm IE, Jakobsen JE, Johansen MG, Purup S, Bolund L, Vajta G, Jorgensen AL (2009) Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res 18:545–558
140. Petersen B, Carnwath JW, Niemann H (2009) The perspectives for porcine-to-human xenografts. Comp Immunol Microbiol Infect Dis 32:91–105
141. Galli C, Perota A, Brunetti D, Lagutina I, Lazzari G, Lucchini F (2010) Genetic engineering including superseding microinjection: new ways to make GM pigs. Xenotransplantation 17(6):397–410
142. Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Starzl TE, Prather RS, Dai Y (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24:435–436

Books

Gordon IR (2003) Laboratory production of cattle embryos, vol Series No. 27, 2nd edn, Biotechnology in agriculture. CAB International, Wallingford

Gordon I (2004) Reproductive technologies in farm animals. CAB International, Wallingford. ISBN 0 85199 862 3

Hafez B, Hafez ESE (2000) Reproduction in farm animals, 7th ed. Lippincott Williams & Wilkins. ISBN 0-683-30577-8

Monk M (1987) Mammalian development, a practical approach. In: Rickwood D, Hames BD (eds) The practical approach series. IRL Press Ltd, Oxford/Washington, DC

Nagy A (2003) Manipulating the mouse embryo : a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. ISBN: 0879695749 0879695919

Robertson E (1987) Teratocarcinoma and embryonic stem cells, a practical approach. In: Rickwood D, Hames BD (eds) The practical approach series. IRL Press Ltd, Oxford/Washington, DC

Transgenic Livestock, Decreasing Environmental Impact of

Serguei P. Golovan[1], Cecil W. Forsberg[2]
[1]Department of Animal and Food Science, University of Delaware, Newark, DE, USA
[2]Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Article Outline

Glossary

Arabinoxylan A polymeric backbone of β(1,4) linked xylose residues with attached C(O)-2,3-linked arabinose residues that is found mainly in cereal grains. It becomes highly viscous when dissolved in the gastrointestinal tract and is not digested by monogastric animals.

Eutrophication A process where water bodies receive excess nutrients especially as phosphorus or nitrogen that stimulate excessive plant and algal growth resulting in reduced water quality.

β-Glucan A polymer found primarily in cereal grains consisting of β (1,3:1,4) linked glucose residues that becomes viscous on solubilization in the gastrointestinal tract and is not digested by monogastric animals.

Glycanase Any enzyme that catalyzes the hydrolysis of a glycan, which includes glucanases and xylanases of all types.

Phytase Any type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid releasing phosphate molecules.

Phytic Acid Inositol hexakisphosphate (or phytate when in the salt form) is the principle storage form of phosphorus in many plants. It accounts for 50–80% of the total phosphorus present and is poorly digested by monogastric animals.

Definition of the Subject

As the world population increases, there is an increasing demand for food including both plant and animal products. The demand for meat and milk are increasing at a faster rate than for plant products because of increased wealth in developing countries. Intensification of livestock production to satisfy the demand is exacerbating the deleterious impact of intensive animal agriculture on the environment and new approaches are needed to reduce the impact. One approach is through the development of transgenic animals that have a smaller environmental footprint. At present there are no transgenic livestock in production. Transgenic livestock that have reduced environmental impact have been developed, but most proposed strategies are at the early stages of development. Physiological modifications that would reduce the impact of livestock include changes to improve feed utilization, increase growth and increase disease resistance, reduce manure output, and decrease greenhouse gas production. Obviously an essential factor is that the changes in physiology of the transgenic animal must have no deleterious effect on health, welfare, performance, and the environment.

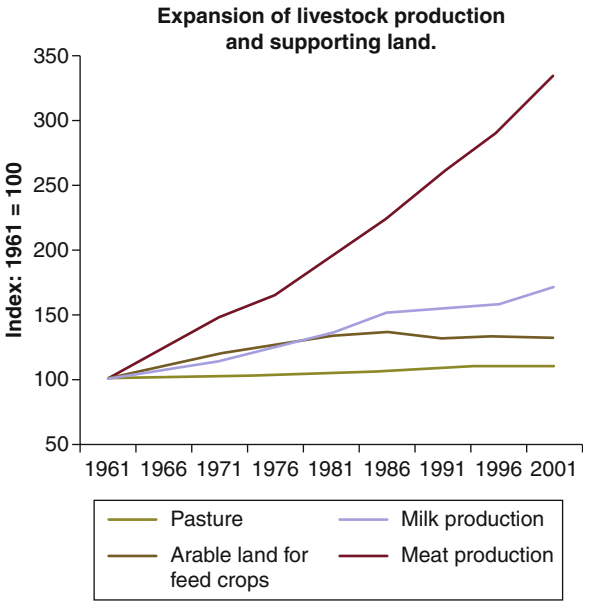
Introduction

Environmental Impact of Livestock

Demand projections point to increases of global meat consumption of 68% and of global milk consumption of 57% of the 2000 base period by 2030 [26, 83]. While agriculture could be considered a "green" industry which uses solar energy to produce food and fiber for human use, it has also become a serious threat to global ecological systems. Currently forest losses in tropical countries is largely due to conversion to agricultural land to increase global grazing and cropland dedicated to the production of feed that already amounts to approximately 70% of all agricultural land [84]. The trends are illustrated by Fig. 1. Livestock production is a major cause of greenhouse gas production, particularly methane production, and as well is a major source of pollution due to the extensive use of pesticides, antibiotics, and nutrients (nitrogen, phosphorus, and minerals) that result in increased

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3



Transgenic Livestock, Decreasing Environmental Impact of. Figure 1
Global expansion of livestock production and supporting land

eutrophication with deterioration in water quality in many countries [87]. The major *challenge for animal agriculture in this century is to sustain and increase meat and milk production without further degrading the environment.*

Production of meat from monogastric animals, mainly swine and poultry, has increased 103% over the period from 1987 to 2007 compared to ruminant meat production that increased by 28% [27]. The shift to monogastric species and continued productivity gains have the potential to reduce environmental impact per unit of animal production in the future, but with overall production increasing, serious pollution concerns remain. The issues and options in addressing the environmental consequences of a growing livestock sector are clearly enunciated in a paper by Gerber et al. [30].

Selective breeding of animals has greatly improved commercial performance of livestock and is especially useful for improvement of *multi-gene traits.* Yet, selective breeding is a slow process, depending on the rate of animal reproduction and can only operate on alleles and genes present in animal population with little control over what to express, where, when, and how much. Continuous selection for a particular trait may result in decreased genetic variability in the locus and decrease ability for further selection. For example, only a few proven bulls produce the majority of offspring in the world Holstein populations and similar reduced breed diversity in US swine and poultry is a cause for concern [56]. It is also difficult to introduce beneficial alleles from rare breeds into existing commercial population because it leads to decreased performance for economically important traits. In contrast, animal genetic engineering allows precise introduction of new alleles from rare breeds, novel genes from evolutionary distant species, or even completely artificial genes with control of the time, place, and amount of gene expression in an animal. Furthermore, a transgenic animal is a self-replicating high-tech animal – it might be expensive to produce but it is cheap to propagate.

Reducing the impact on the environment through animal transgenesis can take many forms (Table 1), for example, improving the overall feed digestion by the animal or reducing the excretion of a specific nutrient such as phosphorus. More subtle means of improving the efficiency of animal production could include enhanced disease resistance or even improving the nutritional quality of the meat since it would increase the product value.

Swine

Growth and Improved Digestion The first transgenic food animal developed that exhibited a trait consistent with a reduced environmental footprint was the transgenic line of pigs developed by Pursel and colleagues [64]. These pigs contained an increased level of bovine growth hormone that gave rise to an 18% increase in feed efficiency and an 11–14% increase in growth rate (Table 1). These improvements obviously decreased manure production per unit of body weight gain. Unfortunately, the pigs exhibited lameness, stress susceptibility, gastric ulcers, and other health problems that negated the unique improvements observed, but the study documented that the problem was due to overproduced growth hormone. In a separate study by Nottle et al. [57], the level of growth hormone was controlled by using an inducible promoter, a modified human metallothionein

Transgenic Livestock, Decreasing Environmental Impact of. Table 1 Genetic modifications in livestock to improve overall performance and reduce environmental impact

Species	Trait	Protein/RNA; Transgene	Reference
Pig	Phosphorus metabolism	Phytase; PSP-APPA	[33]
Pig	Increased growth	Growth hormone; hMTpGH	[57]
Pig	Increased growth	Insulin-like growth factor; (mMT-HIGF-1)	[65]
Pig	Increased lactose in milk	α-lactalbumin; bovine α-lactalbumin	[90]
Pig	Influenza resistance	Mx protein; mMx1-Mx	[53]
Pig, Cattle	Disease resistance	IgA; mouse a and ***x*** chains	[46]
Cattle	Avoids spontaneous prion occurrence	Knockout vectors pBPrP(H)KOneo; pBPrP(H)KOpuro	[73]
Cattle	*Staphylococcus aureus* resistance	Lysostaphin; β-lactoglobulin promoter linked to lysostaphin, neomycin resistance, and GFP	[88]
Cattle	Mastitis resistance	Human lactoferrin in milk	[79]
Goat	Reduced mastitis and healthier kids	Human lysozyme; A_{s1}-casein promoter-hLZcDNA	[38]
Sheep	Visna virus envelope	Visna LTR-env	[14]
Sheep	Ovine prion locus	Homologous recombination	[21]
Sheep	Increased growth	Ovine growth hormone; metallothionein promoter	[1]
Chicken	Influenza virus resistance	Chicken U6 promoter; cRNA binding site influenza virus polymerase	[49]

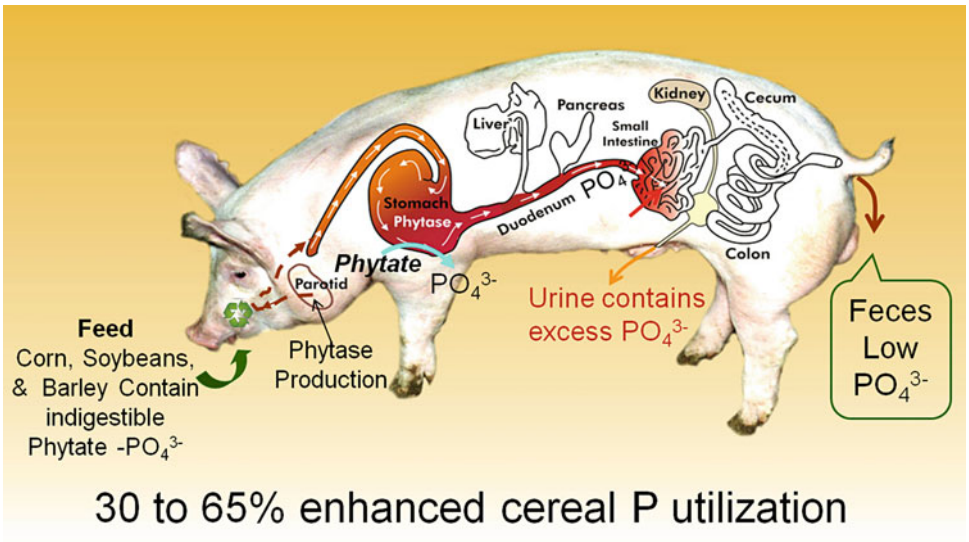
promoter fused to the cDNA sequence for the porcine growth hormone. They documented improved growth of founder animals as compared to non-transgenic littermates, but the study was not carried beyond litter mates. Later, Pursel and colleagues [63] developed transgenic pigs that expressed insulin-like growth factor I (IGF-I) to determine whether directing the expression of IGF-I specifically to striated muscle would enhance lean muscle growth in pigs. However, they observed only a decreased daily rate of fat accretion in the transgenic pigs as compared to the rate in the unmodified pigs. This may be explained by the observation that transgenic modification of a trait that has already undergone intensive selection through traditional breeding and is a metabolic process with multiple effects can have unexpected and in some cases deleterious outcomes [22].

A different approach to enhancing growth rate of pigs was achieved by the introduction of the bovine α-lactalbumin gene into pigs. Sows containing the α-lactalbumin gene produce an increased concentration of lactose in milk during the early lactation that increases the growth rates of piglets [55, 90]. This novel transgenic modification improves efficiency of production by either allowing earlier weaning or more robust piglets at weaning.

Phosphorus is a key nutrient for all living things. At the same time, phosphorus mining damages the environment, and phosphorus-induced eutrophication leads to harmful algal blooms, decreased biodiversity, and changes in species composition. In addition, the global supply of mined phosphorus is running out and some estimate that within 30–40 years there will not be sufficient phosphorus to meet agricultural demand [16, 76]. Manure is a valuable organic fertilizer, but the high phosphorus (P) concentration in relation to the nitrogen (N) content is problematic. A desirable N/P ratio for plant growth is 6.0 for an increment in yield, [42]. This ratio is derived by using the N and P concentrations after losses due to volatilization, which

is primarily an N loss. In contrast, the N/P values for manure from pigs, dairy cattle, and poultry are approximately 0.96, 2.6, and 1.7, respectively. Therefore manures from pigs, dairy cattle, and poultry are 6.3, 2.3, and 3.4-fold enriched in phosphorus in relation to nitrogen utilized by cereal grain crops. This documents the doubly serious impact of excess P relative to N in the manure for land spreading. To decrease phosphorus-induced eutrophication caused by the high phosphorus content of pig manure and to reduce the need for supplemental phosphorus in the diet, Golovan and colleagues [33] developed transgenic pigs expressing an *Escherichia coli* phytase driven by the mouse parotid secretory protein promoter that gave rise to phytase production in the salivary glands (Fig. 2). Phytase, secreted by the parotid gland in the saliva, mixes with the incoming food particles during chewing and hydrolyzes phytate in the acidic environment of the stomach-releasing phosphate that is readily absorbed in the small intestine. Since the dietary phosphorus requirement of the transgenic pigs can be satisfied by the cereal grain diet without inclusion of either supplemental P or supplemental microbial phytase, there is an overall decrease in P excretion in the feces and urine that will enter the environment [33]. A line of these phytase pigs currently in the eighth generation exhibited salivary phytase activities at a levels similar to that of the founding transgenic pig, and excrete 30–65% less phosphorus in the manure depending upon the stage of growth and diet consumed. Trials have documented that the phytase pigs have similar reproductive characteristics, health, and growth rates to that of conventional pigs (Forsberg, unpublished data). The pig parotid secretory protein promoter was also tested [95], but the salivary phytase production in mice was not as efficient as that described by Golovan et al. [32] for the mouse PSP promoter. It would be very instructive to assess the efficacy of the pig PSP promoter driving phytase synthesis in the pig.

Cereal grains and plant protein supplements have an imbalance of amino acids with an excess of nonessential amino acids and lesser amounts of the essential amino acids lysine methionine and threonine [58]. This imbalance results in high excretion of nitrogen from nonessential amino acids, and the swine ammonia emission metric (kg livestock^{-1}) is higher than either beef or dairy cattle [10]. Ammonia emissions are substantially reduced by inclusion in the diet of essential amino acids lysine, methionine, and threonine. Endowing pigs with the selected ability to synthesize essential amino acids in the appropriate proportions would decrease the need for protein



Transgenic Livestock, Decreasing Environmental Impact of. Figure 2
High phytate feed consumption by the Enviropig^{TM}

supplements and would also decrease the excretion of ammonia arising from degradation of nonessential amino acids. To test this thesis, Rees and Hay [71] genetically modified mouse 3T3 cells by the introduction of a chimeric gene containing the coding regions of the *E. coli* gene for aspartokinaseI/homoserine dehydrogenase I and the *Corynebacterium glutamicum* gene for aspartic semialdehyde dehydrogenase subcloned into a Simian virus 40 based mammalian expression vector. These cells produced homoserine. By transfecting these cells with the plasmid pSVthrB/c containing genes coding for homoserine kinase and threonine synthase, the modified cell line expressed the complete pathway for the synthesis of threonine from aspartic acid, and the cell line no longer had a growth requirement for threonine. Therefore, by using the appropriate promoters with these genes it should be possible to produce genetically modified pigs/animals with the innate endogenous capacity to synthesis the essential amino acid threonine. The introduction of genes for the endogenous synthesis of methionine and lysine would be highly beneficial; unfortunately, the development of transgenes appropriately regulated to make each biosynthetic pathway function in tissues will be a daunting task because methionine synthesis requires four additional genes coding for enzymes that convert of homoserine to methionine. Lysine synthesis would require as many as nine genes coding for enzymes in the pathway beginning with aspartic acid (http://www.genome.jp/kegg/pathway/map/map00300). Alternatively, it might be possible to improve utilization of essential amino acids present in feed by increasing ileal digestibility (phytase, cellulase), absorption from gut (amino acid transporters), or even by increasing proportion of gut microorganisms responsible for synthesis of the essential amino acids by developing selective attachment receptors or by suppression of competitors using antimicrobial proteins.

Cereal grains and plant protein supplements contain indigestible structural carbohydrate components including β-glucan and arabinoxylan that are not digested by nonruminant species and are mainly excreted in the manure. It has been shown that supplementation of the diet with exogenous β-glucanase and xylanase improves growth of the piglets [23]. However, supplementation of grower diets with glycanase enzymes has given mixed results. In a study by Nyachoti et al. [59] no beneficial effect was observed by the inclusion of glycanase while Ji et al. [40] observed a distinct beneficial effect on digestion. The beneficial effect of supplemental glucanase was the basis for testing whether this class of enzymes can be expressed in animals to enhance digestion. To test this hypothesis a transgene composed of the mouse pancreas–specific amylase 2.2 promoter and the associated signal sequence was linked to the *Bacillus subtilis* endoglucanase with a 3′polyadenylation sequence. The transgene was introduced into mice by pronuclear microinjection. Offspring containing the transgene expressed the endoglucanase gene in the pancreas and secreted the truncated, but active glucanase into the small intestine [96]. Ohnishi et al. [60] showed that overexpression of the Rab3D upregulated amylase secretion from the pancreatic acini of transgenic mice; therefore it is possible this technique could be used to further enhance glycanase synthesis and secretion from the pancreas into the small intestine. Together with a report by Hall et al. [35] describing the transgenic expression of a *Clostridium thermocellum* thermostable glucanase in the pancreas of the mouse using an elastase promoter/enhancer, these studies show the feasibility of expressing hydrolase genes in the gastrointestinal tracts of livestock.

Health Status Health is a major issue in livestock production documented by the deleterious economic impacts [72] of current and emerging swine zoonoses [82]. Strategies to improve resistance of swine to viral diseases such as foot-and-mouth disease, influenza, and porcine reproductive and respiratory syndrome which depend on host cellular machinery include antisense, decoys, ribozyme, and RNA interference [47, 92]. Recent work on the control of the African swine fever virus through the use of RNA interference may soon show success [41]. To provide resistance against a broad range of bacteria and some viruses, Cheung et al. [11] introduced into mice the porcine protegrin-1 using the cytomegalovirus promoter. Protegrin is an antimicrobial peptide that targets both gram-negative and gram-positive bacteria as well as enveloped viruses. They showed increased resistance to the swine pathogen *Actinobacillus suis*. This protein normally is expressed in neutrophils, and the intent in this study was to assess the effect of more

general expression that would affect the bacteria at an earlier stage in the infection. Introduction of this construct into pigs and subsequent testing will be very informative since subclinical infections in pigs are a major cause of poorer feed efficiency in many environments.

Ruminants

Enhanced Digestion No transgenic ruminants have been reported that have a reduced impact on the environment through enhanced growth characteristics or reduced nutrient excretion. However, it was shown that feeding phytase and cellulases to lactating dairy cows resulted in reduced fecal excretion of dry matter, neutral detergent fiber, and acid detergent fiber, and reduced nitrogen and phosphorus in feces [43]. The beneficial effect of supplemental enzymes was found despite the presence of 101 distinct ruminal microbial phytases [37] and 27,755 putative carbohydrate-active genes, many of which presumably code for ruminal plant cell wall degrading enzymes [36]. Because added phytase enhances phosphorus utilization, transgenic expression of a phytase in the salivary glands of the cow using a promoter such as the indigenous salivary Bsp30a protein promoter [66, 91] would seem to be a possible strategy since Bsp30a is produced at a high concentration in saliva. No information is available on the Bsp30a promoter at this time; therefore background work would be necessary before testing this hypothesis.

Whether there would be interest in exploring the salivary production of glycanases in ruminants is questionable since variable results have been obtained by supplementation of diets with cellulases [5, 6, 62].

Methane from livestock accounts for approximately 37% of the methane produced by human-related activities, and the single largest source being enteric fermentation, mainly in ruminant livestock [30]. To reduce methane production in cattle, one option proposed was to generate transgenic cattle that produce salivary antibodies against rumen methanogens which subsequently bind to and inhibit their action in the rumen [44]. The same authors also identified the prophage φ-mru that is able to lyse methanogen cells. Secretion of anti-methanogenic proteins in the saliva would seem feasible from a physiological perspective as there are several strong salivary promoters [91]. However, before such experiments are undertaken it obviously would be prudent to conduct feeding trials and testing for effects on the methanogenic population and to monitor the effect on the animal.

Health Disease is an ongoing drag on the efficiency in the production of ruminant animals [72]. Mastitis has a serious impact on milk production by ruminants [4]. To eliminate the deleterious effect of mastitis in dairy cows, Wall et al. [88] expressed lysostaphin in the milk. This antimicrobial protein enhanced the resistance to *Staphylococcus aureus* and provisionally could help maintain high milk production as a consequence of reduced mastitis if the genetic modification were accepted for commercial production. In a similar vein Maga et al. [51] demonstrated that human lysozyme expressed in the mammary gland of transgenic dairy goats inhibited the growth of bacteria that cause mastitis and this occurs without an effect on health or performance [38]. In a different approach Simojoki et al. [80] genetically modified dairy cows to secrete human lactoferrin in the milk. Again, it enhanced resistance to mastitis infections. Other avenues to improved performance could be through prion knockouts [73] and developing resistance to viruses (infectious bovine rhinotracheitis, bovine respiratory syncytial viruses, parainfluenza, bovine viral diarrhea, rabies, and foot-and-mouth disease) through the application of an RNA interference-based approach [47].

Poultry

Enhanced Digestion Inclusion of phytase, glucanase, and xylanase in the poultry diet enhances feed digestibility. Phytase was shown to enhance availability of phosphorus and other minerals, and in addition, to enhance amino acid digestibility [18]. The chicken is reported to have a weak endogenous magnesium-stimulated phytase activity associated with the intestinal brush boarder [50], but apparently contributes little to overall phytate digestion. Initial work has been done to develop a secretory competent form of the enzyme with higher activity for expression in poultry [12], although no further work has been reported. Use of the salivary glands of poultry as a site for transgenic

phytase production is a possibility since these glands have been identified [61, 75]. However, based on the low amylase activity of the salivary glands reported for both chickens and turkeys [39], it is questionable whether a suitable promoter is available and a sufficient capacity for synthesis is possible to provide the quantity of enzyme necessary. Another possible site for secretion of hydrolytic enzymes is in the proventriculus using the chitinase promoter [85]; however, this would initially require the cloning and characterization of the promoter. Introduction of any transgene probably would involve use of either a combinatorial cis-regulatory element [77] or a lentiviral vector [49].

Arabinoxylanases alleviate viscosity-induced diffusion constraints associated with diets containing wheat, rye, barley, and triticale. Glucanases have a similar effect digesting β-glucans present and small amounts of amorphous cellulose. These glycanases were shown to enhance the amino acid digestibility [18]. The positive action of these feed enzymes opens the possibility for genetic modification.

Health Subclinical disease in poultry dramatically increases the environmental impact of the birds. For example, subclinical necrotic enteritis in broiler chickens results in a 12% reduction in body weight and an 11% decrease in feed utilization efficiency [81]. Other subclinical diseases undoubtedly have a similar impact; therefore, improving health substantively reduced the environmental footprint of poultry. The application of interference-based gene silencing may be an effective strategy for control of Marek's disease, infectious bursal disease, avian leucosis, Rous sarcoma virus, and avian influenza [47]. The report by Lyall et al. [49] on the production of transgenic chickens resistant to avian influenza viruses is an excellent example of the use of RNA interference to reduce mortalities of producing birds.

Issues with Expression of Novel Hydrolases in the Gastrointestinal Tract

Novel gene products that either have been expressed in the gastrointestinal tract of mammals, or that may be considered for expression are summarized in Table 2. Many phytases have been characterized and considered as potential feed enzymes [67], although only three phytases are illustrated as examples, the *E. coli* phytase expressed in the Enviropig^{TM}, the Avian phytase and the rat phytase, the only phytases so far identified in mammals, but has low activity. A variety of xylanases and glucanases are listed. It includes high-activity glucanases and xylanases, bifunctional xylanase/glucanase enzymes where the same catalytic domain hydrolyzes both substrates and chimeric enzymes. The chimeric enzymes can have any combination of catalytic domains, for example, phytase and endoglucanase, xylanase and glucanase, or other combinations of domains. The bifunctional and chimeric enzymes have the advantage of providing multiple activities in one protein, which has the advantage of using a single promoter.

There are a variety of factors that need to be considered when deciding upon promoter(s) and gene(s) for expression in the alimentary tract of animals. Ideally the introduced transgene should impose the minimal metabolic load with minimal interference of existing physiological and biochemical pathways of the host.

Transgene (protein or RNA):

1. Preferably the transformation of interest should be performed by a single molecule. If more than one protein/RNA is involved it may be possible to design the transgene which express multiple transgenes (see below).
2. The transgene must code for a low molecular mass stable protein with a high specific activity, and targeted to the correct compartment.
3. Cryptic posttranslational modification sites are often present in proteins isolated from non-mammalian species and must be removed if activity/stability is affected (i.e., lysostaphin glycosylation).
4. The pH and temperature optima should correspond closely to that of the site of action which would be pH 2–5 if it should be active in the stomach/proventriculus, or pH 5–8 if secreted into the small intestine where it would be active [13, 20, 34].
5. The enzyme must be resistant to proteases: pepsin if expressed in the stomach, or resistant to tryptic enzymes if expressed and active in the small intestine. If the enzyme were expressed in the salivary

Transgenic Livestock, Decreasing Environmental Impact of. Table 2 Enzymes with some criteria suitable for gastrointestinal expression

Source	Catalytic properties	Mol. Wt.	pH Opt and Min/Max	Temp (°C) Max Act.	Sp.Act. U/mg^{-1} min^{-1}	Protease resistance
E. coli [31]	Phytase/acid phosphatase	44,708 Da	Opt 4.5/2.5–3 Min 2.0 Max 10	60	2,326/712	Pepsin resistant and trypsin sensitive
Avian histidine phosphatase [74], [12]	Acid phosphatase/phytase	53,000 and 55,000 Da	4.5-7.5	Assay 40	0.5	?
Rat [94]	Phytase and alkaline phosphatase	70,000 and 90,000 Da	Phytase – 7.5 Phosphatase – 10.4	Assay 37	5.6	?
Bacillus [70]	Bifunctional phytase/ endoglucanase	73,000 Da	Phytase – 7 Endoglucanase – 6 Range 4.0 – 8.0	55	Phytase – 8 Endoglucanase – 10	?
F. succinogenes [89]	1,3-1,4-β-glucanase	27,957 Da	Opt 6–8 Min 4.0	50	10,800	?
Bisporus sp. [48]	1,3-1,4-β-glucanase	48,000 Da after deglycosylation	Opt 5.0 Range 1–8	60	4,040	Pepsin and trypsin resistant
Alicyclobacillus sp. [2]	Endo-β-1,4-glucanase	48,000 Da	Opt 2.6 Range 1.8–7.6	65	5,032	Pepsin and trypsin resistant
Alicyclobacillus sp. [3]	Endo-β-1,4-glucanase	60,000 Da	3.4 Range 1.2–8.2	60	?	Pepsin and trypsin resistant
Aspergillus niger [45]	Endo-β-1,4-xylanase	23,000 Da	Opt 5.5	50	3,881	?
N. patriciarum [93]	Endo-β-1,4-xylanase	26,000 Da	Opt 6.0	60	5,000	?
Phialophora. Sp. G5 [97]	Endo- β-1,4-xylanase	55,000 Da	Opt 4.0 Range	70	351	Pepsin and trypsin resistant
Paenibacillus sp. Strain E18 [78]	Bifunctional Endo-β-1,4-xylanase/1,3-1,4-β-glucanase	39,000 Da	Opt 6.5–9.0	50	Oat spelt xylan-358 Barley-β-glucan-133	?
C. thermocellum/Geobacillus thermophilus [24]	Chimeric xylanase-arabinofuranosidase	97,000 Da	Opt 6.0	65		?

glands for activity in the small intestine then it would need to be both pepsin and trypsin resistant, stable at low pH, and highly active at neutral pH.

6. Preferably the enzyme should be isolated from the organism with a Generally Recognized As Safe (GRAS) status and have no significant homology to allergenic, pathogenic, or toxic proteins. It is also preferable that it not exhibit any similarity to known allergens, and it would even be useful to eliminate possible glycosylation sites to avoid the development of an allergenic nature [15].

Regulatory elements:

1. Promoter should be tightly controlled allowing expression of the transgene only in desired tissue at the desired time and at a specific level. Strong ubiquitous promoters commonly used for transgenic work are undesirable as they often result in transgene toxicity and undesirable side effects. An interesting observation in plants was that repetitious use of the same promoter did not lead to transcriptional silencing [54], and this may also apply to mammals.
2. Gene-specific regulatory sequences (5′ and 3′ UTR, polyA, introns, Kozak start sequence, etc.) should be used to optimize the transcription and translation of transgene.
3. The codon usage should be modified to correspond closely to that of highly expressed proteins within the biosynthetic tissue [9, 28].
4. Insulators and locus control regions (LCR), such as the chicken P-globin locus, should be used to isolate transgene from effect of neighboring chromatin and stabilize the expression level across multiple generations [8].

Multi-gene Expression

The obvious path for the development of transgenic animals is the expression of multiple genes to further enhance the value of an animal. Regulatory agencies undoubtedly will have a preference for the expression by multiple novel genes in an animal produced by crosses of "previously approved" animals with a single transgene to avoid confusion that could result from the interaction of transgenic traits. Whether these will require a further regulatory submission remains to be determined. Since it will be desirable to develop lines of transgenic livestock homozygous for multiple novel traits, a new approach will be needed to eliminate numerous crosses and continued testing for the presence of each gene. The approved transgenes could also be introduced into new breeds/species by cotransformation, or sequential transformation of established transgenic lines in the same species. Cotransformation could be done with unlinked constructs using different promoters when different ratios of protein products are required. Multiple transgenes can also be housed on a mammalian artificial chromosome. In cases where the same proportion of the protein product is needed, multicistronic constructs with multiple internal *ribosome* entry site (IRES), or even as a single translation product in which individual proteins are separated by cellular protease sites, *could be used* [52].

A proven strategy for multi-transgenic animal production is to use the recent adaptation of the 2A system from foot-and-mouth disease virus [20, 25]. With this approach the open reading frame consists of multiple individual cDNAs separated by sequences encoding 2A and furin cleavage sites. A single complex mRNA is produced and translated into a single complex polypeptide that is spontaneously cleaved into individual exogenous proteins at the 2A sites in the endoplasmic reticulum. This results in equimolar ratios of each of the individual transgenic proteins emerging from the Golgi. This expression system has been demonstrated to produce transgenic animals with up to four transgenes being expressed from a single transcript [86].

Sequential introduction of transgenes would benefit from establishing transgene locus which allows high and stable expression and into which multiple transgenes can be introduced and removed as necessary using site-specific recombination technologies such as Cre/lox, Flp/frt, φC31 integrase, or Gateway systems [7].

A refinement to further speed up the production of homozygous populations is through the application for flow cytometry [29]. Flow cytometric separation of X and Y chromosome-bearing spermatozoa has been demonstrated to be effective in cattle and pigs and resulted in the birth of healthy offspring of the predetermined gender [68, 69].

Future Directions

Intensive selection by current breeding practices have developed livestock that are growing and producing near their physiological upper limits, and any further improvements may enhance stress leading to disease susceptibility. Reduction in the environmental footprint of livestock will come through: identification and elimination of bottlenecks in metabolic and physiological pathways, introduction of novel traits that enable the digestion of dietary components previously not digested (e.g., phytate, glycans), through the introduction of novel metabolic pathways for synthesis of essential nutrients (e.g., amino acids), and through enhanced disease resistance. These novel transgenic animals will only be accepted by farmers if they are as robust as the currently available conventional livestock. Furthermore, these new genetically engineered animals will only enter the human food chain once consumers are satisfied that the meat products are absolutely safe, since they have the choice between conventional and transgenic products. Therefore, the central issue is the confidence consumers have in the national regulatory system. With the expected increase in human population and increasing environmental constraints, greater acceptance of transgenic animals is anticipated in line with the acceptance of transgenic plant products.

Bibliography

1. Adams NR, Briegel JR, Ward KA (2002) The impact of a transgene for ovine growth hormone on the performance of two breeds of sheep. J Anim Sci 80:2325–2333
2. Bai Y, Wang J, Zhang Z, Shi P, Luo H, Huang H, Feng Y, Yao B (2010a) Extremely acidic β−1,4-glucanase, CelA4, from thermoacidophilic *Alicyclobacillus* sp. A4 with high protease resistance and potential as a pig feed additive. J Agric Food Chem 58:1970–1975
3. Bai Y, Wang J, Zhang Z, Shi P, Luo H, Huang H, Luo C, Yao B (2010b) Expression of an extremely acidic β-1,4-glucanase from thermoacidophilic *Alicyclobacillus* sp. A4 in *Pichia pastoris* is improved by truncating the gene sequence. Microb Cell Fact 9:33
4. Barkema HW, Green MJ, Bradley AJ, Zadoks RN (2009) Invited review: the role of contagious disease in udder health. J Dairy Sci 92:4717–4729
5. Beauchemin KA, Colombatto D, Morgavi DP (2004a) A rationale for the development of feed enzyme products for ruminants. Can J Anim Sci 84:23–36
6. Beauchemin KA, Colombatto D, Morgavi DP, Yang WZ, Rode LM (2004b) Mode of action of exogenous cell wall degrading enzymes for ruminants. Can J Anim Sci 84:13–22
7. Brown WRA, Lee NCO, Xu Z, Smith MCM (2011) Serine recombinases as tools for genome engineering. Methods 53:372–379
8. Bulger M, Groudine M (2010) Enhancers: the abundance and function of regulatory sequences beyond promoters. Dev Biol 339:250–257
9. Cannarrozzi G, Schraudolph NN, Faty M, von Rohr RP, Friberg MT, Roth AC, Gonnet P, Gonnet G, Barral Y (2010) A role for codon order in translation dynamics. Cell 141:355–367
10. Carew R (2010) Ammonia emissions from livestock industries in Canada: feasibility of abatement strategies. Environ Pollut 158:2618–2626
11. Cheung QC, Turner PV, Song C, Wu D, Cai HY, Macinnes JI, Li J (2008) Enhanced resistance to bacterial infection in protegrin-1 transgenic mice. Antimicrob Agents Chemother 52:1812–1819
12. Cho J, Choi K, Darden T, Reynolds PR, Petitte JN, Shears SB (2006) Avian multiple inositol polyphosphate phosphatase is an active phytase that can be engineered to help ameliorate the planet's "phosphate crisis". J Biotechnol 126:248–259
13. Clemens ET, Stevens CE, Southworth M (1975) Sites of organic acid production and pattern of digesta movement in the gastrointestinal tract of swine. J Nutr 105:759–768
14. Clements JE, Wall RJ, Narayan O, Hauer D, Schoborg R, Sheffer D, Powell A, Carruth LM, Zink MC, Rexroad CE (1994) Development of transgenic sheep that express the Visna virus envelope gene. Virology 200:370–380
15. CODEX 2008 Report of the seventh session of the Codex*Ad Hoc* intergovernmental task force on foods derived from biotechnology. http://www.codexalimentarius. net/web/archives.jsp?year = 08
16. Cordell D, Drangert JO, White S (2009) The story of phosphorus: global food security and food for thought. Glob Environ Change 19:292–305
17. Cowieson AJ, Bedford MR, Selle PH, Ravindran V (2009) Phytate and microbial phytase: implications for endogenous nitrogen losses and nutrient availability. World's Poult Sci J 65:401–417
18. Cowieson AJ, Bedford MR, Ravindran V (2010) Interactions between xylanase and glucanase in maize-soy-based diets for broilers. Brit Poult Sci 51:246–257
19. Dahlke F, Ribeiro AML, Kessler AM, Lima AR, Maiorka A (2003) Effects of corn particle size and physical form of the diet on the gastrointestinal structures of broiler chickens. Braz J Poult Sci 5:61–67
20. D'Apice AJ, Cowan PJ (2008) Gene-modified pigs. Xenotransplantation 15:87–90
21. Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, de Sousa P, Travers A, Wilmut I, Clark AJ (2001) Deletion of the α(1,3)galactosyl transferase (*GGTA1*) gene and the prion protein (*PrP*) gene in sheep. Nat Biotechnol 19:559–562
22. Devlin RH, Sakhrani D, Tymchuk WE, Rise ML, Goh B (2009) Domestication and growth hormone transgenesis cause similar

changes in gene expression in coho salmon (Oncorhynchus kisutch). Proc Natl Acad Sci USA 106:3047–3052
23. Fan CL, Han XY, Xu ZR, Wang LJ, Shi LR (2009) Effects of β-glucanase and xylanase supplementation on gastrointestinal digestive enzyme activities of weaned piglets fed a barley-based diet. J Anim Physiol Anim Nutr 93:271–276
24. Fan Z, Wagschal K, Lee CC, Kong Q, Shen KA, Maiti IB, Yuan L (2009) The construction and characterization of two xylan-degrading chimeric enzymes. Biotechnol Bioeng 102:684–692
25. Fang J, Qian JJ, Yi S, Harding TC, Tu GH, VanRoey M, Jooss K (2005) Stable antibody expression at therapeutic levels using the 2A peptide. Nat Biotechnol 23:584–590
26. Food and Agriculture Organization (2006) World agriculture: towards 2030/2050-interim report. Food and Agriculture Organization, Rome
27. Food and Agriculture Organization (2009) State of food and agriculture – Livestock in the balance. Food and Agriculture Organization, Rome
28. Fredrick K, Ibba M (2010) How the sequence of a gene can tune its translation. Cell 141:227–229
29. Garner DL (2006) Flow cytometric sexing of mammalian sperm. Theriogenology 65:943–957
30. Gerber PJ, Vellinga TV, Steinfeld H (2010) Issues and options in addressing the environmental consequences of livestock sector's growth. Meat Sci 84:244–247
31. Golovan S, Wang G, Zhang J, Forsberg CW (2000) Characterization and overproduction of the *Escherichia coli appA* encoded bifunctional enzyme which exhibits both phytase and acid phosphatase activities. Can J Microbiol 46:59–71
32. Golovan SP, Hayes MA, Phillips JP, Forsberg CW (2001) Transgenic mice expressing bacterial phytase as a model for phosphorus pollution control. Nat Biotechnol 19:429–433
33. Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney D, Plante C, Pollard J, Fan MZ, Hayes MA, Laursen J, Hjorth JP, Hacker RR, Phillips JP, Forsberg CW (2001) Pigs expressing salivary phytase produce low phosphorus manure. Nat Biotechnol 19:741–745
34. Guinotte F, Gautron J, Soumarmon A, Robert JC, Peranzi G, Nys Y (1993) Gastric acid secretion in the chicken: effect of histamine H_2 antagonists and H+/K(+)-ATPase inhibitors on gastro-intestinal pH and of sexual maturity calcium carbonate level and particle size on proventricular H+/K + ATPase activity. Comp Biochem Physiol Comp Physiol 106:319–327
35. Hall J, Ali S, Surani MA, Hazlewood GP, Clark AJ, Simons JP, Hirst BH, Gilbert HJ (1993) Manipulation of the repertoire of digestive enzymes secreted into the gastrointestinal tract of transgenic mice. Nat Biotechnol 11:376–379
36. Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, Luo S, Clark DS, Chen F, Zhang T, Mackie RI, Pennacchio LA, Tringe SG, Visel A, Woyke T, Wang Z, Rubin EM (2011) Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science 331:463–467
37. Huang H, Zhang R, Fu D, Luo J, Li Z, Luo H, Shi P, Yang P, Diao Q, Yao B (2011) Diversity, abundance and characterization of ruminal cysteine phytases suggest their important role in phytate degradation. Environ Microbiol 13:747–757
38. Jackson KA, Berg JM, Murray JD, Maga EA (2010) Evaluating the fitness of human lysozyme transgenic dairy goats: growth and reproductive traits. Transgenic Res 19:977–986
39. Jerrett SA, Goodge WR (1973) Evidence for amylase in avian salivary glands. J Morphol 139:27–45
40. Ji F, Casper DP, Brown PK, Spangler DA, Haydon KD, Pettigrew JE (2008) Effects of dietary supplementation of an enzyme blend on the ileal and fecal digestibility of nutrients in growing pigs. J Anim Sci 86:1533–1543
41. Keita D, Heath L, Albina E (2010) Control of African swine fever virus replication by small interfering RNA targeting the A151R and VP72 genes. Antivir Ther 15:727–736
42. Kellogg RL, Lander CH, Moffitt DC, Gollehon N (2000) Manure nutrients relative to the capacity of cropland and pastureland to assimilate nutrients: spatial and temporal trends for the United States. http://www.nrcs. usda. gov/technical/NRI/pubs/manntr. html 1–140
43. Knowlton KF, Taylor MS, Hill SR, Cobb C, Wilson KF (2007) Manure nutrient excretion by lactating cows fed exogenous phytase and cellulase. J Dairy Sci 90:4356–4360
44. Leahy SC, Kelly WJ, Altermann E, Ronimus RS, Yeoman CJ, Pacheco DM, Li D, Kong Z, McTavish S, Sang C, Lambie SC, Janssen PH, Dey D, Attwood GT (2010) The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. PLoS One 5:e8926
45. Levasseur A, Asther M, Record E (2005) Overproduction and characterization of xylanase B from *Asperguillus niger*. Can J Microbiol 51:177–183
46. Lo D, Pursel V, Linton PJ, Sandgren E, Behringer R, Rexroad C, Palmiter RD, Brinster RL (1991) Expression of mouse IgA by transgenic mice, pigs and sheep. Eur J Immunol 21:1001–1006
47. Long CR, Tessanne KJ, Golding MC (2010) Applications of RNA interference-based gene silencing in animal agriculture. Reprod Fertil Dev 22:47–58
48. Luo H, Yang J, Yang P, Li J, Huang H, Shi P, Bai Y, Wang Y, Fan Y, Yao B (2010) Gene cloning and expression of a new acidic family 7 endo-β-1,3-1,4-glucanase from the acidophilic fungus *Bispora* sp. MEY-1. Appl Microbiol Biotechnol 85:1015–1023
49. Lyall J, Irvine RM, Sherman A, McKinley TJ, Néñz A, Purdie A, Outtrim L, Brown IH, Rolleston-Smith G, Sang H, Tiley L (2011) Suppression of Avian influenza transmission in genetically modified chickens. Science 331:223–226
50. Maenz DD, Classen HL (1998) Phytase activity in the small intestinal brush border membrane of the chicken. Poult Sci 77:557–563
51. Maga EA, Cullor JS, Smith W, Anderson GB, Murray JD (2006) Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. Foodborne Pathog Dis 3:384–392

52. Martinez-Salas E (1999) Internal ribosome entry site biology and its use in expression vectors. Curr Opin Biotechnol 10:458–464
53. Muller M, Brem G (1994) Transgenic strategies to increase disease resistance in livestock. Reprod Fertil Dev 6: 605–613
54. Naqvi S, Farre G, Sanahuja G, Capell T, Zhu C, Christou P (2010) When more is better: multigene engineering in plants. Trends Plant Sci 15:48–56
55. Noble MS, Rodriguez-Zas S, Cook JB, Bleck GT, Hurley WL, Wheeler MB (2002) Lactational performance of first-parity transgenic gilts expressing bovine α-lactalbumin in their milk. J Anim Sci 80:1090–1096
56. Notter DR (1999) The importance of genetic diversity in livestock populations of the future. J Anim Sci 77:61–69
57. Nottle MB, Nagashima H, Verma PJ, Du ZT, Grupen CG, McIlfatrick SM, Ashman RJ, Harding MP, Giannakis C, Wigley PL, Lyons IG, Harrison DT, Luxford BG, Campbell RG, Crawford RJ, Robins AJ (1999) Production and analysis of transgenic pigs containing a metallothionein porcine growth hormone gene construct. In: Transgenic animals in agriculture. Commonwealth Agriculture Bureau International, London, pp 145–156
58. NRC (1998) Nutrient requirements of swine. National Academy Press, Washington, DC
59. Nyachoti CM, Arntfield SD, Guenter W, Cenkowski S, Opapeju FO (2006) Effect of micronized pea and enzyme supplementation on nutrient utilization and manure output in growing pigs. J Anim Sci 84:2150–2156
60. Ohnishi H, Samuelson LC, Yule DI, Ernst SA, Williams JA (1997) Overexpression of Rab3D enhances regulated amylase secretion from pancreatic acini of transgenic mice. J Clin Invest 100:3044–3052
61. Olmedo LA, Samar ME, Avila RE, de Crosa MG, Dettin L (2000) Avian minor salivary glands: an ultrastructural study of the secretory granules in mucous and seromucous cells. Acta Odontol Latinoam 13:87–99
62. Peters A, Lebzien P, Meyer U, Borchert U, Bulang M, Flachowsky G (2010) Effect of exogenous fibrolytic enzymes on ruminal fermentation and nutrient digestion in dairy cows. Arch Anim Nutr 64:221–237
63. Pursel VG, Pinkert CA, Miller KF, Bolt DJ, Campbell RG, Palmiter RD, Brinster RL, Hammer RE (1989) Genetic engineering of livestock. Science 244:1281–1287
64. Pursel VG, Wall RJ, Michell AD, Elsasser TH, Solomon MB, Coleman ME, DeMayo F, Schwartz RJ (1999) Transgenic animals in agriculture. In: Expression of insulin-like growth factor-1 in skeletal muscle of transgenic swine. CABI Publishing, New York, pp 131–144
65. Pursel VG, Mitchell AD, Bee G, Elsasser TH, McMurtry JP, Wall RJ, Coleman ME, Schwartz RJ (2004) Growth and tissue accretion rates of swine expressing an insulin-like growth factor I transgene. Anim Biotechnol 15:33–45
66. Rajan GH, Morris CA, Carruthers VR, Wilkins RJ, Wheeler TT (1996) The relative abundance of a salivary protein, bSP30, is correlated with susceptibility to bloat in cattle herds selected for high or low bloat susceptibility. Anim Genet 27:407–414
67. Rao DECS, Rao KV, Reddy TP, Reddy VD (2009) Molecular characterization, physicochemical properties, known and potential applications of phytases: an overview. Crit Rev Biotechnol 29:182–198
68. Rath D, Johnson LA (2008) Application and commercialization of flow cytometrically sex-sorted semen. Reprod Domest Anim 43(Suppl 2):338–346
69. Rath D, Ruiz S, Sieg B (2003) Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen. Vet Rec 152:400–401
70. Reddy VA, Venu K, Rao DE, Rao KV, Reddy VD (2009) Chimeric gene construct coding for bi-functional enzyme endowed with endoglucanase and phytase activities. Arch Microbiol 191:171–175
71. Rees WD, Hay SM (1995) The biosynthesis of threonine by mammalian cells: expression of a complete bacterial biosynthetic pathway in an animal cell. Biochem J 309(Pt 3):999–1007
72. Rich KM, Perry BD (2010) The economic and poverty impacts of animal diseases in developing countries: New roles, new demands for economics and epidemiology. Prev Vet Med 97:1–8
73. Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, Sathiyaseelan J, Wu H, Matsushita H, Koster J, Kato S, Ishida I, Soto C, Robl JM, Kuroiwa Y (2007) Production of cattle lacking prion protein. Nat Biotechnol 25:132–138
74. Romano PR, Wang J, O'Keefe RJ, Puzas JE, Rosier RN, Reynolds PR (1998) HiPER1, a phosphatase of the endoplasmic reticulum with a role in chondrocyte maturation. J Cell Sci 111:803–813
75. Samar ME, Avila RE, Esteban FJ, Olmedo L, Dettin L, Massone A, Pedrosa JA, Peinado MA (2002) Histochemical and ultrastructural study of the chicken salivary palatine glands. Acta Histochem 104:199–207
76. Schröder JJ, Smit AL, Cordell D, Rosemarin A (2011) Improved phosphorus use efficiency in agriculture: a key requirement for its sustainable use. Chemosphere 84(6):822–831
77. Seo HW, Kim TM, Choi JW, Han BK, Song G, Han JY (2010) Evaluation of combinatorial cis-regulatory elements for stable gene expression in chicken cells. BMC Biotechnol 10:69
78. Shi P, Tian J, Yuan T, Liu X, Huang H, Bai Y, Yang P, Chen X, Wu N, Yao B (2010) *Paenibacillus* sp. strain E18 bifunctional xylanase-glucanase with a single catalytic domain. Appl Environ Microbiol 76:3620–3624
79. Simojoki H, Orro T, Taponen S, Pyorala S (2009) Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*. Vet Microbiol 134:95–99
80. Simojoki H, Hyvonen P, Orro T, Pyorala S (2010) High concentration of human lactoferrin in milk of rhLf-transgenic cows relieves signs of bovine experimental Staphylococcus chromogenes intramammary infection. Vet Immunol Immunopathol 136:265–271

81. Skinner JT, Bauer S, Young V, Pauling G, Wilson J (2010) An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis 54:1237–1240
82. Smith TC, Harper AL, Nair R, Wardyn SE, Hanson BM, Ferguson DD, Dressler AE (2011) Emerging swine zoonoses. Vector Borne Zoonotic Dis 11:1–10
83. Steinfeld H, Gerber P (2010) Livestock production and the global environment: consume less or produce better? Proc Natl Acad Sci USA 107:18237–18238
84. Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M, de Hann C (2006) Livestock's long shadow – Evnironmental issues and options. FAO, Rome
85. Suzuki M, Fujimoto W, Goto M, Morimatsu M, Syuto B, Iwanaga T (2002) Cellular expression of gut chitinase mRNA in the gastrointestinal tract of mice and chickens. J Histochem Cytochem 50:1081–1089
86. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA (2004) Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 22:589–594
87. Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental changes. Science 292:281–284
88. Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells K, Talbot N, Hawk HW (2005) Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. Nat Biotechnol 23:445–451
89. Wen TN, Chen JL, Lee SH, Yang NS, Shyur LF (2005) A truncated *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase with improved enzymatic activity and thermotolerance. Biochemistry 44: 9197–9205
90. Wheeler MB, Bleck GT, Donovan SM (2001) Transgenic alteration of sow milk to improve piglet growth and health. Reprod Suppl 58:313–324
91. Wheeler TT, Hood K, Oden K, McCracken J, Morris CA (2003) Bovine parotid secretory protein: structure, expression and relatedness to other BPI (bactericidal/permeability-increasing protein)-like proteins. Biochem Soc Trans 31:781–784
92. Wise TG, Schafer DS, Lowenthal JW, Doran TJ (2008) The use of RNAi and transgenics to develop viral disease resistant livestock. Dev Biol (Basel) 132:377–382
93. Xue GP, Denman SE, Glassop D, Johnson JS, Dierens LM, Gobius KS, Aylward JH (1995) Modification of a xylanase cDNA isolated from an anaerobic fungus *Neocallimastix patriciarum* for high-level expression in *Escherichia coli*. J Biotechnol 38:269–277
94. Yang W-J, Matsuda Y, Sano S, Masutani H, Nakagawa H (1991) Purification and characterization of phytase from rat intestinal mucosa. Biochim Biophys Acta 1075:75–82
95. Yin HF, Fan BL, Yang B, Liu YF, Luo J, Tian XH, Li N (2006) Cloning of pig parotid secretory protein gene upstream promoter and the establishment of a transgenic mouse model expressing bacterial phytase for agricultural phosphorus pollution control. J Anim Sci 84:513–519
96. Zhang JX, Meidinger R, Forsberg CW, Krell PJ, Phillips JP (1999) Expression and processing of a bacterial endoglucanase in transgenic mice. Arch Biochem Biophys 367:317–321
97. Zhang F, Shi P, Bai Y, Luo H, Yuan T, Huang H, Yang P, Miao L, Yao B (2011) An acid and highly thermostable xylanase from *Phialophora* sp. G5. Appl Microbiol Biotechnol 89:1851–1858

Transgenic Livestock, Enhanced Nutritional Quality in

Götz Laible
AgResearch, Ruakura Research Centre, Hamilton, New Zealand

Article Outline

Glossary

Antimicrobial A substance with the ability to kill microbes.

Bacteriolytic Causing the lysis of bacteria.

Bacteriostatic Term to describe a substance that inhibits bacterial growth.

Casein micelle Protein particle consisting of aggregated casein proteins in colloidal suspension.

Circadian rhythm A daily cycle controlling the biological processes in living organisms.

Dominant-negative molecule A mutant molecule capable of interacting with the wild-type form to produce an inactive complex.

Endogenous Internally derived or synthesized.

Endopeptidase Proteolytic enzyme that can only break peptide bonds within the molecule but not at the termini.

Gastrointestinal Term referring to the digestive tract.

Genome Entirety of an organism's hereditary information.

Glycomacropeptide κ-casein-derived peptide released by rennet during cheese manufacture.

Homologous recombination Exchange of genetic material between two DNA fragments by crossing over in a region with sequence homology.

In vitro Experimental procedure conducted artificially.

Knockdown Attenuation of gene function usually resulting in diminished amount of synthesis of the protein that the gene encodes.

Knockin Integration of a new gene which replaces an endogenous gene.

Knockout Functional disruption of a specific gene of an organism commonly achieved by a partial or complete deletion of the gene sequence.

Pathogen/pathogenic Infectious agent/disease causing.

Phenotype/phenotypic A measurable characteristic of an animal such as hair color growth rate, or degree of carcass marbling. These traits are the product of genetics and the environment.

Phenylketonuria Genetic disorder in which the essential amino acid phenylalanine cannot be metabolized potentially causing mental retardation.

Pleiotropic effects The phenomena of a single gene having influences on multiple traits.

Prion An infectious protein particle.

Promoter A regulatory DNA sequence that controls the transcription of a particular gene.

RNA interference A sequence-specific gene-silencing process in which double-stranded RNAs trigger the destruction of specific RNAs.

Somatic cell count Quantification of somatic cells found in milk as an indicator for the quality of the milk.

Transgene An exogenous gene introduced into the genome of another organism.

Transgenic An animal plant or microbe whose genetic material has been altered using an artificial process.

Unsaturated (saturated) fatty acids Fatty acid molecules with (no) double bonds.

Whey Liquid that remains after separation of the solid fraction when cheese is made.

Definition of the Subject and Its Importance

Since the domestication of animals several millennia ago, which enabled the transition from hunter-gatherer to farming communities, humans have shaped livestock according to specific needs for food, both in quantity and quality. Industrialization and rapid population growths prompted immense changes to

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

farming systems, human lifestyle, and food choices, and is predicted to result in drastic climate changes within a relative short time period. Thus, current food production systems face new challenges in this rapidly changing world, in particular demands for safe and healthier food to address modern health concerns and the desire for longevity. In the past, traditional breeding and selection schemes have been invaluable for the incremental improvement of food animals. However, the process is slow and undirected and is not well suited for enhancing specific nutritional characteristics. The relatively new transgenic technology offers the potential for enhancing existing or introducing entirely novel characteristics at unprecedented rates and magnitudes and could provide substantial benefits for consumers in the form of healthier and safer food. Nevertheless, the complexity of food production traits should not be underestimated. The accurate modification of the appropriate gene(s) to generate desired phenotypes able to deliver enhanced food products remains challenging and requires careful testing and evaluation. Despite the unrivaled opportunities for innovative functional foods and the progress realized with transgenic crops, lack of acceptance due to concerns associated with the technological novelty of transgenic livestock has so far greatly limited even the research-driven evaluation of new concepts aimed at food applications.

Introduction

Livestock animals are farmed for human food production and are a primary source of dietary proteins, lipids, carbohydrates, vitamins, and minerals from milk and meat. The development of efficient farming production systems has played a crucial role to satisfy the elementary requirement for a stable food source to meet the daily nutritional needs of a multibillion human population. While milk and meat represent in general, a high-quality food source for humans, the effects of modernization and associated substantial changes to the farming systems and lifestyle of people create new challenges for providing a healthy diet of high nutritional quality. The increased understanding of how human nutritional requirements relate to health and longevity provides new opportunities to optimize the nutritional quality of food products and thus improve human health and well-being. Animal fat, which is an ingredient of all animal food products, can serve as an example to illustrate this correlation. They are relatively high in saturated fats (which have been strongly associated with cardiovascular and coronary heart disease), but low in beneficial, unsaturated fats. Thus, higher levels of unsaturated fats and lower levels of saturated fats would improve the nutritional quality of animal-derived food products. Efforts to increase the ratio of unsaturated to saturated fat have received considerable attention. However, attempts to alter the composition of milk and meat to improve its nutritional quality by conventional breeding and selection strategies have proved to be difficult. Traditional breeding and marker-assisted selection schemes are limited to the random combination of some but not all superior allelic gene variants existing within the gene pool of the livestock species. In contrast, transgenic animal technology allows for a more targeted approach with the prospect to introduce specific genes which are known to impact on defined phenotypic traits to enhance existing characteristics. Unlike traditional breeding and selection, the genetic improvement of livestock by genetic engineering is not restricted by the species barrier and can utilize the gene pool of other species to introduce entirely novel and unique characteristics. Moreover, transgenic technology is a versatile platform technology that can employ additive strategies to introduce a new gene function (gain of function), delete gene functions (knockout, loss of function), replace an endogenous gene function with a different one (knockin, exchange of function), or precisely control when and where the genetic alteration is applied (inducible gene expression and conditional knockout). Thus, it holds great promise as a new tool that can deliver solutions for many of the problems concerning the efficient production of high-quality foods from animals.

Despite mounting lifestyle-related health problems, food security, and environmental pressures which need to be urgently addressed, negative public perception, ethical concerns, regulatory uncertainties, and an associated reluctance to fund this line of research have been major factors that have so far greatly limited the international research efforts to evaluate the potential of the transgenic technology for enhancing food production of livestock.

While transgenic plants have been commercialized since 1996 [1], transgenic livestock applications for food production have so far been restricted to a few proof-of-concept studies that aim to improve the nutritional quality of milk and meat and will be outlined in detail in the following sections.

Milk with Enhanced Nutritional Qualities

Milk is an important food with high protein content. The protein fraction determines many of the functional properties and provides opportunities for nutritional enhancement through additional health benefits, increased food safety, and improved processing properties for dairy food manufacture.

One approach has been the introduction of new antimicrobial properties into milk, to provide passive immunity to consumers. Lyzozyme (LZ) is a naturally occurring antimicrobial protein, present in milk, saliva, and tears of mammals, where it forms part of the defense system against bacterial infections. However, it is found at three orders of magnitude higher concentration in human milk as compared to dairy milk. The introduction of an expression construct for human LZ resulted in LZ levels in the milk of the transgenic goats equivalent to about 68% of the level found in human milk [2]. The consumption of the human LZ-containing goat milk by pigs exerted beneficial effects and improved their gastrointestinal health [3, 4]. Although the milk has not been tested in humans, the promising results suggest similar gastrointestinal benefits could be possible for humans. In addition, the elevated LZ levels increased general food safety properties of the milk by reducing the bacterial growth that causes cold-spoilage thus prolonging its shelf life. Following a similar strategy, recombinant human lactoferrin (LF) has been overexpressed in the milk of dairy cattle [5]. LF is a minor milk protein with antimicrobial and anti-inflammatory properties, which can support the innate defense mechanisms. Although bovine LF has similar functions, it is present in milk at very low levels. Its human counterpart has been fully adapted to human requirements during evolution and is expected to be better suited for human health applications, in particular, at elevated levels. The transgenic cows were shown to produce milk that contains human LF at concentrations much higher than the natural level of bovine LF. These new characteristics indicate that this functional food might offer new health benefits such as increased protection against infections and healthier intestinal microflora. A conceptually related approach aims to boost the immune system through food-mediated delivery of neutralizing monoclonal antibodies to target specific pathogenic microorganisms of the digestive tract. Thus, consumption of antibody-enriched milk could provide instant protection from infections which severely affect those with increased susceptibility, including the elderly, infants, and patients with illnesses. Proof-of-concept studies in mice successfully demonstrated the potential of this approach. The expression of high antibody levels in milk was able to confer complete protection for the suckling young when challenged with an otherwise lethal dose of virus [6]. Whether the health-enhancing attributes of the functional proteins described above can indeed be realized as food-mediated health benefits in humans is presently unknown and still needs to be assessed.

Although milk from dairy cows is a very common human food, it is known to trigger allergic reactions in some people, a phenomenon that is particularly problematic in infants. Not present in human milk, β-lactoglobulin has been identified as a major whey protein in the milk of dairy species and is thought to be a major allergen in the milk of cows. Reduction of this allergenic protein in dairy milk would make it more amenable for susceptible individuals. This could be achieved by either the disruption of the β-lactoglobulin gene using homologous recombination, often referred to as knockout, or the specific knockdown of its expression by RNA interference (RNAi). However, gene knockouts, a standard approach for the functional characterization of genes in mice, proved to be difficult in farm animals due to low recombination efficiencies in primary livestock cells. Thus RNAi technology might provide a more straightforward approach to unravel the role of β-lactoglobulin and reduce the allergenicity of cow's milk.

Altering the milk composition by altering the levels and ratios of the main endogenous milk proteins has been suggested to enhance the nutritional quality of milk and its processing characteristics, including increased heat stability, calcium content, and cheese manufacture [7, 8]. A drastic change of milk composition has been achieved recently with the introduction of

additional β- and κ-casein genes in transgenic cattle [9]. The milk derived from these cows showed a two- to threefold increase in κ-casein and a minor change for β-casein. These changes affected the physical appearance of the milk, which showed a distinctive color change from ordinary white to yellow for the modified milk [10]. Because κ-casein is located on the surface of the casein micelles, any increase in κ-casein is expected to reduce the size of the casein micelles [11]. The reduced size of the micelles affects the light scattering properties and is thought to be the reason for the observed color change in the high κ-casein milk [10]. Although the effects on the processing characteristics of this novel milk still remain to be determined, trial cheese production resulted in cheese with increased levels of essential amino acids and beneficial minerals and thus improved nutritional quality.

Furthermore, due to the higher κ-casein content, this speciality milk is an improved source of κ-casein-derived bioactive peptides [12] such as glycomacropeptide (GMP) which has been associated with a wide range of health-promoting activities including protection against toxins, bacteria, and viruses, suppression of gastrointestinal inflammation, and modulation of the immune system [13]. GMP is one of the few naturally occurring proteins that lacks the amino acid phenylalanine, which makes it a safe source of dietary protein for people suffering from the genetic disorder phenylketonuria (PKU). They cannot metabolize phenylalanine, and normally need to avoid high-protein foods such as dairy products to prevent the detrimental accumulation of phenylalanine metabolites.

An extension to this, highlighting the unique ability of the transgenic technology to provide novel foods tailored for specific dietary requirements, is the development of transgenic rabbits that produce a milk protein suitable as dietary replacement for PKU sufferers [14].

The carbohydrate component of dairy milk has been another target for modifying milk composition to improve health attributes. The milk sugar lactose causes intestinal disorders in lactose-intolerant people who lack adequate intestinal lactose-hydrolyzing enzyme activity to sufficiently digest lactose after milk consumption [15]. Two strategies to reduce the milk sugar lactose content were evaluated in transgenic mouse models which could provide an elegant alternative to expensive postharvest milk processing. The complete disruption of the expression of α-lactalbumin, an essential component of the lactose synthetase complex, resulted in lactose-free milk [16]. However, because lactose is the main osmotic regulator of milk secretion, it strongly affected production and secretion of the milk, which was highly concentrated and the transgenic mice were unable to sustain lactation. In comparison to the knockout strategy, knockdown of α-lactalbumin expression through RNAi could offer better control, to achieve an acceptable reduction of the lactose and water content of milk, without impacting on its vital attributes. Thus, it might provide an opportunity to reduce the lactose content and at the same time significantly lower transportation costs of liquid milk.

A more successful approach addressing lactose intolerance, which cleverly dissected the issues of osmolarity and lactose levels, targeted the expression of a mammalian lactose hydrolyzing enzyme to the mammary gland [17]. Milk lactose was reduced by 50–85% without affecting osmolarity, due to the conversion of the produced lactose into the osmotically active monosaccharides, glucose, and galactose. Although the transgenic mouse results are promising, and it was speculated that a similar reduction of lactose in bovine milk could ameliorate lactose intolerance, they can only provide an indication due to substantial species-specific differences in milk composition. So far, this approach has not been transferred to livestock and still awaits verification of its merit in dairy cows.

Beside protein and sugar, fat is another important nutritional component of milk which has a significant impact on human health and provides opportunities for the improvement of dairy products. Animal fats are very rich in saturated fatty acids (SFA) which are considered “unhealthy” and have been associated with cardiovascular and coronary heart disease. A major focus is therefore to decrease the unhealthy fats in favor of the healthier unsaturated fats. This would have the added benefit of decreasing the hardness of milk fat resulting in softer butter, with improved spreadability. As the majority of the long-chain fatty acids in milk fat and in particular unsaturated fatty acids (UFAs) originate from food sources, an effective approach to improve milk fat composition might be to tailor the diet of dairy cows through the use of particular forage species or feed additives [18, 19]. Transgenic

technology on the other hand provides the ability of a more directed approach, by modulating endogenous enzymes involved in lipid metabolism. The mammary gland-specific expression in transgenic goats of the rat stearoyl-CoA desaturase (SCD), an enzyme involved in converting SFAs into mono-UFAs, resulted in an increase of mono-UFAs and decreased levels of medium chain SFAs [20]. However, this beneficial change in the milk fat composition was only transient in this particular study. It was most prominent in early lactation which was thought to be a consequence of high instability of the SCD-encoding mRNA and the resulting low expression levels.

Another concept has been to lower the total fat content by disrupting acetyl-coenzyme A carboxylase to prevent de novo fatty acid synthesis in the mammary gland, which accounts for about 50% of the milk fat [7]. While low-fat liquid milk is desirable from a consumer perspective, for the cow it could reduce the feed energy requirements and improve body condition which is likely to translate into lower milk production costs and improved conception rates during early lactation.

A more extreme variation of the concept of adjusting the activity of endogenous enzymes involved in lipid metabolism is the introduction of novel enzymatic activities normally not found in mammals. This can provide livestock animals with the ability to endogenously synthesize essential poly-UFAs (PUFA) and transform their food products into enriched sources. As a result, food products derived from these animals could offer a range of additional PUFA-specific health benefits such as reduced risk for the development of coronary heart disease and a healthy immune system [21]. This strategy was first tested in transgenic mice with the introduction of the fat-1 gene encoding the Caenorhabditis elegans n-3 fatty acid desaturase, an enzyme activity that is absent in vertebrates and renders transgenic animals capable of producing omega-3 FAs through conversion of dietary derived n-6 FAs. Omega-3 is a class of beneficial n-3 PUFAs that is associated with a lower risk of morbidity and mortality from atherosclerosis and coronary heart disease. Milk-specific expression of the desaturase in transgenic mice resulted in elevated levels of long-chain n-3 PUFAs and a concomitant decrease in n-6 PUFAs, although this was most pronounced in phospholipids which are a minor fraction of milk fat, and to a lesser degree in the milk triacylglycerides [22]. Pups consuming the n-3 PUFA enriched milk accumulated the n-3 PUFA docohexaenoic acid in their brains, a compound which has been associated with cognitive performance [23]. The constitutive expression using a humanized fat-1 gene resulted in essentially similar qualitative changes of the n-3 and n-6 PUFAs in milk but at a higher magnitude [24]. So far, the concept has been transferred to pigs but not to any of the dairy species. Unfortunately, changes to the milk fat composition were not investigated in these pigs because the main focus for the study was on the fat content of meat and will be discussed in the next section.

Meat with Enhanced Nutritional Qualities

The introduction of a novel FA desaturase activity to genetically complement mammals with the ability to endogenously synthesize essential PUFA and improve the nutritional characteristics of meat was pioneered in a transgenic mouse model mentioned in the previous section [24]. Mice were engineered by integrating a humanized version of the fat-1 gene into their genomes. Expression of the Caenorhabditis elegans n-3 FA desaturase in these transgenic mice resulted in muscles that were highly enriched in the beneficial omega-3 FAs. While all organs and tissues of the transgenic mice showed a marked reduction of the n-6 to n-3 FA ratio, the effect was greatest for skeletal muscle where the ratio dropped from a value of ~50 for conventional mice to close to 1 for transgenic mice. Considering the health concerns associated with the typically high n-6 to n-3 ratio of Western diets, consumption of food products from omega-3 enriched livestock may provide an opportunity to improve diets. When the approach was recently applied to pigs, the constitutive expression of the fat-1 transgene essentially replicated these results with tail samples from the transgenic pigs found to be highly enriched in omega-3 FAs, in particular for two of the most potent n-3 FAs mainly found in fish [25]. Although, the nutritional consequences still need to be investigated in greater detail, transgenic

food animals enriched in omega-3 FAs may provide a more economical, safe, and sustainable means to fortify meat than the current practice of feeding animals with fish meal and satisfy the growing demand for omega-3 fatty acids in human nutrition [26].

Following a similar concept, pigs were engineered for the endogenous production of the essential PUFA linoleic acid by introducing the FAD2 gene encoding the Δ12 FA desaturase from spinach [27]. Expression of the plant desaturase was selectively targeted to adipocytes and resulted in transgenic pigs showing a 20% increase in linoleic acid content of their white adipose tissue. These results further substantiate the prospect for improving the fatty acid composition of domestic animals through transgenic technology. Meat produced by transgenic pigs could ultimately provide an alternative source for essential FAs, which may ameliorate lifestyle-related health concerns.

Early transgenic livestock studies were mainly focused on modifying body composition for enhanced meat production by stimulating muscle growth via the introduction of genes for growth factors such as growth hormone (GH) or insulin-like growth factor I (IGF-I), a concept that has been highly successful in proof-of-concept studies in transgenic mice [28–31]. Although pigs responded with remarkable growth enhancement following the administration of exogenous GH [32], the initial studies with transgenic GH pigs [33] and sheep [34, 35] were disappointing. The transgenic animals achieved only slightly increased growth rates and were plagued by a range of deleterious side effects due to high systemic GH levels. These were a consequence of poor regulatory control of the transgene activity [33, 36]. Targeting the transgene expression to skeletal muscle [37] or applying conditional expression strategies with the ability to switch it on or off [38] essentially overcame the problem of generating adverse health effects in the transgenic animals and resulted in more desirable effects on growth rate and body composition. Pigs engineered for the tissue-specific expression of human IGF-I in skeletal muscle produced approximately 10% more carcass lean tissue and 20% less total carcass fat. Unexpectedly, the effects showed a strong gender bias with the body composition of transgenic males remaining comparable to conventional boars [37]. Transgenic sheep overexpressing ovine GH, controlled by a zinc-inducible promoter, grew significantly faster, and had a leaner body composition [39]. However, the transgenic sheep had higher parasite fecal egg counts, a potential indication of a compromised immune system.

The modest success of improving growth characteristics in sheep and pigs with growth factor-encoding transgenes is in complete contrast to what has been achieved with GH-enhanced transgenic fish using all piscine DNA constructs [40–43]. In the majority of fish species, the GH transgene resulted in dramatic growth acceleration with up to 35-fold increased growth rates in transgenic loach and salmonids and produced fish that reach double the normal body size in half the normal time [44]. However, some of these growth-enhanced fish showed undesirable pleiotropic effects such as altered skin color, modified skull shape, decreased fertility, and decreased viability. Intriguingly, the astounding growth phenotypes were only achievable in wild fish and could not be replicated in domesticated fish [45]. This may indicate that there is a biological ceiling for further growth enhancement by GH in highly selected domesticated animals, which could explain why the approach achieved only slightly accelerated growth rates in transgenic GH pigs and sheep. Thus, for livestock, the approach, at least in its current form, appears unlikely to deliver on its promise to substantially increase meat production which has to be achieved without adversely impacting on the health of the transgenic animals. The only beneficial outcome realized was some nutritional enhancement through the production of leaner meat, which may provide new leads to further improve the nutritional quality of meat.

A promising alternative strategy to boost growth performance with the prospect of greater control might be the direct manipulation of key regulators of skeletal muscle development. Myostatin is a crucial negative regulator of muscle growth, whose function was revealed with a mouse knockout model which showed two to three times the muscle mass of wild-type mice [46]. These mice closely resembled the double-muscling phenotype of some cattle breeds characterized by a 20% increase in muscle mass associated with a reduction in fat tissue. Analysis of the myostatin gene revealed that these breeds are carriers of natural mutations that result in loss of myostatin function as the underlying cause for

the double-muscle phenotype in cattle [47, 48]. The bulkier conformation of these animals is, however, causing major calving difficulties in these breeds, which are associated with significant welfare concerns. Transgenic technology provides the possibility to avoid these concerns by restricting the effects on the myostatin pathway to only postnatal stages of major muscle growth. The feasibility of such an approach was already demonstrated with a conditional myostatin knockout in mice resulting in postnatal disruption of the myostatin gene and comparable increase in muscle mass to a constitutive knockout [49]. However, additive strategies to interfere with the myostatin pathway including the expression of dominant-negative molecules [50, 51] or RNAi [52, 53] might offer even more flexibility with the ability to control the degree of increased muscle mass. Using site-specific recombination techniques, transgenic mice were generated with a muscle-specific expression cassette for a dominant-negative myostatin pro-domain integrated into the male-specific Y-chromosome [54]. Males of these lines showed a 5–20% increase in skeletal muscle mass; because females do not have a Y-chromosome, all females were non-transgenic and not affected in their growth characteristics. Combined with a postnatal or inducible expression strategy, interference with myostatin function targeted to males only could improve the efficiency of current cattle production systems with the concomitant production of bulls with superior meat production ability and elite dairy cows.

Safer Food from Livestock with Enhanced Health Characteristics

Any improvement to the health status of food animals has an immediate positive effect on food safety, which ultimately will lead to safer food products of superior quality. Moreover, healthier livestock would deliver a whole range of additional beneficial attributes, including improved animal welfare, reduced reliance on animal remedies, reduced risk for disease transmission to humans, and improved reproductive performance and production. While conventional breeding programs with the aim of reducing susceptibility of livestock to pests and diseases have not been very successful, the prospect of complementing traditional disease control measures with transgenic technology is particularly appealing, because it provides new, targeted strategies for improved disease control and animal health [55, 56].

One of the most costly diseases in agriculture is a bacterial infection of the mammary gland known as mastitis. The disease causes a significant reduction of milk yields and renders the milk produced by infected cows unsuitable and unsafe for human consumption. In its nonclinical appearance, the main indicator is a high somatic cell count in milk. Mastitis, however, has the potential to severely affect the health of infected animals and can cause death or require euthanasia on animal welfare grounds. In dairy cattle, about one third of clinical mastitis cases are caused by Staphylococcus aureus infection, a pathogen which, due to its ability of intracellular survival, is particularly difficult to control with conventional antibiotic therapies. Transgenic technology can provide alternative mastitis prevention strategies and in one such approach, the expression of a bacteriolytic enzyme in mammary gland cells, has already been evaluated.

Lysostaphin is a bacterial enzyme with endopeptidase activity that cleaves crucial cell wall components of staphylococci resulting in the lysis of the bacteria. Its potential as an effective antimicrobial agent for the treatment of mastitis was first demonstrated in a mouse model where the mammary-specific expression of lysostaphin conferred a protective effect against Staphylococcus aureus infections [57]. Recently, the concept has been successfully extended to cattle. The transgenic cows, which produced lysostaphin in their milk, recapitulated the mouse results and showed a high degree of protection when challenged with the pathogen [58].

Two other proteins with antimicrobial properties, LF and LZ have long been suggested as strong candidates to confer resistance of mastitis in cattle [59]. Although conceptually similar to the lysostaphin approach, cows overexpressing the antimicrobial protein human LF in their milk appeared to gain no improved protection against mastitis when challenged with an Escherichia coli strain isolated from cows with clinical mastitis [60]. This was rather unexpected, considering that these transgenic cows produced much greater levels of human LF in milk compared to the milk enriched with lysostaphin. Serving as a model for dairy cattle, human LZ has been expressed in the mammary gland of goats [2]. The potent bacteriostatic properties of the milk produced by these goats against two mastitis causing bacteria strongly

suggests that human LZ might be another excellent candidate for conferring resistance to mastitis [61]. Particularly, in light of the human LF results, direct experimental validation of the protective potential will be required to determine the effectiveness of human LZ in preventing mastitis.

A different concept to introduce disease-resistant properties into livestock attempts to strengthen the immune system through the expression of pathogen-specific antibodies and thus providing instant immunity without prior exposure to this particular pathogen. The first studies in livestock involved the expression of mouse monoclonal antibodies in transgenic rabbits, sheep, and pigs but were met with little success because of unexpected problems to express fully functional antibodies [62, 63].

An extension of this approach has been the targeted production of viral-neutralizing monoclonal antibodies in milk, to provide passive immunity and protection from viral infections, which can severely affect suckling neonates. A mouse model expressing a coronavirus specific monoclonal antibody in milk demonstrated that the sustained production of high antibody levels throughout lactation could successfully neutralize the viral infectivity [64]. Moreover, in a similar study, suckling young were challenged with a lethal dose of murine hepatitis virus (MHV). Ingestion of MHV-specific antibody-enriched milk produced by the transgenic mice induced full protection against the viral infection in the pups [6].

The disruption of the virus entry mechanism proved to be an equally potent strategy. Transgenic mice engineered for the expression of a soluble form of a porcine herpes virus receptor, gained effectively full resistance against pseudorabies virus (PRV) infections [65]. Furthermore, the transgenic mice displayed superior protection levels compared to mice vaccinated with an attenuated PRV strain, demonstrating that this transgenic approach may offer superior efficiency for the control of pseudorabies in livestock [66].

The introduction of a specific disease resistance gene MX1 from mouse, known to confer resistance to influenza viruses in mice was another approach evaluated for its potential to protect livestock animals from viral infections. Similar to other pioneering studies prior to the mid-1990s, difficulties to adequately control Mx1 expression in transgenic pigs hampered the approach, which ultimately failed to achieve viral protection in pigs [67]. For now, the real potential of this approach remains unknown but the identification of antiviral Mx alleles from livestock species [68–70] and new tools for the improved control of transgene expression levels, such as the recently developed auto-regulative tetracycline-responsive expression cassette [71], may encourage a reevaluation of MX alleles for their potential to confer disease resistance in domesticated animals.

Rather than attempting to provide additional protective attributes, transgenic technology also enables the direct targeting of endogenous genes implicated in disease pathways. Such a concept has been applied to produce livestock animals which are resistant to the fatal neurodegenerative prion diseases or transmissible spongiform encephalopathies. This family of diseases, which include scrapie and the "mad cow disease" bovine spongiform encephalopathy, or BSE for short, is characterized by an accumulation of a misfolded isoform of the cellular prion protein (PrP) that acts as a novel infectious agent. Proof-of-concept studies in the mouse [72, 73], either introduction of mutated prion protein genes [74], gene knockout [75–77], or RNAi-mediated knockdown of PrP expression [78] demonstrated the possibility to produce animals that are resistant to prion diseases. Resistant livestock, certain to be free of such diseases would eliminate the risk for potential transmission of the disease to humans and provide additional safeguards for the food chain. However, some mouse studies raised concerns that the loss of the normal cellular function of PrP may adversely affect the animals by interfering with the circadian rhythm [79], synaptic functions [80, 81], learning [82], or loss of movement coordination [83]. When these findings were further investigated, the underlying molecular cause was not the loss of PrP function but an interference with the expression pattern of an adjacent gene as a result of only partially deleting the PrP locus [84–86].

Initial studies in sheep [75] and goats [77] indicated that animals heterozygous for the PrP knockout display, at least in young animals, a normal phenotype. These findings could now be confirmed with homozygous knockout cattle with deletions of the entire PrP gene [87]. In vitro assays with brain tissue homogenates derived from these PrP deficient cattle

demonstrated resistance to prion propagation while the cattle were apparently normal in all aspects analyzed.

The recent development of RNAi technology, based on the expression of a short RNA molecule that can interfere with the activity of a specific target gene offers new opportunities to directly attack pathogens by disrupting their lifecycles. It is particularly attractive as an antiviral strategy, where RNAi can be used to target viral transcripts and suppress viral infection [88]. Because RNAi is a sequence-specific process, it will only affect the virus but not the host animal. Validated by the successful application of RNAi to control viral diseases in mouse model systems [89–91], the concept is applicable to a wide range of significant pathogens such as the RNA viruses that constitute over two thirds of the Office International Des Epizooties (OIE) list-A pathogens [88]. Recent outbreaks of viral diseases in farmed animals with immense socioeconomic and animal welfare costs such as foot and mouth disease or with the risk to cross the species barrier into humans such as Severe Acute Respiratory Syndrome (SARS), avian influenza and swine flu highlight the importance for improving current intervention strategies. Several studies are presently undertaken to evaluate the effectiveness of RNAi-mediated viral resistance in domesticated animals including chickens, pigs, cattle, sheep, and horses.

Future Directions

Transgenic livestock technology has made tremendous progress since its humble beginnings in 1985 [92]. Poor control of the transgene activity presented a main problem in early studies. Today, it is possible to integrate a transgene into a specific site of the genome and provide accurate control from the endogenous regulatory sequence elements. In addition, molecular switches have been developed which enable to switch the expression of a transgene on or off and can be combined to form sophisticated gene control networks [93]. In parallel, the understanding of how gene function relates to phenotypes is rapidly growing and has been further accelerated with the sequencing of the genomes of livestock species [94–96]. Thus, the application of transgenic technology to improve food production holds much promise for the future. Yet, transgenic technology should not be seen in isolation and as sole answer to all questions. The greatest benefit will result from combining transgenic strategies with other effective technologies. At present, however, most consumers consider transgenic technology for food applications as risky and unsafe and thus not acceptable [97]. While this may be predominantly driven by a general unease about an unfamiliar new technology, it had profound effects on politicians, regulators, and research investors and has largely prevented its application. With the mounting pressure on food safety and security, food and lifestyle-related health problems in the context of the rapidly increasing population and a changing climate, calls are becoming louder that this promising technology can no longer be ignored [98]. To gain consumer acceptance, it will be crucial to engage into an open dialog with the public that balances perceived against real risks. Only if a robust risk benefit assessment forms the bases for a decision on the acceptability of food-producing transgenic livestock will there be a chance to realize the benefits of the technology.

Bibliography

Primary Literature

1. James C (2009) Global status of commercialized biotech/GM crops: 2009. ISAAA Brief No. 41. ISAAA, Ithaca, NY
2. Maga EA, Shoemaker CF, Rowe JD, BonDurant RH et al (2006) Production and processing of milk from transgenic goats expressing human lysozyme in the mammary gland. J Dairy Sci 89:518–524
3. Brundige DR, Maga EA, Klasing KC, Murray JD (2008) Lysozyme transgenic goats' milk influences gastrointestinal morphology in young pigs. J Nutr 138:921–926
4. Brundige DR, Maga EA, Klasing KC, Murray JD (2010) Consumption of pasteurized human lysozyme transgenic goats' milk alters serum metabolite profile in young pigs. Transgenic Res 19:563–574
5. van Berkel PH, Welling MM, Geerts M, van Veen HA et al (2002) Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nat Biotechnol 20:484–487
6. Kolb AF, Pewe L, Webster J, Perlman S et al (2001) Virus-neutralizing monoclonal antibody expressed in milk of transgenic mice provides full protection against virus-induced encephalitis. J Virol 75:2803–2809
7. Wall RJ, Kerr DE, Bondioli KR (1997) Transgenic dairy cattle: genetic engineering on a large scale. J Dairy Sci 80:2213–2224

8. Zuelke KA (1998) Transgenic modification of cows milk for value-added processing. Reprod Fertil Dev 10:671–676
9. Brophy B, Smolenski G, Wheeler T, Wells D et al (2003) Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. Nat Biotechnol 21:157–162
10. Laible G, Brophy B, Knighton D, Wells DN (2007) Compositional analysis of dairy products derived from clones and cloned transgenic cattle. Theriogenology 67:166–177
11. Farrell HM, Malin EL, Brown EM, Qi PX (2006) Casein micelle structure: what can be learned from milk synthesis and structural biology? Curr Opin Coll Interface Sci 11:135–147
12. Clare DA, Swaisgood HE (2000) Bioactive milk peptides: a prospectus. J Dairy Sci 83:1187–1195
13. Brody EP (2000) Biological activities of bovine glycomacropeptide. Br J Nutr 84(suppl 1):S39–S46
14. Baranyi M, Hiripi L, Szabo L, Catunda AP et al (2007) Isolation and some effects of functional, low-phenylalanine kappa-casein expressed in the milk of transgenic rabbits. J Biotechnol 128:383–392
15. Sahi T (1994) Genetics and epidemiology of adult-type hypolactasia. Scand J Gastroenterol Suppl 202:7–20
16. Stinnakre MG, Vilotte JL, Soulier S, Mercier JC (1994) Creation and phenotypic analysis of alpha-lactalbumin-deficient mice. Proc Natl Acad Sci USA 91:6544–6548
17. Jost B, Vilotte JL, Duluc I, Rodeau JL et al (1999) Production of low-lactose milk by ectopic expression of intestinal lactase in the mouse mammary gland. Nat Biotechnol 17:160–164
18. Kay JK, Roche JR, Kolver ES, Thomson NA et al (2005) A comparison between feeding systems (pasture and TMR) and the effect of vitamin E supplementation on plasma and milk fatty acid profiles in dairy cows. J Dairy Res 72:322–332
19. Palmquist DL, Beaulieu AD, Barbano DM (1993) Feed and animal factors influencing milk fat composition. J Dairy Sci 76:1753–1771
20. Reh WA, Maga EA, Collette NM, Moyer A et al (2004) Hot topic: using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition. J Dairy Sci 87:3510–3514
21. Simopoulos AP (1999) Essential fatty acids in health and chronic disease. Am J Clin Nutr 70:560S–569S
22. Kao BT, Lewis KA, DePeters EJ, Van Eenennaam AL (2006) Endogenous production and elevated levels of long-chain n-3 fatty acids in the milk of transgenic mice. J Dairy Sci 89:3195–3201
23. McCann JC, Ames BN (2005) Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. Am J Clin Nutr 82:281–295
24. Kang JX, Wang JD, Wu L, Kang ZB (2004) Transgenic mice – Fat-1 mice convert n-6 to n-3 fatty acids. Nature 427:504
25. Lai L, Kang JX, Li R, Wang J et al (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24:435–436
26. Kang JX, Leaf A (2007) Why the omega-3 piggy should go to market. Nat Biotechnol 25:505
27. Saeki K, Matsumoto K, Kinoshita M, Suzuki I et al (2004) Functional expression of a Delta12 fatty acid desaturase gene from spinach in transgenic pigs. Proc Natl Acad Sci USA 101:6361–6366
28. Hammer RE, Brinster RL, Rosenfeld MG, Evans RM et al (1985) Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. Nature 315:413–416
29. Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ et al (1988) Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 123: 2827–2833
30. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME et al (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611–615
31. Palmiter RD, Norstedt G, Gelinas RE, Hammer RE et al (1983) Metallothionein-human GH fusion genes stimulate growth of mice. Science 222:809–814
32. Chung CS, Etherton TD, Wiggins JP (1985) Stimulation of swine growth by porcine growth hormone. J Anim Sci 60:118–130
33. Pursel VG, Pinkert CA, Miller KF, Bolt DJ et al (1989) Genetic engineering of livestock. Science 244:1281–1288
34. Murray JD, Nancarrow CD, Marshall JT, Hazelton IG et al (1989) Production of transgenic merino sheep by microinjection of ovine metallothionein-ovine growth hormone fusion genes. Reprod Fertil Dev 1:147–155
35. Rexroad CE Jr, Hammer RE, Bolt DJ, Mayo KE et al (1989) Production of transgenic sheep with growth-regulating genes. Mol Reprod Dev 1:164–169
36. Pursel VG, Rexroad CE Jr (1993) Recent progress in the transgenic modification of swine and sheep. Mol Reprod Dev 36:251–254
37. Pursel VG, Wall RJ, Mitchell AD, Elasser TH et al (1999) Expression of insulin-like growth factor-1 in skeletal muscle of transgenic swine. In: Murray JD, Anderson GB, Oberbauer AM, McGloughlin MM (eds) Transgenic animals in agriculture. CABI, Oxon, UK, pp 131–144
38. Nottle MB, Nagashima H, Verma PJ, Du ZT et al (1999) Production and analysis of transgenic pigs containing a metallothionein porcine growth hormone gene construct. In: Murray JD, Anderson GB, Oberbauer AM, McGloughlin MM (eds) Transgenic animals in agriculture. CABI, Oxon, UK, pp 145–156
39. Adams NR, Briegel JR, Ward KA (2002) The impact of a transgene for ovine growth hormone on the performance of two breeds of sheep. J Anim Sci 80:2325–2333
40. Devlin RH, Yesaki YT, Biagi CA, Donaldson EM et al (1994) Extraordinary salmon growth. Nature 371:209–210

41. Du SJ, Gong Z, Fletcher GL, Shears MA et al (1992) Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. Biotechnology 10:176–181
42. Nam Y, Noh J, Cho Y, Cho H et al (2001) Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach Misgurnus mizolepis. Transgenic Res 10:353–362
43. Rahman MA, Maclean N (1999) Growth performance of transgenic tilapia containing an exogenous piscine growth hormone gene. In: McAndrew B, Penman D, Hulata G (eds) Aquaculture 173:1/4, 333–346. 34 ref
44. Zbikowska HM (2003) Fish can be first-advances in fish transgenesis for commercial applications. Transgenic Res 12:379–389
45. Devlin RH, Biagi CA, Yesaki TY, Smailus DE et al (2001) Growth of domesticated transgenic fish. Nature 409:781–782
46. McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387:83–90
47. Kambadur R, Sharma M, Smith TP, Bass JJ (1997) Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res 7:910–916
48. McPherron AC, Lee S-J (1997) Double muscling in cattle during to mutations in the myostatin gene. Proc Natl Acad Sci USA 94:12457–12461
49. Grobet L, Pirottin D, Farnir F, Poncelet D et al (2003) Modulating skeletal muscle mass by postnatal, muscle-specific inactivation of the myostatin gene. Genesis 35:227–238
50. Lee S-J, Reed LA, Davies MV, Girgenrath S et al (2005) Regulation of muscle growth by multiple ligands signaling through activin type II receptors. PNAS 102:18117–18122
51. Yang J, Ratovitski T, Brady J, Solomon M et al (2001) Expression of myostatin pro domain results in muscular transgenic mice. Mol Reprod Dev 60:351–361
52. Jain H, Singh S, Kadam M, Sarkhel BC (2010) Knockdown of the myostatin gene by RNA interference in caprine fibroblast cells. J Biotechnol 145:99–102
53. Magee TR, Artaza JN, Ferrini MG, Vernet D et al (2006) Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. J Gene Med 8:1171–1181
54. Pirottin D, Grobet L, Adamantidis A, Farnir F et al (2005) Transgenic engineering of male-specific muscular hypertrophy. Proc Natl Acad Sci USA 102:6413–6418
55. Muller M, Brem G (1998) Transgenic approaches to the increase of disease resistance in farm animals. Revue Scientifique Et Technique (International Office Of Epizootics) 17:365–378
56. Whitelaw CB, Sang HM (2005) Disease-resistant genetically modified animals. Rev Sci Tech 24:275–283
57. Kerr DE, Plaut K, Bramley AJ, Williamson CM et al (2001) Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. Nat Biotechnol 19:66–70
58. Wall RJ, Powell AM, Paape MJ, Kerr DE et al (2005) Genetically enhanced cows resist intramammary Staphylococcus aureus infection. Nat Biotechnol 23:445–451
59. Seyfert HM, Henke M, Interthal H, Klussmann U et al (1996) Defining candidate genes for mastitis resistance in cattle: The role of lactoferrin and lysozyme. J Anim Breed Genet 113:269–276
60. Hyvonen P, Suojala L, Orro T, Haaranen J et al (2006) Transgenic cows that produce recombinant human lactoferrin in milk are not protected from experimental Escherichia coli intramammary infection. Infect Immun 74:6206–6212
61. Maga EA, Cullor JS, Smith W, Anderson GB et al (2006) Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk. Foodborne Pathog Dis 3:384–392
62. Lo D, Pursel V, Linton PJ, Sandgren E et al (1991) Expression of Mouse Iga by transgenic mice pigs And sheep. Eur J Immunol 21:1001–1006
63. Weidle UH, Lenz H, Brem G (1991) Genes encoding a mouse monoclonal antibody are expressed in transgenic mice, rabbits and pigs. Gene 98:185
64. Castilla J, Pintado B, Sola I, Sanchez-Morgado JM et al (1998) Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. Nat Biotechnol 16:349–354
65. Ono E, Amagai K, Taharaguchi S, Tomioka Y et al (2004) Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock. Proc Natl Acad Sci USA 101:16150–16155
66. Ono E, Tomioka Y, Taharaguchi S, Cherel P (2006) Comparison of protection levels against pseudorabies virus infection of transgenic mice expressing a soluble form of porcine nectin-1/HveC and vaccinated mice. Vet Microbiol 114:327–330
67. Mueller M, Brenig B, Winnacker E-L, Brem G (1992) Transgenic pigs carry cDNA copies encoding the murine Mx1 protein which confers resistance to influenza virus infection. Gene 121:263–270
68. Asano A, Ko JH, Morozumi T, Hamashima N et al (2002) Polymorphisms and the antiviral property of porcine Mx1 protein. J Vet Med Sci 64:1085–1089
69. Baise E, Pire G, Leroy M, Gerardin J et al (2004) Conditional expression of Type I interferon-induced bovine Mx1 GTPase in a stable transgenic vero cell line interferes with replication of vesicular stomatitis virus. J Interferon Cytokine Res 24:513–521
70. Leroy M, Pire G, Baise E, Desmecht D (2006) Expression of the interferon-alpha/beta-inducible bovine Mx1 dynamin interferes with replication of rabies virus. Neurobiol Dis 21:515

71. Kues WA, Schwinzer R, Wirth D, Verhoeyen E et al (2006) Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. FASEB J 20:1200–1202
72. Bueler H, Aguzzi A, Sailer A, Greiner RA et al (1993) Mice devoid of PrP are resistant to scrapie. Cell 73:1339–1347
73. Perrier V, Kaneko K, Safar J, Vergara J et al (2002) Dominant-negative inhibition of prion replication in transgenic mice. Proc Natl Acad Sci USA 99:13079–13084
74. Cyranoski D (2003) Koreans rustle up madness-resistant cows. Nature 426:743
75. Denning C, Burl S, Ainslie A, Bracken J et al (2001) Deletion of the alpha(1, 3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat Biotechnol 19:559–562
76. Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J et al (2004) Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet 36:775–780
77. Yu G, Chen J, Yu H, Liu S et al (2006) Functional disruption of the prion protein gene in cloned goats. J Gen Virol 87:1019–1027
78. Golding MC, Long CR, Carmell MA, Hannon GJ et al (2006) Suppression of prion protein in livestock by RNA interference. Proc Natl Acad Sci USA 103:5285–5290
79. Tobler I, Gaus SE, Deboer T, Achermann P et al (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. Nature 380:639
80. Collinge J, Whittington MA, Sidle KCL, Smith CJ et al (1994) Prion protein is necessary for normal synaptic function. Nature 370:295
81. Mallucci GR, Ratte S, Asante EA, Linehan J (2002) Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. [erratum appears in EMBO J 2002 Mar 1;21(5):1240]. EMBO J 21:202–210
82. Criado JR, Sanchez-Alavez M, Conti B, Giacchino JL et al (2005) Mice devoid of prion protein have cognitive deficits that are rescued by reconstitution of PrP in neurons. Neurobiol Dis 19:255
83. Sakaguchi S, Katamine S, Nishida N, Moriuchi R et al (1996) Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. Nature 380:528
84. Li A, Sakaguchi S, Atarashi R, Roy BC et al (2000) Identification of a novel gene encoding a PrP-like protein expressed as chimeric transcripts fused to PrP exon 1/2 in ataxic mouse line with a disrupted PrP gene. Cell Mol Neurobiol 20:553–567
85. Moore RC, Lee IY, Silverman GL, Harrison PM et al (1999) Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein doppel. J Mol Biol 292:797
86. Rossi D, Cozzio A, Flechsig E, Klein MA et al (2001) Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. EMBO J 20:694–702
87. Richt JA, Kasinathan P, Hamir AN, Castilla J et al (2007) Production of cattle lacking prion protein. Nat Biotechnol 25:132
88. Clark J, Whitelaw B (2003) A future for transgenic livestock. Nat Rev Genet 4:825–833
89. Carmona S, Ely A, Crowther C, Moolla N et al (2006) Effective inhibition of HBV replication in vivo by anti-HBx short hairpin RNAs. Mol Ther 13:411–421
90. McCaffrey AP, Nakai H, Pandey K, Huang Z et al (2003) Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol 21:639–644
91. Tompkins SM, Lo CY, Tumpey TM, Epstein SL (2004) Protection against lethal influenza virus challenge by RNA interference in vivo. Proc Natl Acad Sci USA 101:8682–8686
92. Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ et al (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
93. Greber D, Fussenegger M (2007) Mammalian synthetic biology: engineering of sophisticated gene networks. J Biotechnol 130:329–345
94. Amaral AJ, Megens HJ, Kerstens HH, Heuven HC et al (2009) Application of massive parallel sequencing to whole genome SNP discovery in the porcine genome. BMC Genomics 10:374
95. Elsik CG, Tellam RL, Worley KC, Gibbs RA et al (2009) The genome sequence of taurine cattle: a window to ruminant biology and evolution. Science 324:522–528
96. Wallis JW, Aerts J, Groenen MA, Crooijmans RP et al (2004) A physical map of the chicken genome. Nature 432:761–764
97. Pardo R, Engelhard M, Hagen K, Jorgensen RB et al (2009) The role of means and goals in technology acceptance. A differentiated landscape of public perceptions of pharming. EMBO Rep 10:1069–1075
98. Fedoroff NV, Battisti DS, Beachy RN, Cooper PJ et al (2010) Radically rethinking agriculture for the 21st century. Science 327:833–834

Books and Reviews

Council for Agricultural Science and Technology (CAST) (2003) Biotechnology in animal agriculture: an overview. Issue Paper 23. CAST, Ames, Lowa

Council for Agricultural Science and Technology (CAST) (2009) Animal productivity and genetic diversity: cloned and transgenic animals. Issue Paper 43. CAST, Ames, Lowa

Gottlieb S, Wheeler MB (2008) Genetically engineered animals and public health: compelling benefits for health care, nutrition, the environment and animal welfare. www.bio.org/foodag/animals/ge_animal_benefits.pdf

Heller KJ (2006) Genetically engineered food: methods and detection. Wiley-VHC, Weinheim

Jimenez-Flores R, Richardson T (1988) Genetic engineering of the caseins to modify the behavior of milk during processing: a review. J Dairy Sci 71:2640–2654

Swain DL, Charmley E, Steel JW, Coffey SG (2007) Redesigning animal agriculture: the challenge for the 21st century. CABI, Wallingford, UK

Transgenic Livestock, Ethical Concerns and Debate

Mickey Gjerris[1], Reinhard Huber[2], Jesper Lassen[1], I Anna S. Olsson[2], Peter Sandøe[1]
[1]Danish Centre for Bioethics and Risk Assessment, Institute of Food and Resource Economics, University of Copenhagen, Frederiksberg, Denmark
[2]Laboratory Animal Science Group, IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal

Article Outline

Glossary

Animal bioreactor Transgenic animal that produces recombinant proteins in its milk, egg white, blood, urine, or seminal plasma.

Antibody Protein produced as part of the immune reaction to render harmless a foreign substance (e.g., bacteria) entering the body of an organism.

Cloning (a) Production of exact copies (clones) of a gene/genes (gene cloning). The DNA strand containing the gene of interest is cut into suitably sized pieces (fragmentation) and the gene of interest is linked to a piece of DNA (cloning vector). This vector is then introduced into cells (transfection) which are cultured in vitro and then screened for the presence of the gene of interest. (b) Production of genetically identical organisms by somatic cell nuclear transfer (SCNT). It involves the introduction of the nucleus of a somatic cell from the organism to be cloned into an enucleated egg cell. The resulting cell divides after activation (application of electric shock) into an embryo which may then be implanted into a surrogate mother (reproductive cloning) or used to establish a tissue culture (therapeutic cloning).

Embryonic germ cell Pluripotent stem cells derived from early germ cells with properties similar to embryonic stem cells.

Embryonic stem cell Cell derived from an early embryo that is not differentiated by itself but may divide either to form (a) other stem cells or (b) cells that differentiate into specialized cell types.

Gene copy number Genes naturally exist in varying number of copies in the genome. In relation to genetic modification, gene copy number refers to the number of copies of a transgene that integrate into the host genome.

Gene expression The assembly of a product (mainly protein) based on the information coded in a gene.

Gene targeting The modification of a certain endogenous gene of an organism based on homologous recombination.

Germ cell Cell that produces gametes (egg cells in females, sperm in males).

Heterozygous Organism/cell in which the two chromosomes in a pair contain different alleles (alternative forms of a gene) at a given locus. The dominant allele will determine the phenotype.

Homologous recombination The exchange of genetic information between two similar or identical strands of DNA (often during meiosis, i.e., the formation of gametes). This process is used for the introduction of DNA sequences into the genome of organisms by gene targeting.

Homozygous Organism/cell in which the two chromosomes in a pair contain identical alleles (alternative forms of a gene) at a given locus. The alleles may either be dominant or recessive.

Hemizygous Organism/cell with only the given allele present at the given locus of only one of the chromosomes in a pair.

Knockout The replacement of a functioning endogenous gene with an inoperable version.

Lentiviruses Viruses that are able to infect both dividing and nondividing cells and are therefore used as tools as vectors for gene delivery.

Marker-assisted selection Method allowing the selection of breeding animals based on their genotype rather than their phenotype. Regions of the genome

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

that control certain production traits are mapped and DNA markers that control production traits are identified. Animals whose genome contains the desired markers are selected for further breeding.

Motivation The internal state of an animal which makes it behave in a certain way; the overall summation of all internal and external factors affecting decision-making.

Pronucleus The nucleus of either a sperm (male p.) or an egg (female p.) cell before their fusion inside the egg cell in the process of fertilization. At pronuclear microinjection, the DNA containing the transgene is injected into one of the pronuclei which fuse. After implantation of the egg into a female, the resulting embryo develops into a hemizygous transgenic animal which must be bred to obtain homozygous transgenic animals.

Recombinant protein Proteins that are expressed based on recombinant DNA (rDNA). rDNA is obtained by genetic engineering techniques, i.e., the ´artificial´ combination of information contained in the transgene and in the host genome.

Somatic nuclear cell transfer See Cloning.

Subjective experience Conscious mental state, an experience that the individual is aware of.

Viral vector Virus-based vehicle to introduce genetic information into cells making use of the capacity of viruses to transfer their genome into the cells they infect. For transgenesis purposes, they are rendered replication-deficient to avoid replication once the transfer of the genetic information of interest into the cell has taken place.

Definition of the Subject

Humans have been able to manipulate the genomes of livestock through selective breeding for centuries; however, direct intervention has become possible only through the development of transgenic technology over the past 3 decades. So far, genetically modified animals have mainly been developed for basic research and biomedicine, but they are slowly beginning to enter the agricultural production system. In this article, livestock is defined to be all species used within the agricultural and aquacultural system. Note that such animals can be genetically modified for use within basic and medical research as well.

Most people would readily agree that there is a difference between what humans *can* do and what they *ought* to do. Equally, most people would happily acknowledge that it is good to do the morally right thing. However, the harmony usually ends there, because although it is easy to agree that a good thing should be promoted, it is often hard to reach consensus on what that good thing is, how it can be promoted, and to what extent the end justifies the means. There may also be disagreement over what counts as a bad outcome or about whether there are acts which are wrong in themselves. As soon as questions as these are discussed whether in private or in public, people are engaging in an ethical discussion – a discussion in which the aim is to reach agreement on what is good and bad and what is right and wrong.

Such ethical discussions have closely shadowed developments within biotechnology over the past 30 years. A range of applications of biotechnology to animals, including reproductive technologies, genetic modification, and cloning, has prompted concern about the ethical limits of human use of animals. It is probably an understatement to say that discussion has so far led to no consensus in the public sphere, but it would also be an overstatement to say that the debate has led to unanimous agreement. What *has* emerged, among other things, is a clearer understanding of the basic ethical assumptions driving the different viewpoints, together with greater attention to the ethical duties of humans toward animals.

The technological challenge of animal biotechnology is: What *can* be done? This question is closely followed by the question: What *ought* to be done? As with any other human activity, this question needs to be answered if decisions on what to do are to be made. That is both the blessing and the curse of being an ethical creature equipped with a conscience. Humans cannot just act and use the possibilities that lie before them; they have to take responsibility for the values that are promoted through their choices. At the same time, there are huge disagreements about what the relationship between *can do* and *ought to do* in the area of transgenic animals is. One of the consequences of this is that ethics cannot be pursued as a mere afterthought to the technology or as something of concern only to opponents of the technology.

The subject of this article is the ethical concerns about transgenic livestock. While the strict scientific definition of "transgenesis" implies that genetic material is transferred between species [1, 2], the term is often used interchangeably with genetic modification to cover a wider range of techniques in which the animal genome is manipulated – e.g., including the inactivation of genes. For livestock applications, transgenesis proper is the predominant kind of genetic modification, and so, in this chapter, "transgenic" is used only to refer to the transfer between species. Examples of genetic modification will be used additionally to clarify important points. The goal is to make the different areas of concern that arise in the ethical debates transparent, and to discuss different ways of approaching a public dialogue on these matters.

Introduction

Gene technology and other forms of modern biotechnology have given rise to ethical controversies since they were first introduced in the early 1980s. This has led to a growing awareness among both scientists and politicians that successful implementation of biotechnology in democratic and pluralistic societies requires ethical concerns to be discussed and taken seriously. Numerous and extensive attempts have thus been made over the past 3 decades to engage stakeholders and the public in general in discussion of the ethical aspects of biotechnology.

Already, the earliest transgenic animals in the 1980s were the subject of controversy [3–6]. However, it was probably with the creation of the cloned sheep Dolly in 1997 that animal biotechnology became widely known to the public in the industrialized world [7]. Looking at both studies of public opinion on the subject and the ethical literature, one can see that the concerns here can be divided into distinct groups. Some relate to possible impacts on humans, either through health-related risks, environmental consequences, or socio-economic changes. Some revolve around the possible conflicts arising from the possibility of patenting living beings. Others revolve around animal welfare, and different concepts of that, while the last group expresses more general concerns about the (un)naturalness of the technologies, and possible violations of the integrity of the animal species. One discussion that arches over all of these issues and has been an important determinant in framing the ethical debate as a whole focuses on how best to regulate the area.

Sociological studies have revealed public skepticism about transgenic animals. Considerations of risk, usefulness, animal welfare, and other moral values play a substantial role in the public evaluation of the technologies. Based to some extent on these studies, many attempts have been made to engage the public and stakeholders such as scientists, politicians, legislators, industry, animal welfare organizations, consumers, and others in society-wide discussions of how the new technologies can be implemented in a way that will be acceptable for a majority of citizens. Whether or not these attempts have been successful obviously depends on the criteria of success applied. What can be said, however, is that they have not led to general agreement about the development and use of transgenic animals.

In this area of ethical thinking and public discourse, it is necessary to be aware of the open-ended nature of the discussion. From a scientific point of view, there is a world of difference between a knockout pig that has had a gene disabled to better serve as a organ donor for humans (xenotransplantation) [8] and a goat that has had a human gene inserted that expresses itself in the mammary glands thus enabling human proteins to be harvested from the milk [9]. But from the point of view of the nonexpert, these differences might seem ethically unimportant if the concern in play is the possible violation of the species' integrity.

There is also a tendency among experts to look primarily at methods, or techniques, when evaluating biotechnology, whereas members of the public often bring in considerations of usefulness as well. Ethical discussions thus differ from technical discussions, and it is necessary to critically evaluate the amount of scientific knowledge and expertise needed within different layers of the ethical discussion. Obviously, science and scientific thinking cannot be left out of ethical discussions of science and its technical applications, but neither should their importance be overstressed [10].

Similarly, it can be problematic if the subject of the ethical discussion becomes too narrow. There is a tendency in the current research environment for ethics to follow natural science into increasingly specialized fields where it becomes difficult to include other areas of science with similar concerns. But at

the level of ethical concerns, it is sometimes helpful to discuss transgenic livestock together with, for example, other issues relating to modern intensive animal production [11]. Thus the concerns that people have about transgenic livestock may to a large extent be about taking an already problematic development even further.

As a prerequisite to any ethical debate about transgenic livestock, it is of course necessary to make it clear what the subject of the discussion is, and what areas of application are involved. This ensures an informed debate in which misunderstandings resulting from unclarity are avoided.

Science and Applications

From Early Research to Maturing Technology

Humans have manipulated the genetic makeup of livestock through the process of domestication for thousands of years. Scientifically based farm animal breeding began in the early twentieth century, and, particularly since the 1950s, it has had a profound effect on most livestock species. Various modern biotechnologies – particularly reproductive technologies – have gradually been adopted since the 1980s.

A comparatively recent technology in this process of development is genetic engineering/modification; this emerged in the 1980s and has evolved considerably since. Genetic modification involves the direct manipulation of the genome of a group of founder animals, unlike traditional breeding, where animals are selected on the basis of their phenotype and then mated in order to approach a certain breeding goal gradually. There is a degree of vagueness in the definition of the techniques used to generate genetically modified organisms. Genetic modification, in the broadest sense, has been defined as an "altering of the genetic material in that [the GM] organism in a way that does not occur naturally by mating or natural recombination or both" [12]; this covers a wide array of technologies.

Genetic modification and transgenesis are sometimes used synonymously, but the term "transgenesis" is often defined more narrowly to apply to just one aspect of genetic modification: transgenic animals carry foreign genetic information (i.e., information from another species) and transmit this information to their progeny, because the transgene is integrated in the germ cells of the animal [1]. Genetic modification may involve further modification of the genome (moving, deleting, multiplying, modifying genes) within an organism – e.g., the creation of knockout animals. Creating a knockout animal involves the targeted inactivation of a gene. In this process, the protein usually produced as a consequence of the genetic information in question is no longer produced. This is achieved by replacing an active gene with an inactive version, thus preventing the gene in question from being expressed [13]. Knockout applications are common in mice used for fundamental and biomedical research; however, in livestock species, transgenesis is the most common form of genetic modification, although even here, knockout applications do exist (e.g., mini-pigs which have had genes knocked out in connection with xenotransplantation research) [14].

The main projected advantages of transgenesis over established breeding techniques include the possibility of introducing genes that do not exist in the genome of the parents (outside the species), and the opportunity to target desirable traits without coselecting genetically coupled undesirable ones. The foreign gene is introduced in early stage embryos (first cell stage) or in cells that participate later in the complete development of the animal [13]. These embryos develop into transgenic founder animals, which can then be used in further breeding. Regardless of the particular method used, the main steps in producing transgenic animals are as follows [15]:

- Identifying the trait of interest and localizing the corresponding gene in the donor species genome
- Multiplying the gene through cloning
- Production of a suitable construct for gene transfer
- Gene transfer
- Proof of integration of the foreign gene
- Proof of expression of the foreign gene in the host animal
- Demonstrating that offspring of founder animals inherit the foreign gene
- Selective breeding to diffuse the foreign gene into the relevant population of animals

Some of the technology to create transgenic livestock is based on methods developed for use in mice: the first transgenic mammals created by pronuclear microinjection in 1980 were mice [16]. This method

was adapted in 1985 to create the first transgenic livestock (rabbits, sheep, pigs) [17].

In further developing the technologies, researchers have tried to address technical limitations, and to develop alternatives to pronuclear injection with improved efficiency (i.e., proportion of newborn animals carrying the transgene), predictability, and control of gene expression. Predictability refers to where in the host genome the transgene is integrated. When technologies in which the integration site is random are used, there may be unexpected expression patterns as a result of interference with the host genome. Technologies allowing targeted integration increase predictability by giving greater control of gene expression. Whether or not the number of copies can be controlled is also relevant, as copy number influences the level and stability of transgene expression [18].

Table 1 provides an overview of the most prominent methods of transgenesis, noting the availability for different species as well as the predictability, efficiency, and feasibility.

There are however several practical obstacles to routine production of transgenic livestock [21]. The efficiency of the methods used was very low in the beginning, and although progress has been made here, transgenesis still cannot compete with other methods used for the genetic improvement of livestock (e.g., selective breeding in combination with artificial insemination and marker-assisted selection) on cost efficiency, practicality, and public/political acceptance. Furthermore, many traits of interest in livestock are controlled by more than one gene and are therefore difficult to improve by transgenesis. The introduction of transgenesis in livestock breeding is further complicated by the fact that, typically at any rate, very few transgenic animals are generated in the first generation. This makes diffusion into a breeding population without corresponding inbreeding depression difficult. It also means that, since the phenotypic uniformity of transgenic offspring is often limited, the evaluation of gene expression in transgenic livestock would require a large number of animals.

Fish have been popular models for research into transgenesis as they are easy to maintain/handle and highly fertile. They have easily manipulable, large eggs, which are fertilized and develop externally; fish species also usually tolerate genetic manipulation well during early development (unlike, say, rats). The most

Transgenic Livestock, Ethical Concerns and Debate. Table 1 Summary of existing transgenic approaches (Adapted from [19, 20])

Transgenic method	Availability	Integration site(s)[a]	Number of copies per site	Efficiency[b]/ technical difficulty	Note
Microinjection	Most mammals[c]	Random	Variable	Low/medium	First method
Somatic cell nuclear transfer	Most mammals	Can be determined	Often single copy	Low/high	>1997
Lentivirus-based	Most mammals	Random and multiple	Single copy	High/low	Late 1990s
Sperm manipulation	Most mammals[c]	Random	Variable	Moderate/low	Successful application in few laboratories so far
ES/EG[d] cells	Mice and rats, not (yet) available in livestock	Predetermined	Often single copy	Low/NA[e]	Recently successful in Chicken (EG [91])

[a]Determination of the site of integration or random integration in the genome

[b]Arbitrary scale: low, <5%; moderate, >5%; high, >80%

[c]All mammals tested so far

[d]Embryonic stem/Embryonic Germ

[e]Not Applicable as not used in livestock (so far)

common technique used for gene transfer is still pronuclear microinjection but, as with other livestock species, research into increased efficiency has led to the development/adaptation of new methods, including electroporation (i.e., improving the permeability of the cell membrane for DNA macromolecules by applying short electric pulses), sperm-mediated gene transfer, direct gene transfer into tissues of adult fish (via intramuscular injection of DNA or "gene gun delivery"), and the use of viral vectors [22–24].

Risks are attached to the technology, naturally, and sometimes these are unpredictable. They include the occurrence of undesirable effects of transgene expression, which should be addressed in food safety assessment schemes (like in other emerging novel foods). In particular, safety assessment should be performed regarding toxic/antinutritional effects, allergenic potential, bioactivity (e.g., hormones active *post* consumption) [25]. For a thorough discussion of different risks associated with transgenic livestock, please see "The Ethical Issuessection ."

Applications of Genetic Modification to Livestock

Several explorations of potential applications of genetic modification to livestock species have been published [1, 20, 26–29], and agricultural purposes have been examined in particular [2, 30]. The authors of these studies usually distinguish between biomedical and agricultural applications. The basis of this distinction seems to be that applications in the agricultural domain facilitate the direct consumption of products from transgenic animals/their offspring by humans, while in the biomedical field, applications target human health via the development of pharmaceuticals, antibodies, transferable organs/tissues, or by studies into basic biological processes and disease mechanisms. There are products for direct consumption, such as functional foods, which however may fall into both categories [31].

The history of R&D here shows that – apart from scientific interest in basic biological questions – transgenic livestock research has undergone changes of focus and has followed funding and fashion. In this process, biomedical applications such as xenotransplantation, animals as "bioreactors" and disease models, and among agricultural applications the "Enviropig®" have emerged.

Initial developments in transgenic farm animals focused on the improvement of one of the classical production-related traits: growth [17]. The animals involved have become popularly known as "Beltsville pigs." Ever since, there have been reports of procedures involving transgenesis for specific problems with, and questions about, livestock production (for a recent review [2]).

There have been attempts to use the technologies to improve animal health and disease resistance. There are various ways of achieving this, including passive immunization of young animals against viruses through antibody expression in milk and improvement of early growth and/or viability in piglets through increased sow milk yield when overexpressing bovine alpha-lactalbumin. Food quality and the improved usability of animal products for human consumption (e.g., the expression of human lactoferrin in milk to obtain positive effects on the immune system, reduction of lactose to circumvent intolerance, carcass and meat composition alteration toward leaner meat, and meat with higher content of omega-3 fatty acids) can also be mentioned in this context. Another production-related application aims at diminishing the "ecological footprint" of industrial pig production: this involves the expression of a bacterial phytase gene in the saliva of pigs which leads to reduced phosphorous excretion.

Moving to the broader and related area of genetic modification, gene knockout has been employed in efforts to induce disease resistance (prion diseases) [1]; it has also been used to create pigs that do not express alpha 1,3 GT in an attempt to overcome organ rejection after xenotransplantation.

Apart from its applications in biomedicine and basic biological research, transgenic technology has been used in fish to improve production parameters such as growth and food conversion, adaptation to cold environments (lowering the freezing point of body fluids through the expression of antifreeze proteins), disease resistance and prevention of the introduction of transgenes into wild populations (sterility). As with land farm animals, in 1985, the very first successful application of transgenic technology reported in fish aimed at increased growth by introducing a gene for human growth hormone (to be expressed, in this case, in goldfish). For an extensive review of applications see [24].

In a relatively recent review paper, tentative projections as to when various applications of transgenic livestock will be applied in the field are made [20]. Most of the biomedical and agricultural applications projected by Niemann and colleagues to be introduced some time beyond 2005 are still at the research stage in 2010; and, of the applications listed, only recombinant pharmaceutical proteins have actually reached the market: in 2006 the European Commission, and in 2009 the FDA in the USA, gave approval for the drug ATryn® (a recombinant form of human antithrombin, an agent that prevents blood-clotting) to be produced in the milk of transgenic goats for pharmaceutical markets. Growth-enhanced transgenic fish are often presented as the most promising candidate for the introduction of transgenic animals for food production [23, 32].

Future Perspectives

The idea of using transgenic animals in food production is controversial not only with the public, but also within the scientific community. The success of transgenic livestock for agricultural purposes has failed to meet initial expectations not least as a result of its practical limitations as compared with other breeding tools [1].

Scientists differ considerably in their assessments of the impact transgenic livestock will have on animal agriculture, and in the time-frame within which this impact will unfold. These attitudes also reflect opinions about the usefulness and feasibility of the technology; in general, biomedical applications usually meet with more optimism than agricultural applications. The following excerpts from relevant publications that can be seen in Table 2 illustrate the range of views:

Animal biotechnology is embedded in a social context and therefore affected by general trends in the societies in which it unfolds. This also means that one should be neither excessively optimistic about altruistic motives behind the technology nor excessively suspicious about possible egoistic and narrow motives. In general, one should bear in mind – when listening to all the problems that the technology might solve and how soon it will revolutionize human lives – that in western capitalist society, the worlds of research and business, science and technology, and knowledge-building and knowledge-using, have converged. Scientists are not necessarily independent and altruistic, but human beings working in a marketplace like everybody else. They are, in other words, stakeholders. A certain amount of sound skepticism in evaluating the claims they make is therefore probably not going to be wasted [33].

It must at the same time be remembered that the potential of the technology is enormous and the range of applications almost endless – in theory, at any rate. But as the last 25–30 years have shown, it is a long way from having an idea to making it work. Sometimes, it seems that the more that is learned through science, the more it is realized how little is known, because the world in general, and molecular biology specifically, turns out to be much more complicated than initially believed [34]. When trying to assess the potential applications of transgenic livestock, there is, therefore, a large and inescapable uncertainty.

Public Perceptions of Transgenic Technologies

This section describes public perceptions of transgenic animals. As a point of departure, it should be noted that while experts, in the nature of their profession, tend to assess transgenic animals as an isolated phenomenon, the public often see these animals within a wider context. Thus most lay persons will see transgenic animals as parts of modern biotechnology. Respecting this, this section first places public perceptions of transgenic animals within a wider context of other applications of biotechnology within the agricultural and medical fields; and, second, present various aspects of the public perceptions of transgenic animals. Another aspect of the contextuality of public perceptions is the fact that perceptions should be interpreted, or understood, in the particular context in which they arise. Perceptions reflect deeper rooted cultural and/or religious values as well as passing material settings – and so they will vary with continents, countries, and religions. The following descriptions examine public attitudes in both Europe and North America, but the main focus is on Europe.

Accounts of public perceptions have two main empirical sources: qualitative interview studies or quantitative surveys. The former, which involve group interviews or individual interviews, are effective at delivering a deeper insight into the arguments and values behind specific views, but as a result of the

Transgenic Livestock, Ethical Concerns and Debate. Table 2 Examples of scientists' views on impact of transgenic livestock and relevant time frame

	Impact	Time frame
Optimistic/clear-cut predictions	"Transgenic technology allows for the stable introduction of exogenous genetic information into livestock genomes. With its ability to enhance existing or introduce entirely novel characteristics at unprecedented magnitude and speed, this emerging technology is expected to have a profound impact on the genetic improvement of livestock in the future." [2] "The overwhelming challenge to agriculture in the next century will be to feed a huge population while creating and maintaining sustainable agricultural systems. Biotechnology, including transgenic livestock, will play a key role in meeting this challenge by allowing greater precision in resource use and product design." [30]	"Sixteen years ago, transgenic animal technology was invented, and, since then, an industry has formed to exploit that technology. In the next 16 years, products created by that technology will likely be in the hands of consumers." [26]
Moderate optimism/"some time in the future"	"New methods for modifying the genome will underpin a resurgence of research using transgenic livestock. This will not only increase our understanding of basic biology in commercial species, but might also lead to the generation of animals that are more resistant to infectious disease." [1]	"We anticipate that in the near future, genetically modified animals will play a significant role in the biomedical field but that agricultural applications will develop more slowly due to the complexity of many economically important traits and to current resistance to the concept of engineered farm animals." [15] "While various transgenic concepts for the genetic improvement of livestock animals for agriculture are being evaluated the integration of this technology into practical farming systems remains some distance in the future." [2]
Critical/uncertain about time-frame	"It is not clear at all that the benefits that genetic engineering has produced hitherto compensate the potential risks of widely using these products in animal production. Putting the question in a crude way: Are we risking our health for a 5% of the benefits in milk production? Should we use transgenic pigs for a 33% less manure spread? In general, every advance of molecular genetics has been received with high expectations in the field of animal production, but the consequent application, if any, has been much more modest." [1]	"Over the past few years several commercial ventures have withdrawn from transgenic biopharming for various, usually financial, reasons. So, even though much of the groundwork has been doneit is unclear what the future holds for this use [agricultural applications] of transgenic livestock. [. . .] It will take both a better understanding of the genomes of livestock, with the anticipated increase in candidate genes to choose from, and a major practical success before transgenic technology seriously challenges genetic improvement of livestock through selection for most conventional traits." [1] "When a procedure is challenged for some present reason, it is not uncommon to argue that in the future all these problems will be solved. [. . .] In the opinion of the author of this paper, the general attitude towards genetic engineering in domestic livestock should be reconsidered." [21]

limited number of participants, the interviews give a poor picture of the representation and structure of perceptions across the public at large. Quantitative surveys, by contrast, often involve 1,000 or more respondents. Their strength, as a tool, lies in the ability to produce a picture of the distribution of perceptions within a population in a country or a region.

One of the most frequently cited sources of information about public perceptions of new biotechnologies is the Eurobarometer surveys. These have been carried out regularly and simultaneously in all EU-member countries, since 1989. Since the Eurobarometers contain a number of core questions which, with small variations, have been included in all surveys, they provide real insight into the longitudinal development of public perceptions of modern biotechnology in Europe. As a supplement – but indeed not as systematically and regularly – interview studies have been carried out. These, however, are often designed to study a specific aspect of modern biotechnology, and this aspect, or topic, is seldom transgenic animals.

Perceptions of Transgenic Animals as an Application of Modern Biotechnology

A number of interesting findings emerge in the Eurobarometer surveys [35–38]. First, the surveys demonstrate that the new biotechnologies are not assessed and rejected, or accepted, en bloc; rather the public makes a balanced judgment about different applications by weighing the relevant pros and cons. One distinct result of the surveys has been the finding that if these pros and cons are categorized as matters of risk, usefulness, and moral concerns, both risks and utilities are important, but moral concerns has a veto-like character [39].

Second, the studies show that perceptions depend on the type of application. Relatively speaking, applications within the medical domain are generally accepted, whereas food-related applications are viewed with considerably more skepticism. Qualitative studies have demonstrated that this differentiation between applications is linked to the kinds of usefulness provided by the different applications [40, 41]. Thus more acceptable applications are seen as useful, in a societal sense, in virtue of their potential contribution to, for example, the alleviation of hunger, human suffering, or environmental problems. The less acceptable applications, by contrast, are generally useful only in an economic way or to the individual.

Within this general picture, it has been found in North America as well as in Europe that applications including transgenic animals tend to be approached with skepticism [41, 42]. In the 1996 and 2002 Eurobarometers, for example, transgenic animals produced for xenotransplantation were, together with GM foods and GM crops, assessed as one of the least acceptable of seven applications [37, 43]. Despite the fact that xenotransplantation belongs to the medical area it is viewed with some skepticism by the public. One explanation of this may be another somewhat consistent finding. This is the existence of an "organism scale" – something not only indicated by the Eurobarometers, but also found in interview studies [41, 42, 44]. On this scale, acceptance is related to the distance (as it were) between the genetically manipulated organism and humans. Apes and some other mammals are located at the top of the scale, and plants and microorganisms are at the bottom. Thus the fact that the use of transgenic animals in xenotransplantation involve organisms (animals) located toward the top of the scale may contribute to the public skepticism.

Perceptions of Transgenic Animals

This section provides more details about the specific concerns present in public perceptions of transgenic animals. The presentation is structured using the now common distinction between risks, usefulness, and ethics/other moral concerns.

Transgenic Animals as a Risk In the sociology of risk, the concept of risk most often refers to any side effect of an action, practice, or technology [45]. Here, however, public perceptions of risks of transgenic animals follow the convention within studies of biotechnology, where risks are solely understood as the (unwanted) consequences for human health and the environment.

The data on transgenic animals as a risk is somewhat limited. Eurobarometer surveys include perceptions of risk related to transgenic animals in 1996 (xenotransplants and research animals) and again in 2002 (xenotransplants). In 1996 as well as in 2002,

almost 60% of the respondents to some extent agreed that the use of transgenic animals in xenotransplantation was risky [43, 46]. It is, however, impossible to determine whether this relatively high perception of risk refers to xenotransplantation as a strategy or to the transgenic animals themselves. The fact that 60% also regarded the less complex question of transgenic research animals as risky in 1996, however, indicates a considerable concern about the risks.

A qualitative study carried out in Denmark in 2002 gives an indication of the worries lying behind this risk perception [44]. First, it was found, during the exploratory interviews, that human health problems arising from the consumption of meat from transgenic or otherwise manipulated animals were not prominent issues. In discussions of GM foods in general, it transpired that there were no particular short-term concerns, mainly because people trusted the authorities and their risk assessments – but also that, by contrast, in the long run, there was some skepticism, partly because participants were not sure if the experts could foresee the long-term risks to human health. Here, references were made to the BSE crisis, which was taken to have demonstrated experts' inability to predict health consequences. In this context, some also raised concerns about the fact that the natural order of things is challenged through the use of modern biotechnologies. This natural, or god-given, order is seen as incorporating an inherent safety mechanism that is bypassed by the use of genetic technology.

Other interview studies have found similarly low levels of concern about the risks associated with the use of transgenic animals in xenotransplantation [47]. The environmental risks also seem to be considered low: essentially, it is believed that it is possible to prevent transgenic animals (unlike microorganisms and plants) from escaping and spreading their genes.

Transgenic Animals and Usefulness As noted above usefulness has several aspects: societal, individual/self-interested, and economic. Within this spectrum, the Eurobarometer surveys aim to determine the level of perceived societal usefulness by asking how useful for society the respondents find different applications. Results show that in both 1996 and 2002, about 60% of the Europeans surveyed judged xenotransplantation useful to society, and in 1996, a somewhat smaller proportion (52%) found transgenic research animals useful. It should be stressed, however, that both applications fall within the medical area, which is known to be generally more acceptable. The interviews in Denmark confirmed this general picture, sometimes pointing to the purpose (e.g., fighting obesity) being noble, but the strategy (e.g., transgenic leaner meat from pigs) wrong, since there are well known and less controversial alternatives (e.g., obesity-curbing diets) that should be pursued first. Interview participants, however, often found themselves trapped in a dilemma between the highly valued principle that transgenic animals must serve a societal purpose, and their own interests; and sometimes this resulted in pragmatic acceptance of applications in their own interest, despite their principled rejection of the same applications.

Transgenic Animals and Other Moral Concerns In most other situations where the human use of animals is on the agenda, animal welfare is a major theme. It is striking that there is little evidence that welfare, conceived of, in the narrow sense, involving a direct effect on the well-being of the animals, is important in public perceptions of transgenic animals. In interview studies this aspect seems to be conspicuous by its absence. Instead, participants raise questions about animal integrity, or about what can be justified with regard to human treatment of animals [39, 44] – a finding that by and large is confirmed by an OECD review from 2008 [48]. In particular, the question of integrity seems to be important in the assessment of transgenic animals, and to contribute to the establishment of the ethical limits to the degree of manipulation that can be exercised over other living beings. Rather than providing final answers to the question of where such limits should be drawn, qualitative studies allow the public to voice questions such as: "Is human life worth more than that of an animal? (. . .) Which is more morally acceptable: waiting for a 17-year-old motorcyclist to die or using a pig as a 'spare parts bin'" [39].

Despite such statements, in which animals are valued as such, a zoo-sociological classification [49] can, it seems, be discerned in the few studies of public perceptions of animal biotechnology. According to this classification of animals, there are different limits to what you can do to different species of animal.

The classification seems to reflect the idea that the closer an animal resembles humans, the better are the reasons (or the greater the usefulness) required to justify manipulating it. Hence, fish are not as important as cows, and cows are less important than primates.

The Ethical Issues

In this section, the ethical concerns typically raised in published discussions of animal biotechnology and in debates in the public sphere will be analyzed. The concerns will be organized into three main categories: risks to humans, risks to animals, and other moral concerns. In this way, the section follows the findings of the sociological studies and can be seen as an attempt to provide an ethical analysis of the values underlying public perceptions of biotechnology at the same time as being an assessment of the kinds of risk the development of transgenic animals present.

It is important to realize that ethical categories do not come out of the blue. The fact that many discussions of transgenic animals center on risks is no coincidence; it reflects the fact that the regulation of biotechnology is cantered on risks, as was mentioned earlier. There are differences in the way biotechnology is regulated in different parts of the world. However, the various regulatory regimes governing transgenic livestock more or less share the following requirements: (1) the livestock must not present a risk to the environment; (2) nor may it present a risk to human health; and (3) nor may it present a risk to animal health. If these three requirements are fulfilled, there is, in most regulatory regimes, no legitimate reason to set up limits to the development and use of transgenic livestock. In light of this regulatory situation, it is no wonder that discussions of transgenic livestock, like discussions regarding other forms of biotechnology, take risk issues as their starting point.

Risks to Humans

The risks to humans are uncontroversial in the sense that everybody involved in the discussion acknowledges that, to the extent that the technology presents serious risks to humans, this is a serious matter. Following the literature on the subject, it can be said that with regard to human health and the environment, transgenic technology so far does not seem to carry significant risks to humans, whereas the socio-economic impact of the technology on such matters as food prices, agricultural production systems, and financial interests are much harder to assess.

Health Risks to humans are most often equated with the risks to human health presented by products from transgenic animals consumed as medicine or food. Only a very limited amount of research has been done in this area, since very few products have been developed. There is, however, a substantial literature on what risks should be taken into consideration when such products are evaluated. In the medical area, it is recommended that the risk assessment should follow the conventional testing of newly developed drugs. In the food area, risks arising from the modification of proteins, allergenicity, toxicology and nutritional value, and so on, will be important parameters [50].

One concern relates to the possibility that the genes transferred might cause allergic reactions in people. If a gene from a plant is transferred into an animal and that gene is responsible for the production of a protein that makes some people allergic to the plant, then that gene could very well make the same people allergic to the meat and/or milk from the animal. There are clearly ways of avoiding the most obvious problems here – by choosing genes that are not known to produce allergenic proteins, or by labeling all products from transgenic animals. But the whole area of food allergy is scientifically complicated, with many unknowns. Basically, it would seem that the risks in this area can be managed, but for each potential product careful consideration will have to be given to the question which genes should be used for modifying which animals [51].

Another issue of importance is whether the physical composition of the food product will change and thus possibly alter the nutritional value of the product. This result could be intentional or unintentional. One might attempt to genetically modify an animal to change the nutritional value of its meat and milk. It could be the amount or composition of fat in pigs, as in the case of the omega-3 pig [52] or the amount of lactoferrin in milk [53]. In such cases, it would be necessary to evaluate how these changes might affect human health in comparison with traditional products. But the changes could be unintended. Thus they might be caused by changes in genes that are not directly linked

to the nutritional value of an animal. Molecular biology is a very complex science that tells that genes are interconnected and seldom work alone. What happens to one gene can have effects elsewhere. Thus changes in genes related, for instance, to the environmental impact of an animal might have unintended side effects in that they also affect its nutritional value. Furthermore, less than perfect methods of gene transfer could also lead to unintended genetic changes. Only through effective testing and control procedures can such problems be countered in a responsible way. As no genetically modified animals have been approved for the agricultural market yet, and since only few have been developed and tested, it is very hard to say precisely how large a risk this is. It should be clear, however, that this issue will need to be addressed on a case-by-case basis. It will depend on the gene inserted and the animal modified, and it will, in all likelihood, be impossible to say anything general about the matter.

One area of biomedicine where it can be foreseen that the technology presents serious risks to humans has attracted attention. This is the xenotransplantation of pig organs. The worry is that dormant viruses in the pig genome could be transferred to humans through the organs and then "awaken" in the new environment in the human host organism, giving rise to a newly emergent disease against which humans have no natural defense. Notorious examples of emergent diseases transferred from animals to humans are the Spanish Flu, AIDS, and Avian Influenza. In the worst-case scenario just one xenotransplantation "gone wrong" could cause a pandemic [54]. The degree of scientific uncertainty here is rather high, as is generally the case in newly emerged fields of research. However, putting problems raised by the transplantation of live tissue from one species to another to one side, at present, the risks to human health presented by transgenic animals seem to be manageable.

The Environment Some of the ethical concerns connected with transgenic livestock relate particularly to the environment. One such concern is that the animals might escape and breed with wild populations, thus spreading their genes in an uncontrollable way. The most cited example here is that of transgenic fish – e.g., salmon with genetic alterations allowing faster growth. The concerns in this area can either be about the indirect consequences this might have for humans (in this case, economic losses to the fishing industry if transgenic fish cause havoc in the wild-living species that are already under pressure from intense fishing), or direct concerns for the animals and the wider ecosystem [51, 55]. With the exception of fish, individual animals produced by biotechnological methods are rather easy to confine, and this makes it less likely that they will be a hazard for the environment. But here, as with most other concerns discussed so far, it will be necessary to evaluate the animals case by case to estimate the risks: in this case, by trying to anticipate how the animals will interact with the environment and whether this will be a threat to human interests.

Other Concerns for Risks to Human Interests Other concerns focus on the socio-economic changes transgenic animals could bring about, especially if they are ever integrated on a large scale into farming and food production. As the technology is not yet developed, let alone introduced, in this area, it is very hard to guess what will happen. But concerns that the technologies will strengthen the movement toward large-scale farming and could deepen the divide between the developed and the developing world are not unrealistic – witness what usually happens when new technology is introduced and what has happened within the sphere of plant biotechnology. For further discussion of these concerns about the impact of biotechnology on agriculture in general [10].

Another kind of concern for humans that is sometimes raised is that the continuing reification or commodification of nature, where nature is seen exclusively as a resource to be used by humans, will harden human ethical sensitivity in general, thus ultimately causing ethical problems between humans as well. This kind of argument has been laid against the unethical use of animals since the dawn of western philosophy – as, for instance, in the work of Thomas Aquinas (*c.* 1225–1274). The key idea is that by being harsh to animals, humans may end up being harsh to their fellow humans. This idea is closely connected to concerns that humans, by turning what is strange and unknown into biological factories, lose an important part of what it is to be a living being that shares the world with other living beings, thus diminishing the human world [56].

In addition to these concerns, it is often suggested that if the applications of biotechnology are accepted on animals, there might be a gradual change of views on these applications and in the end their use on humans would be found acceptable. In this "slippery slope argument" the problem is not the production of transgenic animals in itself (point A), which is seen as ethically acceptable, but the production of transgenic human beings (point B), which is seen as unacceptable and claimed to be at the bottom of an inescapable slide down a moral slope. Thus point A should be avoided, although it is perfectly acceptable in itself, because it automatically leads to point B: a place that it is in no way desirable to be at.

Risks to Animal Welfare

Transgenic technologies used on livestock may cause welfare problems for the animals. As genetic modification means, by definition, the generation of animals of a genotype and phenotype not presently existing, during research and development, it is necessary to make predictions about the health and welfare of these animals, based on what is known about the gene itself and on previous experience with the technology. Genetically modified animals have so far mainly been used in biological research and as disease models. Usually, the goal of modification is to produce animals that either under- or overexpress certain genes, or which express a mutated, disease-causing human gene. In all of these cases, body function in the organism is in some way disrupted. Although the severity varies, welfare problems often accompany these disruptions.

The situation is different with transgenic livestock. Here, there is no *formal* conflict between the objective of the research and the health of the animals: ideally, animals functioning as bioreactors or xenotransplantation donors, as well as transgenic animals in food production, should be healthy. But this is not always the case, and harmful side effects may arise. For example, the "Beltsville pigs" involved in the earliest experiments suffered from extensive health and welfare problems (stress susceptibility, with only marginal improvement of the desired trait, namely growth [57]).

The problems here may arise at different stages in the development of transgenic animals: the in vitro technologies used at the beginning of the process may have a negative impact on early development by disturbing early gene expression. The site of transgene integration is also relevant, as insertional mutations may lead to the loss of host gene function in the "affected" region. This risk is more frequent with those technologies where there is no control over where the transgene integrates. However, it is primarily a problem at the research stage: as such animals are not desirable as so-called "founder animals" for diffusion of the transgene into a wider population, other individuals will be selected for further breeding.

Finally, the level, and the developmental and temporal control of their expression, may mean that proteins, which are the products of transgene expression, cause problems in transgenic animal [58]. Welfare problems deriving from the activity of the transgene itself will affect much larger numbers of animals, because these problems will accompany the gene as it spreads in the population. This could be the case, for example, if animals are engineered to improve production, and if the greater production results in more prevalent production-related diseases.

Systematic welfare assessment schemes in transgenic livestock are still lacking, even though recommendations on this matter were published almost 10 years ago [58] and have been updated more recently [59]. Conclusions about the welfare of transgenic livestock have often been based, not on comprehensive studies, but rather on small-scale, or anecdotal, reports leading scientists to state, for example, that the transgenic animals "have shown no obvious abnormal phenotype" [60], are "apparently normal" [2], or "developed normally" [20].

Welfare Assessment The requirements of welfare assessment schemes in experimental design should reflect the numbers of affected animals at different stages of a transgenic program (first time generation, an increasing number of animals, and establishment of the production herds). In the early stages, with low numbers, predictions of the welfare of the animals will mostly be based on previous experience with the transgene/gene product, if available, in other species (mice); anecdotal information will be essential. As animal numbers increase quantitative research comparing groups of transgenic and control (nontransgenic) animals should be performed. With the establishment of

production herds it will not be possible to monitor the individual animal; therefore, surveillance and sampling of welfare-relevant effects of transgenesis will become more important [59].

Which aspects of the welfare of transgenic animals are assessed will depend to some extent on how animal welfare is defined. Animal welfare scientists typically base their evaluations on the clinical health and subjective experiences of animals [61]. Often, the subjective experience of animals is taken to be expressed in what animals choose. Recently, this approach has been summarized as follows: "Good welfare is defined as animals being healthy and having what they want" [62].

A broader, and complementary, perspective also includes the animal's opportunity to engage in essential species-specific behavior [61, 63, 64]. This broader perspective is related to an even wider set of concerns about animal biotechnology wherein, the anxiety is not that the technology poses risks to animal welfare, but that it violates animal integrity and basic concepts of naturalness. This will be further discussed in section "Naturalness and Integrity".

The Narrow and the Broad Perspective on Animal Welfare From the narrow perspective defined above, a transgenic application is problematic only if it gives rise to health problems or negative subjective experiences. From the broader perspective, the question of animal welfare is also about the extent to which the animal is allowed to fulfill its species-specific potential. The broader perspective thus points to an additional group of considerations that has to be taken into account when reflecting on animal welfare. It should be noted that the two perspectives are not mutually exclusive. Equally, they will often deliver similar recommendations.

Nevertheless, the two kinds of consideration can, on occasion, be difficult to reconcile in practice; and in that eventuality, it becomes important to clarify what kind of perspective is in play. This can be illustrated with two examples.

First, the battery cage in which laying hens are prevented from performing a range of highly motivated behaviors emerged early on as one of the most emblematic issues in the discussion of farm animal welfare. Following many years of intense debate, accompanied by research to develop and evaluate alternative approaches, policy makers have decided to outlaw this housing system, which will be phased out in the EU by the end of 2012. However, if the problem is defined as the frustration of highly motivated behaviors, an alternative (though as yet hypothetical) solution would be to change the birds, so that they become content with the limited life in a battery cage. From a narrow perspective, there is no ethical objection to denying the animal the opportunity to behave as a bird would do in nature (as battery cage egg production does) as long as this does not negatively affect the subjective welfare of the animals, i.e., lead to negative experiences [64]. Therefore, if birds could be caused to lack motivations other than those that could be satisfied in the cage, this would be a good thing to do from the narrow perspective; it would reduce animal suffering in what is, of course, a highly profitable housing system [65, 66].

For the foreseeable future, this seems to be an academic discussion, as the technology is not, as yet, able to produce such animals – if it ever will be. To begin with, the trait to modify would have a complex genetic background, since the objective must be an animal in which one has eradicated all motivations other than those that can be satisfied in a battery cage. Second, it will be a difficult challenge to ensure that one is indeed modifying the animal into one with a restricted set of motivations rather than an animal that reacts passively, or even with apathy, to adverse conditions [67].

From the broader animal welfare perspective, on the other hand, the very idea that hens should be designed to cope well with battery cages raises serious worries and questions about the natural life of chickens, and about the experiences that constitute such a life. Thus even if it is technically possible, this kind of manipulation is highly questionable from the broader perspective.

If the battery cage case was hypothetical, the blind hen case represents a real situation. A Canadian scientist involved in poultry breeding has bred a blind egg-laying hen [68]. The blindness was thus not inflicted on living chickens, but congenital. According to the researchers, blind hens would be at less risk from feather-pecking and cannibalism, these being welfare problems typically found within free-range production systems. Again, from a narrow welfare perspective, this would seem to be a welcome solution to a rather serious welfare problem.

From the broader perspective, both the notion of deliberately breeding chickens that have such limited potential as to be content with life in a battery cage and the aim of breeding blind hens to solve production problems in the agricultural sector are seen as ethically problematic in ways that might outweigh the advantages of these ideas as perceived from the narrow perspective. Something just seems to be amiss when you deliberately create an animal with less potential than normal [69], whether or not the animal has negative experiences as a result. From this perspective, the task is not to change the animals to fit within the limits of the production system, but to adapt the production system to accommodate the needs of the animals. Implicit in this version of the broader perspective is a certain respect for the natural state of the animal.

It should be said, however, that another version of the broader perspective takes a slightly different view. While agreeing that the natural behavior of the animal is to be respected, the advocate of this view does not consider the natural behavior of the animal to be something static. Just as domesticated animals have been bred to be better adapted to housing in confinement in the past, animals today can be bred, either conventionally or through genetic modification, to be better adapted for modern-day production systems. Thus the fact that one can alter the nature of an animal by genetic modification does not constitute an ethical problem as long as one respects the nature that the animal ends up with [65, 66].

Whether animal welfare is seen from the narrow or one of the broader perspectives, the difference between traditional breeding technologies and the new biotechnological tools seems to be more of a quantitative difference in the potential of applications and associated welfare problems than a qualitative difference that creates entirely new welfare issues. The novelty or technological nature of the changes does not in itself introduce new concerns.

Other Ethical Concerns

From the concerns regarding species-specific behavior within the broader perspective, there is a connection to other concerns that are most often treated outside the discussion of animal welfare. These are concerns about the perceived unnaturalness of the technology and possible violations of the integrity of the animals. Rather than being related to the individual animals themselves, these have to do with a more abstract and wider human perception of what an animal is. They can be said to fall within the scope of animal ethics, but outside the scope of animal welfare.

Naturalness and Integrity Technologies involving the genetic modification of animals are often labeled "unnatural" [44, 70]. As discussed previously, there might be differences in the more technical details over how unnatural the animals are in scientific terms. An animal that has had a gene inserted from another species that it would not be able to share genes with is normally seen as more unnatural than an animal which has had an extra gene from its own species inserted. But it is often argued that from a scientific perspective, this is just a matter of degree and not something requiring the label "unnatural" to be placed on products with which it is associated. There seems to be no qualitative differences between what is going on in traditional breeding and modern biotechnologies. The one follows almost logically from the other, and to label one of them "unnatural" would be to label the other likewise [71]. However, not everyone agrees with this conclusion. It has been argued that the fact that traditional breeding methods are already accepted by at least most of the public, does not necessarily mean that the next, much more advanced, step is also acceptable [72]. The growing attention to the welfare of animals produced through transgenic technologies might even serve as reason to look back on already established, and hitherto innocent, methods of animal breeding with critical eyes [34].

Another criticism of the term "unnatural" is that, although it is intuitively compelling, the normative impact of naturalness suffers from three inherent ambiguities when applied to domesticated animals. First of all, it is almost impossible to point to a stage in the development of such animals that would constitute their natural state and thus be the developmental point that should be respected [64]. Second, even without human interference, animals evolve to adapt to changing environments. Claiming that it is unnatural to change the genomes of animals can therefore, from a scientific point of view, be seen as saying that nature is unnatural. Finally, it needs to be explained why it is

assumed that what is natural is also ethically good. Cancer, earthquakes, and influenza are naturally occurring phenomena, but very few would, for that reason, deem them to be ethically good. For reasons such as these, the notion of naturalness is very often rejected as unclear and insignificant to the ethical debate [73].

These criticisms are very relevant. However, they might be seen as utterly failing to address the fundamental issues at stake in ethical debate about unnaturalness. There seem to be two different viewpoints from which the technologies can be discussed. As Anne Chapman has pointed out, the debate around unnaturalness and biotechnology tends to operate with only two very distinct concepts of nature. Nature is seen either as everything there is (leaving nothing to be unnatural) or as everything that is untouched by humans (in which case all things touched by human activity become unnatural). What is lacking is a middle ground that takes into account the fact that, from an everyday perspective, most people experience the naturalness of things as a gradually developing quality: things can be more or less natural [74]. This difference can very well be expressed in terms of the degree of control that humans exercise over natural processes. Here, it becomes obvious that although the genetic inheritance of animals is changed both through conventional breeding and through transgenic technologies, the latter, being more powerful, are more unnatural.

This understanding of the difference between natural and unnatural as being determined basically by the extent of human control can also be used as an interpretative key that helps to clarify the concerns underlying the claim that transgenic technologies violate the *integrity* of animals.

There are several definitions of the concept of animal integrity available, yet it seems that the notion still eludes clear understanding. The word is derived from the Latin "integer" meaning *wholeness, completed, untouched, unharmed.* Rutger and Heeger [75] define animal integrity as the "wholeness and intactness of the animal and its species-specific balance, as well as the capacity to sustain itself in an environment suitable to the species." De Vries [76] interprets this definition as one mainly aiming at physical, biological, or genetic wholeness. To a large extent, violations of animal integrity will therefore also violate the welfare of the animal [77]; and they will do so irrespective of whether animal welfare is understood from the narrow or one of the broad perspectives.

What is of special interest here, however, is whether practices that do not produce ill health, negative mental states in the animal, or prevent the animal from performing its species-specific behavior can also be seen as violation of integrity. An example, already discussed above, would be breeding animals that fits the production systems better rather than developing housing systems that satisfy the existing behavioral needs of the animals [66]. Again, one might consider changes of composition in dairy cow milk to mimic the content of human milk [78].

The debate about what integrity is, and why it is relevant, mimics the debate about naturalness in important ways. The concept of integrity is often (as in the definition proposed by Rutger and Heeger [75]) tied tightly to biological, empirical features such as behavior, the possession of an unaltered genome, and so on. The concept is then often criticized and questioned, since it makes it unclear why the integrity is especially violated through biotechnology [79].

The notion of integrity is perhaps better understood as one expressing the ethical reservations some experience when animals are commodified to an extent where the distinction between unnatural and natural breaks down, or where the power humans exercise on the animal leaves the animal as nothing more than a resource to fulfill human needs. Integrity is thus something that is experienced directly, but something also that shows up when there is a violation of it [80]. It is experienced in a phenomenological understanding of the animal – not reducing it to an object for examination or a resource to be developed, but affirming the notion of it as another "flesh-of-the-world" entity [81] sharing a "more-than-human-lifeworld" [56] with humans and all the other members of the biotic community. The claim is then that the integrity of the animal is present at this level of experience as the "completeness" of the animal before humans show up with their ability to change animals into something that can satisfy human needs. Self-evidently, an animal can be killed, but life cannot be breathen back into it. In essence, integrity is the experience that the animal is whole, complete, full, finished, when humans encounter it. Humans cannot add to it, only take away [82].

When developed along these lines, the concept of integrity loses it biological foundations and thus becomes impossible to assess from a scientific point of view. Some will probably find that the prescientific nature of the concept disqualifies it automatically; others might find that by interpreting integrity as a "zone of untouchableness" given by the sheer existence of the animal, the moral concerns about interfering in the lives of animals can be seen to make sense, even if they go beyond the animal's welfare interests.

Respect for Autonomy As has already been mentioned, current legislation in most countries is set up to permit transgenic livestock only if this does not pose a risk to the environment and to the animals' health; and to ensure that products originating from transgenic livestock will be allowed to enter the food chain only if they pose no risk to human health. As should be clear from the above analysis, a number of reasons why people might be skeptical about transgenic livestock can be described, and some of these will go beyond what is captured by legislation. First, some people will assess risks differently from the experts giving advice to the authorities. Second, some people will have a wider notion of what counts as a risk than that embedded in the legislation. Finally, third, some people may have moral concerns that go beyond the risk paradigm as shown earlier.

In light of this, one can ask to what extent people should have choice when they buy animal-based products. According to current legislation in the US, there is no requirement to label products derived from GM animals, whereas current EU legislation requires such products to be labeled. Here, then there will be room for a genuine ethical disagreement about whether consumers have the right to be told about the origin of animal products – and retain this right whether or not the products pose a risk. Those opposed to mandatory labeling will typically argue that such labeling may cause unfounded fears in consumers, whereas those in favor of it may argue that consumers ought to have the right to choose for themselves in line with what matters to them; and opinion polls seem to show that many consumers want to know whether or not food products are genetically modified [83].

Public Discussions

The advent of modern biotechnologies, and subsequent ethical debate, has been closely followed by a number of stakeholders: researchers, the biotech industry, politicians devising the legislative framework, NGOs within the areas of environmentalism, animal welfare, animal rights, and so forth. From the outset, it has been clear that the technologies raise ethical issues and that it is necessary to debate these. Equally, after the controversies surrounding the introduction of GM crops and products based on these crops in Europe in the 1990s, it became glaringly apparent that there was widespread skepticism about biotechnology, and that it would be necessary to create some kind of dialogue between stakeholders in the public sphere.

In this section, it is discussed how different motivations for entering this dialogue shape the dialogue itself and the subjects that are deemed relevant within it. As should be apparent from the analysis of ethical concerns provided above, it is not a shortage of subjects to talk about that characterizes the area. As in the section on public perceptions of transgenic animals, it has been necessary to widen the scope and look at the issue at a more general level to capture the trends in the discussions. There is, however, little reason to doubt that ethical discussion of the issues raised by transgenic animals will follow the pattern seen so far in adjacent technologies.

Public Participation and Dialogue

Today "dialogue with the public" is almost a mantra within the biotech community – a community that can be said to consist of all stakeholders, from the most jubilant adherents of the technologies to the most fundamentalist skeptics. The idea of public participation in the preparation of policies for emerging technologies such as biotechnology is very widely accepted. But whereas the general idea of public participation and the concept of dialogue are almost universal within the western world, the content of these notions is far from self-evident [84].

Quite what is meant by "public participation" can, generally speaking, be said to be decided by the reasons for supporting such participation in the first place. There seems to be a continuum from, on the one hand, those who support public participation because

it is a way of assuring the public of some sort of democratic or semi-democratic influence, to those who see public participation as a way of merely legitimizing the technologies in the eyes of the public. In the first case, the goal is to live up to democratic ideals of some sort without influencing the result of the participation. In the second, the point is to get the technologies accepted [85].

The influence of the goal of the public participation on the structure of the debate can most easily be seen when the concept of *dialogue* is considered. There seem to be three different understandings of the concept of dialogue in ethical discussions of biotechnology today. One is (1) a specific variety of (so to speak) *monological* dialogue. This assumes that widespread skepticism about biotechnology is based on the public's lack of knowledge and understanding of the technologies. The remedy to this is therefore knowledge transfer from the sphere of science to the public. As soon as public has been educated the skepticism will disappear.

This way of thinking, sometimes labeled "the knowledge deficit model" [86], is very widespread in the scientific community. One noteworthy example of it can be found in the report *Why clone farm animals? Goals, motives, assumptions, values and concerns among European scientists working with cloning of farm animals* [87]. Here, a group of scientists working on cloning are interviewed about the technology and various aspects of it. All say that it is very important to establish a dialogue with the public about the technology, but when directly asked what subjects would be suited for such a dialogue, they invariably answer that the public should be provided with information about the technology.

The largest problem with the knowledge deficit model is that, although it continues to thrive within the scientific community, it has been refuted in sociological studies of the public perceptions of biotechnology. What happens when citizens are provided with information about the science and possible applications is that they form an opinion about it; but there is no evidence to suggest that knowledge in general erases skepticism [44].

(2) A second form of dialogue is as monological as the first, but the exchange moves in the opposite direction – it is the public rather than the scientists now doing all the talking. Here, the task is to examine public attitudes to biotechnological applications and figure out which will be socially acceptable. The goal is thus to ensure that the technologies pursued will not end up in the same situation as the GMOs in Europe in the 1990s. Very often, the two monological kinds of dialogue are combined, so that consumer preferences and consumer education go hand-in-hand in an attempt to produce social acceptance for the development of the technologies [85].

(3) The third concept of dialogue in the current debate sees dialogue as a way of balancing two very important considerations. One consideration is to respect the other person, whether it is a single person, a group of stakeholders, or society at large. The other is to take responsibility for one's own views of the world and attempt to do what one sincerely believes to be in the best interest of the other (whoever that may be). A classic example of this conflict can be found in the relationship between the physician and the patient. The ethical duty for the physician is to avoid taking all responsibility from the patient (paternalism as tyranny) and yet not leave the patient on his own in trying to decide upon different methods of treatment in the mistaken belief that the task of a physician is just to provide neutral information and at all costs respect the patient's right to self-determinacy (autonomy as denial of responsibility).

The concept of dialogue implies that there are two or more opinions about something, and that the people holding these opinions are willing to discuss them, holding a small window open in the back of their minds to the possibility that they may be wrong. A dialogue where it is from the outset decided that only one part of the dialogue (and that is usually the other part) could end up changing their minds is no dialogue, but could better be described as a caricature of energetic and zealous religious proselytizing.

When promoting public participation in the debates about such things as transgenic animals, it is important to realize that this is always done with a specific goal in mind – and that this goal will always influence the way the dialogue is structured. Similarly, the motivation for entering the dialogue in the first place will influence views on what questions are relevant and who should participate. These differences are also visible within different cultures where, for example, the role of the scientists and nonexperts in the

debates are understood very differently, just as the purpose of the whole exercise is understood quite differently [88].

Possible Goals of Ethical Dialogues

Motivation for entering ethical discussion of biotechnology in general and transgenic livestock in particular varies from stakeholder to stakeholder, although it is probably safe to say that all stakeholders enter the discussion because they think that the information they can bring will convince others of their viewpoints [73]. But besides the urge to win the argument, there are other and more realistic goals of ethical dialogue, and these could provide reasons for entering in the first place.

The first reason is enlightenment or clarification. Ethical debates are where humans meet and exchange their views. This gives the involved parties a chance to explain their views and listen to the explanations of others. Thus the areas of disagreement and the values that underlie them can both be clarified. This need not lead to agreement, of course; it will only do so if some of the disagreements turn out to be based on misunderstandings either of the viewpoints of the opponent or the facts of the issue. But it can lead to a better understanding of the opponent's viewpoints; and that is valuable in itself. It is valuable, first and foremost, because understanding is itself valuable, and second, because it is easier to find areas of consensus and compromise if you have an understanding of the viewpoints of others rather than just finding them irrational or blind to the ethical dimensions of the issue. Understanding why someone has certain opinions makes it easier to respect him or her, and it can create an environment around the debate that makes it easier to move on to the next step [89].

The second possible result of ethical debates is that the decisions eventually reached are socially robust. A socially robust decision is here understood as a decision that seeks to include widely held concerns about a technology while at the same time allowing it to progress in areas that are of significant benefit to society. As mentioned earlier in section "Public Perceptions of Transgenic Technologies," it is apparent from sociological studies that, in biotechnology, there are two scales of importance. One is the scale of organism, the other a scale of applications. The simpler the organism, the more acceptable the technologies become; and the more the application of the technology addresses issues like health, the environment, and aid to the developing world, the more acceptable it becomes [44]. Thus using transgenic technologies to produce livestock that will benefit medical research will, in all likelihood, be more socially acceptable than producing livestock that can improve productivity in agriculture.

By targeting on areas of need, developments in transgenic livestock would therefore be less problematic than they would be if the focus was on areas of economic gain. A socially robust development process will mean that certain applications are left aside in order to gain broad societal acceptance of the progress of the technologies [90].

Future Directions

The technology to produce transgenic animals is here to stay. The genie cannot be put back in the bottle. And as the technology is further developed and refined, it will, in all likelihood, become more and more economically feasible and sustainable to produce animals through these technologies. But the ethical problems will not disappear either. The issue of animal welfare as seen from a narrow perspective will continue to play a role, as will the issues around possible negative impacts on human health and the environment. Even when more data on these risks become available, it should be obvious from the discussion of GM crops that the risk evaluation will continue to be an area of controversy for many years to come.

Discussion of the socio-economic impact of the technologies will intensify. If the animals enter the agricultural sector and the food production chain, there is reason to expect that they will only strengthen the current trends within agriculture, where ever larger units with animals dominate production in a highly technological environment. This will inevitably give rise to ethical debate – but probably debate of a sort that is already taking place about how the agricultural production system should function. In the areas of naturalness and integrity, ethical disagreement will continue to flourish, especially if animals are produced for applications that are not generally regarded as important to society.

All in all, then, it is safe to say that ethical debate about transgenic livestock, and about the development of the technology, will continue to play a significant role in the future. As research and technology development becomes more and more reliant on private funding, it will be necessary to show that the development of transgenic animals will pay off investors. Finally, there is no doubt that the area will continue to be a political and ideological battle scene. It will therefore be necessary to apply methods of public participation that include many viewpoints. These methods should help to ensure socially robust development in a technology, for which the potential to create social and ethical conflicts seem as large as the technological potential.

Bibliography

1. Clark J, Whitelaw B (2003) A future for transgenic livestock. Nat Rev Genet 4:825–833
2. Laible G (2009) Enhancing livestock through genetic engineering – recent advances and future prospects. Comp Immunol Microbiol Infect Dis 32:123–137
3. Lacy WB, Lacy LR, Busch L (1988) Agricultural biotechnology research: practices, consequences, and policy recommendations. Agric Hum Values 5(3):3–14
4. Baile CA, Krestel-Rickert DH (1988) Will society permit the potential of genetic engineering to advance the frontiers of biology? J Anim Sci 66:2125–2130
5. Fox MV (1989) Genetic engineering and animal welfare. Appl Anim Behav Sci 22(2):105–113
6. Gavora JS, Lister EE (1989) Practical and ethical considerations of agricultural research assistance for the third world. J Agric Environ Ethics 2(4):307–322
7. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813
8. Cooper DKC, Dorling A, Pierson RN III, Rees M, Seebach J, Yazer M, Ohdan H, Awwad M, Ayares D (2007) [Alpha]1, 3-galactosyltransferase gene-knockout pigs for xenotransplantation: where do we go from here? Transplantation 84(1):1–7
9. Kling J (2009) First US approval for a transgenic animal drug. Nat Biotechnol 27:302–304
10. Thompson PB (1997) Food biotechnology in ethical perspective. Blackie Academic & Professional, London
11. Gamborg C, Gjerris M (2009) The price of responsibility. In: Gjerris M, Gamborg C, Olesen JE, Wolf J (eds) Earth on fire. Climate change from a philosophical and ethical perspective. University of Copenhagen, Copenhagen, Open access. Available at www.earthonfire.foi.dk
12. European Commission (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC – Commission Declaration. European Commission, Brussels
13. Houdebine LM (2009) Methods to generate transgenic animals. In: Engelhard M, Hagen K, Boysen M (eds) Genetic engineering in livestock: new applications and interdisciplinary perspectives. Springer-Verlag, Berlin, pp 31–48
14. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS (2002) Production of α-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295(5557):1089–1092
15. Niemann H, Kues W, Carnwath JW (2007) Transgenic farm animals: current status and perspectives for agriculture and biomedicine. In: Engelhard M, Hagen K, Boysen M (eds) Symposium on new application of livestock genetic engineering. Springer-Verlag, Berlin, pp 1–30
16. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA Biol Sci 77:7380–7384
17. Hammer RE, Pursel VG, Rexroad CE, Wall R, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
18. Kong QR, Wu ML, Huan YJ, Zhang L, Liu HY, Bou G, Luo YB, Mu YS, Liu ZH (2009) Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. PLoS ONE 4:10
19. Le Provost F, Lillico S, Passet B, Young R, Whitelaw B, Vilotte JL (2009) Zinc finger nuclease technology heralds a new era in mammalian transgenesis. Trends Biotechnol 28:134–141
20. Niemann H, Kues W, Carnwath JW (2005) Transgenic farm animals: present and future. Rev Sci Tech 24(1):285–298
21. Blasco A (2008) The role of genetic engineering in livestock production. Livest Sci 113:191–201
22. Foresti F (2000) Biotechnology and fish culture. Hydrobiologia 420:45–47
23. Zbikowska HM (2003) Fish can be first – advances in fish transgenesis for commercial applications. Transgenic Res 12:379–389
24. Rasmussen RS, Morrissey MT (2007) Biotechnology in aquaculture: transgenics and polyploidy. Compr Rev Food Sci Food Saf 6:2–16
25. Kelly L (2005) The safety assessment of foods from transgenic and cloned animals using the comparative approach. Rev Sci Tech 24:61–74
26. Wall RJ (1996) Transgenic livestock: progress and prospects for the future. Theriogenology 45(1):57–68
27. Wolf E, Schernthaner W, Zakhartchenko V, Prelle K, Stojkovic M, Brem G (2000) Transgenic technology in farm animals – progress and perspectives. Exp Physiol 85:615–625
28. Niemann H, Kues WA (2003) Application of transgenesis in livestock for agriculture and biomedicine. Anim Reprod Sci 79:291–317
29. Wheeler MB (2007) Agricultural applications for transgenic livestock. Trends Biotechnol 25:204–210

30. Bremel RD, Homan EJ, Howard TH (2001) Current and future promises of transgenesis for agricultural livestock in a global marketplace. J Dairy Sci 84:E1–E8
31. Prather RS (2006) Cloned transgenic heart-healthy pork? Transgenic Res 15:405–407
32. Hallerman EM, McLean E, Fleming IA (2007) Effects of growth hormone transgenes on the behaviour and welfare of aquacultured fishes: a review identifying research needs. Appl Anim Behav Sci 104:265–294
33. Nightingale P, Martin P (2004) The myth of the biotech revolution. Trends Biotechnol 22(1):564–569
34. Gjerris M, Olsson A, Sandøe P (2006) Animal biotechnology and animal welfare. In: Council of Europe (ed) Ethical eye – animal welfare. Council of Europe, Strasbourg, pp 8–110
35. Durant J, Bauer J, Gaskell G (1998) Biotechnology in the public sphere. Science Museum, London
36. Gaskell G, Bauer M (2002) Biotechnology 1996–2000: the years of controversy. Science Museum, London
37. Gaskell G, Allum N, Stares S (2003) Europeans and biotechnology in 2002. Eurobarometer 58.0
38. Gaskell G, Allansdottir A, Allum N, Corchero C, Fischler C, Hampel J, Jackson J, Kronberger N, Mejlgaard N, Revuelta G, Schreiner C, Stares S, Torgersen H, Wagner W (2006) Europeans and biotechnology in 2005: patterns and trends. Eurobarometer 64.3
39. Wagner W, Kronberger N, Allum N, Cheveigné de S, Diego C, Gaskell G, Heinßen M, Midden CJH, Ødegaard M, Öhman S, Rizzo B, Rusanen T, Stathopoulou A (2002) Pandora's genes – images of genes and nature. In: Bauer MW, Gaskell G (eds) Biotechnology. The making of a global controversy. Cambridge University Press, Cambridge, pp 244–276
40. Lassen J, Jamison A (2006) Genetic technologies meet the public: the discourses of concern. Sci Technol Hum Values 31(1):8–28
41. Einsiedel EF (2005) Public perceptions of transgenic animals. Rev Sci Tech 24(1):149–157
42. Frewer LJ, Howard C, Shepherd R (1997) Public concerns in the United Kingdom about general and specific applications of genetic engineering: risk, benefit, and ethics. Sci Technol Hum Values 22(1):98–124
43. Gaskell G, Bauer MW, Durant J (1998) Public perceptions of biotechnology in 1996: eurobarometer 46.1. In: Durant J, Bauer MW, Gaskell G (eds) Biotechnology in the public sphere. A European sourcebook. Science Museum, London, pp 189–214
44. Lassen J, Gjerris M, Sandøe P (2006) After dolly – ethical limits to the use of biotechnology on farm animals. Theriogenology 65:992–1004
45. Beck U (1992) Risk society. Towards a new modernity. Sage, London
46. European Commission (2002) Eurobarometer 58.0. European Commission
47. Dahl K, Sandøe P, Johnsen PF, Lassen J, Hansen AK (2003) Outline of a risk assessment: the welfare of future xeno-donor pigs. Anim Welfare 12:219–237
48. Rigaud N (2008) Biotechnology: ethical and social debates. OECD, Paris
49. Arluke A, Sanders CR (1996) Regarding animals. Temple University Press, Philadelphia
50. National Research Council and Institute of Medicine of the National Academies (2004) Safety of genetically engineered foods. Approaches to assessing unintended health effects. National Academy of Science, Washington
51. The Royal Society of Canada (2001) Elements of precaution: recommendations for the regulation of food biotechnology in Canada. An expert panel report on the future of food biotechnology. The Royal Society of Canada, Ottawa
52. Lai L, Jing X, Kang X, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Starz TE, Prather RS, Dai Y (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24:435–436
53. Wall RJ, Kerr DE, Bondioli KR (1997) Transgenic dairy cattle: genetic engineering on a large scale. J Dairy Sci 80(9):2213–2224
54. Fishman JA, Patience C (2004) Xenotransplantation: infectious risks revisited. Am J Transplant 2004(4):1383–1390
55. PEW Initiative on Food and Biotechnology (2003) Future fish. Issues in science and regulation of transgenic fish. PEW Initiative on Food and Biotechnology, Washington
56. Abram D (1996) The spell of the sensuous. Vintage Books, New York
57. Pursel VG, Pinkert CA, Miller KF, Bolt DJ, Campbell RG, Palmiter RD, Brinster RL, Hammer RE (1989) Genetic engineering of livestock. Science 244:1281–1288
58. Van Reenen CG, Meuwissen THE, Hopster H, Oldenbroek K, Kruip TAM, Blokhuis HJ (2001) Transgenesis may affect farm animal welfare: a case for systematic risk assessment. J Anim Sci 79:1763–1779
59. Van Reenen CG (2007) Assessing the welfare of transgenic farm animals. In: Engelhard M, Hagen K, Boysen M (eds) Symposium on new application of livestock genetic engineering. Springer-Verlag, Berlin, pp 119–143
60. Wheeler MB (2002) Production of transgenic livestock: promise fulfilled. In: Sixth international workshop on the biology of lactation in farm animals, Quebec, 2002. American Society of Animal Science, pp 32–37
61. Duncan IJH, Fraser D (1997) Understanding animal welfare. In: Appleby MC, Hughes BO (eds) Animal welfare. CAB International, Wallingford, pp 19–31
62. Dawkins MS (2004) Using behavior to assess welfare. Anim Welfare 13:3–7
63. Fraser D, Weary DM, Pajor EA, Milligan BN (1997) A scientific conception of animal welfare that reflects ethical concerns. Anim Welfare 6:187–205
64. Appleby MC, Sandøe P (2002) Philosophical debate on the nature of well-being: implications for animal welfare. Anim Welfare 11(3):283–294
65. Rollin BE (1995) Frankenstein syndrome: ethical and social issues in the genetic engineering of animals. Cambridge University Press, Cambridge

66. Shriver A (2009) Knocking out pain in livestock: can technology succeed where morality has stalled? Neuroethics 2(3):115–124
67. D'Eath RB, Conington J, Lawrence AB, Olsson IAS, Sandøe P (2010) Breeding for behavioural change in farm animals: practical, economic and ethical considerations. Anim Welfare 19(5):17–27
68. Ali A, Cheng KM (1985) Early egg production in genetically blind (rc/rc) chickens in comparison with sighted (Rc+/rc) controls. Poult Sci 64(5):789–794
69. Lassen J, Sandøe P, Forkman B (2006) Happy pigs are dirty! – conflicting perspectives on animal welfare. Livest Sci 103(3):221–230
70. Verhoog H (2003) Naturalness and the genetic modification of animals. Trends Biotechnol 21(7):294–297
71. Sandøe P, Holtug N, Simonsen HB (1996) Ethical limits to domestication. J Agric Environ Ethics 19(5):469–493
72. Task Group on Public Perceptions of Biotechnology (1999) Ethical aspects of agricultural biotechnology. European Federation of Biotechnology, Den Haag
73. Reiss MJ, Straughan R (2000) Improving nature. The science and ethics of genetic engineering. Cambridge University Press, Cambridge
74. Chapman A (2005) Genetic engineering: the unnatural argument. Techné 9(2):81–93
75. Rutger B, Heeger R (1999) Inherent worth and respect for animal integrity. In: Dol M, van Vlissingen MF, Kasanmoentalib S, Visser T, Zwart H (eds) Recognizing the intrinsic value of nature. Van Gorcum, Assen, pp 41–53
76. De Vries R (2006) Genetic engineering and the integrity of animals. J Agric Environ Ethics 19(5):469–493
77. Vorstenbosch J (1993) The concept of integrity. Its significance for the ethical discussion on biotechnology and animals. Livest Prod Sci 36:109–112
78. Gottlieb S, Wheeler MD (2008) Genetically engineered animals and public health: compelling benefits for health care, nutrition, the environment, and animal welfare. BIO – The Biotehnology Organisation, Washington
79. Bovenkerk B, Brom FWA, Van Den Bergh BJ (2002) Brave new birds: the use of 'animal integrity' in animal ethics. Hastings Cent Rep 32:16–22
80. Cooper DE (1998) Intervention, humility and animal integrity. In: Holland A, Johnson A (eds) Animal biotechnology and ethics. Chapman & Hall, London, pp 145–155
81. Merleau-Ponty M (1969) The visible and the invisible. Northwestern University Press, Evanston
82. Løgstrup KE (1995) Vidde og prægnans. Sprogfilosofiske betragtninger. Metafysik I. 2. udg. Gyldendal, København
83. Roosen J, Lusk JL, Fox JA (2001) Consumer demand for and attitudes toward alternative beef labeling strategies in France, Germany, and the UK. Agribusiness 19(1):77–90
84. Nielsen AP, Lassen J, Sandøe P (2004) Involving the public – participatory methods and democratic ideals. Glob Bioeth 17:191–201
85. Gjerris M (2008) The three teachings of biotechnology. In: David K, Thompson P (eds) What can nanotechnology learn from biotechnology? Elsevier, Burlington, pp 91–106
86. Bauer MW, Bonfadelli H (2002) Controversy, media coverage and public knowledge. In: Bauer MW, Gaskell G (eds) Biotechnology. The making of a global controversy. Cambridge University Press, Cambridge, pp 149–175
87. Meyer G (2005) Why clone farm animals? Goals, motives, assumptions, values and concerns among European scientists working with cloning of farm animals, Project report 8. Danish Centre for Bioethics and Risk Assessment, Frederiksberg
88. Nielsen AP, Lassen J, Sandøe P (2007) Democracy at its best? The consensus conference in a cross-national perspective. J Agric Environ Ethics 20:13–35
89. Gjerris M (2009) This is not a hammer – on ethics and technology. In: Bedau M, Parke E (eds) Our future with protocells: the social and ethical implications of the creation of living technology. MIT Press, Boston, pp 291–306
90. Gjerris M, Sandøe P (2006) Farm animal cloning: the role of the concept of animal integrity in debating and regulating the technology. In: Kaiser M, Lien ME (eds) Ethics and the politics of food: preprints of the 6. Congress of the European Society for Agricultural and Food Ethics. Academic, Wageningen, pp 320–324
91. van de Lavoir MC, Mather-Love C, Leighton P, Diamond JH, Heyer BS, Roberts R, Zhu L, Winters-Digiacinto P, Kerchner A, Gessaro T, Swanberg S, Delany ME, Etches RJ (2006) High-grade transgenic somatic chimeras from chicken embryonic stem cells. Mech Dev 123:31–41

Transgenic Technologies and Increased Livestock Fertility

F. Xavier Donadeu, Cheryl J. Ashworth
The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Scotland, UK

Article Outline

Glossary

Bone morphogenetic proteins (BMPs) Group of molecules belonging to the transforming growth factor family that were initially discovered by their ability to induce the formation of bone and cartilage. BMPs provide pivotal morphogenetic signals orchestrating tissue architecture throughout the body.

Calving The act of delivering a calf at the end of normal bovine pregnancy.

Energy balance In reference to animal physiology, it is the relation between energy intake from nutrient consumption and energy expenditure to maintain body functions.

Estrus The stage of the estrous cycle when a female is sexually receptive and during which ovulation occurs.

Fertility The ability to produce healthy offspring.

First-service conception rate Refers to the percentage of animals in a herd that become pregnant upon insemination on the first estrous cycle after parturition.

Genetic selection/selective breeding The process of breeding animals or plants with the goal of enhancing particular genetic traits.

Heterozygous Refers to an individual having two different alleles for a given trait.

Homozygous Refers to an individual having identical paternally and maternally inherited alleles for a given trait.

Knockin Integration of a new gene which replaces an endogenous gene.

Knockout Functional disruption of a specific gene of an organism, commonly achieved by a partial or complete deletion of the gene sequence.

Livestock Domesticated animals raised to produce commodities such as food, fiber and labor.

Mutation A change in the DNA sequence of a gene which can result in changes in the sequence and function of the encoded protein.

Ovarian follicle The structural and functional unit in the ovary within which an oocyte grows and matures until it is expelled during ovulation.

Ovulation The process by which mature ovarian follicles rupture under the influence of luteinizing hormone to release an oocyte into the oviduct.

Phenotype/phenotypic A measurable characteristic of an animal such as hair color or growth rate, and which is the product of genetics and the environment.

Point mutation A mutation involving a single nucleotide base pair in a DNA sequence.

Production trait A genetically determined characteristic of an individual of a livestock species which relates to its capacity to provide a certain product (milk, meat, fiber, eggs, work) which contributes directly to the value of the animal for the farmer.

Promoter A regulatory DNA sequence that controls the transcription of a particular gene.

Reproductive performance The productivity of an animal or herd in terms of offspring produced.

Reproductive/breeding efficiency Related to the capacity of an individual to successfully carry out all reproductive processes from gamete formation to fertilization, establishment of pregnancy and delivery of healthy offspring.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

Seasonal reproduction Refers to the dependence of reproductive activity on seasonal factors, most notably changes in day length, in certain species.

Single nucleotide polymorphism DNA sequence variations involving alteration of a single nucleotide in a genome sequence.

Transgene An exogenous gene introduced into the genome of another organism.

Transgenic An animal, plant or microbe whose genetic material has been altered using an artificial process.

Zinc finger nucleases (ZFNs) Synthetic proteins consisting of an engineered DNA-binding motif characterized by multiple finger-like protrusions (finger domain) and which has been fused to the cleavage domain of the *FokI* restriction endonuclease. ZFNs can be used to induce double-stranded breaks in specific DNA sequences and thereby promote site-specific homologous recombination and targeted manipulation of genomic loci in a variety of different cell types.

Definition of the Subject and Its Importance

The possibility of using transgenic technology to promote desirable traits, including enhanced reproductive efficiency, in livestock species has attracted considerable interest since transgenic animals were first produced. Almost 20 years ago McEvoy and associates [1] proposed that transgenic technologies could be used to increase reproductive output in food producing animals by increasing litter size in livestock or egg-laying capacity in poultry, and by reducing the limitations associated with seasonal breeding. During the intervening 2 decades, the uptake of transgenic technologies in livestock production has been limited because of technical, regulatory and ethical constraints [2]. However, the availability of novel technologies, such as Zinc finger nucleases (ZFNs) [3], which allow for greater precision and control, together with a growing need to increase livestock productivity to meet the requirements of a growing world population will likely help to realize current aspirations on use of transgenesis in animal reproduction. The application of transgenic technologies in laboratory animals has been invaluable in considerably furthering our understanding of numerous reproductive processes including gonad development and function [4–6], oogenesis and early embryogenesis [7, 8], and trophoblast gene function [9, 10], and through this increased understanding will lead to new strategies to improve reproductive efficiency of livestock species.

Introduction

Reproductive efficiency of domestic animals is critical to the sustainability of modern livestock industries. Thousands of years of domestication led to changes in the reproductive physiology of animals, for example, a dramatic reduction in the influence of season on reproduction in cattle and pigs. During modern times, intensification of farming systems coupled with the implementation of programs of genetic improvement aimed at selection for specific (non-reproductive) productivity traits had further effects on reproductive physiology often with negative consequences to breeding efficiency, with ultimately an impact on the overall productivity and sustainability of livestock industries. Meeting the increasing world demands for animal products will require increased production of healthy offspring from livestock while coping with a decrease in the availability of natural resources to maintain these animals. Achieving this within a reasonable time frame will require the use of strategies alternative, or in addition, to traditional breeding and genetic selection. This entry will describe current limitations in reproductive performance of livestock. The potential and limitations of transgenic technologies to address such problems will then be described and specific illustrative examples will be provided.

The Problem of Low Fertility in Modern Livestock

At its 2009 World Food Summit, the United Nations Food and Agricultural Organization recognized that agricultural output will need to increase by 70% by 2050 in order to feed the world's population which is expected to exceed 9 billion in this timeframe (FAO 2009, ftp://ftp.fao.org/docrep/fao/Meeting/018/k6050e.pdf). A significant portion of that output will be derived from the demand for animal protein, especially in developing countries as they become more affluent. Considering increasing production constrains derived from climate change and competing demands on resources, the key target for the future of livestock

production will be to maximize the number of offspring produced by each female animal that are also fit for purpose. This will involve three challenges, namely, to increase the numbers of offspring that a female can produce, to optimize the in utero conditions under which fetuses develop so to maximize development of appropriate phenotypes, and to bias offspring sex to reflect animal usage. Meeting these challenges will require continued advances of traditional methods and approaches, such as husbandry and genetics, as well as the broad uptake of newer and emerging biotechnological solutions such as transgenesis.

An important point to be made is that these challenges will need to be accomplished in the face of a persistent decrease in livestock fertility associated with modern farming systems in western countries, particularly affecting the dairy, pig and chicken meat industries [11–13]. In these animals, specific production traits (milk, meat) have been genetically selected at the expense of a reduction in non-selected traits such as fertility. In many cases an even more important contribution to low fertility in modern livestock herds has resulted from industry consolidation into fewer, larger production units which are managed intensively toward the primary objective of maximal economic gain.

A clear example of this is the dairy cattle industry, where a steady increase in milk productivity over the past few decades has been associated with a progressive decline in fertility to current first-service conception rates below 40%, resulting in extended calving intervals and premature culling [11, 14, 15]. According to the UK's Dairy Science Forum (2008), poor fertility in dairy herds is estimated to cost to the UK industry alone above £300 million a year, in addition to being a major welfare issue. Under current management schemes, to maximize milk production cows need to conceive soon after parturition so that the interval between lactations is minimized and a target of one-calf-a-year can be achieved. This requires timely restoration of gonadotropin secretion and ovarian activity after calving, together with adequate uterine clearance and repair, if successful follicle maturation and ovulation followed by fertilization and establishment of pregnancy is to take place by about 90–100 days [16, 17]. However, dairy cattle have been selected to efficiently partition nutrients toward milk [18] production thus creating a state of negative energy balance after parturition where reinitiation of high milk production will occur preferentially to restoration of reproductive function conducive to establishment of pregnancy [19].

Even more profound effects on reproductive activity in cattle but also in other livestock [20–22] are derived from suboptimal management of animals under intensive farming conditions including under nutrition, stress and general poor health reflected, for example, in a high incidence of lameness and mastitis, two major conditions associated with low reproductive performance of dairy cattle [11, 14]. Repeated calving itself poses an enormous risk to sustained reproductive performance because of the incidence of parturition-related or postpartum complications (e.g., dystocia, retained fetal membranes, endometritis). Poor management, together with suboptimal ovarian function during postpartum, also leads to poor estrus detection in large herds of dairy cows and other livestock leading to a dramatic decrease in the efficiency of artificial insemination-based programs [23]. Finally, several other factors can have a large impact on overall female health including subclinical infections and environmental factors such as heat stress which has direct effects on follicle development, oocyte quality and embryo development [24, 25].

Poor management is a primary cause of low reproductive performance of livestock in developing countries. As an example, the domestic buffalo constitutes an essential farming resource to millions of families in developing countries, particularly Asia. This species has been traditionally regarded as having low reproductive efficiency, including late attainment of puberty, a seasonal pattern of reproduction, poor expression of estrus, low conception rates and long calving intervals. To a large extent, these characteristics can be attributed to environmental and managerial factors including the absence of a year-round nutrient supply, harsh environmental conditions and suboptimal human intervention [26, 27]. Different studies have shown that buffalo fertility can improve considerably by exercising proper management and feeding and that this can be enhanced by genetic selection [27, 28].

In some instances, the problem is not a lack of nutrients but over nutrition. This is the case in the broiler chicken, in which overfeeding of growth-selected animals results in excessive recruitment of ovarian follicles leading to simultaneous ovulation of multiple follicles. This alters the timing of egg laying and results in poor eggshell quality [29]. This problem has been addressed by nutrient restriction of animals which is necessary to achieve reasonable levels of egg production. This is a significant production and welfare issue as nutrient-restricted broiler breeders show clear evidence of physiological stress as well as an increased incidence of abnormal behaviors, among other problems [30].

Limitations on the reproductive performance of livestock can also be imposed by naturally evolved traits. This is the case in sheep, goats and horses, in which ovulatory activity is restricted to certain periods of the year to ensure that offspring are born at a time when food resources are plentiful [31]. Another example is the inability to selectively breed for the desired gender for a given production trait (for example females only for milk and males only for meat production) which within livestock industries leads to considerable waste and welfare concerns due to the need to cull the less useful gender.

The Need and Potential of Transgenic Technologies to Improve Reproductive Performance of Livestock Species

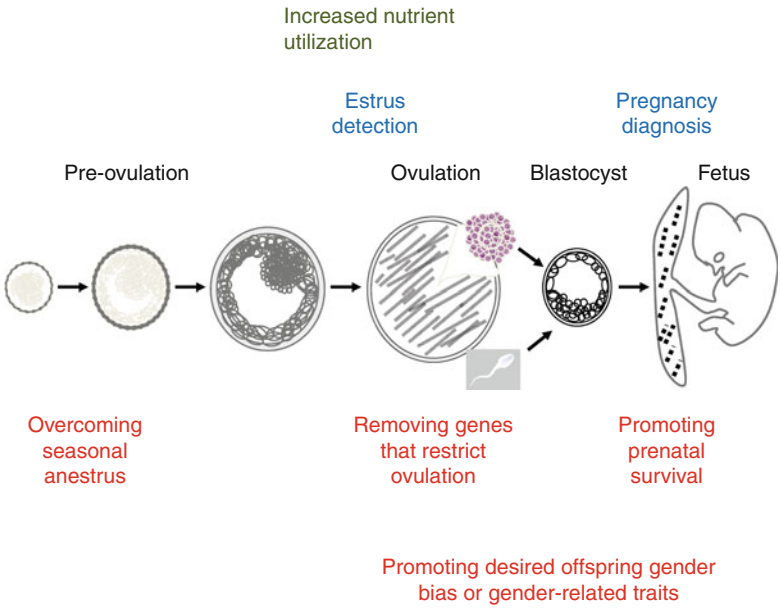
As outlined above, declining fertility trends in livestock can be partially reversed by improved management. This may include pharmacological control of reproductive function which, although effective in the short term, is nonetheless expensive and has limited benefits as not all animals respond adequately to hormone treatments, particularly if these are not associated with improvements in more basic aspects of management [32–34].

Interventions aimed at increasing general herd health and welfare and reducing stress can be very effective in substantially improving fertility, particularly if they include close monitoring of the reproductive status of individual animals. Optimal implementation of such measures, however, can be costly and in some cases may not be feasible and/or economically viable, particularly in developing countries.

Optimum nutrition is also an important aspect of good management, particularly as it relates to prevention of low energy balance during postpartum and other critical reproductive states [15, 19]. It has been shown that the extent to which reproductive performance can be increased through nutrition is limited [35] and that a given diet can have different effects on different aspects of reproduction, as exemplified by the administration of diets which stimulate plasma insulin levels in postpartum cattle [36]. Such diets can effectively stimulate follicle development but at the same time have negative effects on the oocyte, justifying the need for carefully tailoring diet composition to specific reproductive functions if optimum fertility is to be achieved.

Although selection of livestock for certain production traits has been associated with a loss of fertility, studies with dairy cattle have shown that this loss can be reversed, through genome-wide selection on multiple traits, without completely compromising milk production [35]. However, eliminating the fertility problem in livestock through genetics would require that reproductive traits be included as a significant component of selection indexes and the desired outcome may not be achieved in decades. Alternatively, genetic gain could be aimed at reducing the impact of poor fertility on productivity, for example, by selecting for extended lactation periods in dairy cattle [14], although in many instances this may not be economically feasible. In addition, it is unlikely that existing genetic variation in modern livestock lines will continue to generate the rate of gain obtained in the past. On that note, the use of transgenic technology to induce modifications in selected genes followed by conventional breeding and selection would offer an attractive alternative to effectively introduce desirable fertility traits in livestock, particularly in cattle and sheep which have long generation times and small offspring numbers.

Transgenic approaches have been used in livestock for the purposes of increasing productivity, conferring disease resistance and increasing environmental sustainability [2]. Similar approaches could be used to increase fertility (Fig. 1). These could be aimed at improving reproductive function directly (for example,



Transgenic Technologies and Increased Livestock Fertility. Figure 1
Schematic diagram showing stages of the reproductive process where transgenic technologies could impact. The examples presented include strategies to directly improve reproductive function (in *red*), to indirectly favor reproductive success (in *green*), or to facilitate reproductive management (in *blue*)

by removing genes that naturally suppress ovulation) or indirectly (for example, by improving energy utilization by reproductive tissues) or at facilitating reproductive management of livestock (for example, by inserting reporter genes that could facilitate estrus detection or early pregnancy diagnosis). To achieve this, gene knockin or knockout approaches could be used to modify whole genes or large portions of these genes. Alternatively, novel ZFN technology allows for targeted or untargeted gene mutagenesis to be induced to selectively modify gene function in animals [37–39]. This approach would be more likely to be accepted by the public than the insertion of foreign genes into livestock genomes and it could also provide more predictable results, particularly if natural or induced mutations of the gene(s) of interest already existed in the same or a different species.

Successful application of transgenesis in livestock fertility will ultimately require a more thorough understanding of reproductive physiology in these species. Transgenic studies aimed at targeting fertility genes in livestock have not been reported and therefore potential gene targets would have to be obtained from data on naturally occurring mutations or single nucleotide polymorphisms associated with fertility, or from results of experimental gene targeting in rodents, which can already provide a substantial list of candidate genes [40]. It must be stressed that, because of significant species differences, considerable caution should be used when attempting to extrapolate results from genetically modified mice into other species. A clear example of this is the failure of genetic deficiencies in bone morphogenetic protein (BMP) 15 or growth differentiation factor (GDF) 9, which are naturally associated with multiple ovulation in sheep, to increase the ovulatory response in mice [41]. In addition, it is essential to remember that most reproductive traits are controlled by several genes acting in concert and they may not therefore be easily altered by targeting a single gene. Finally, genetic modification in mice can reportedly be associated with a reduction of fertility in vitro [42], an observation that would need to be properly addressed.

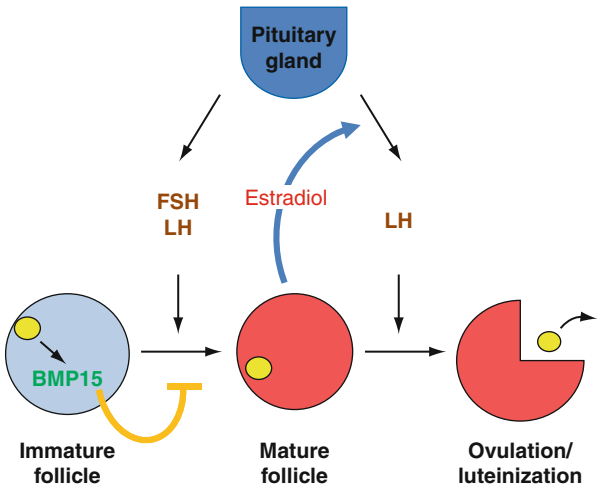
The choice of a particular gene modification aimed at improving livestock fertility would need to be based on different considerations. Optimally, a gene should be

targeted only in a specific cell type or organ of interest. This could be achieved with the use of cell-specific gene promoters to drive the targeting event. Choosing a gene target with naturally restricted expression, if possible restricted to the cell type of interest, would be technically simpler and more efficient. In addition, small-scale mutagenesis rather than whole gene replacement or insertion of a new gene would be preferred to achieve the desired phenotype effects. This is now possible with remarkable efficiency in rodents and pigs by using ZFN technology [37–39]. Lastly, the intended mutation would optimally exist naturally and have been characterized thus allowing more accurate prediction of the phenotype in transgenic offspring.

Potential Targets for Transgenic Improvement of Livestock Fertility

Among the genes that could be targeted to increase fertility in livestock, certain members of the BMP family meet all three criteria described above for an optimum target. In this section, these genes will be first considered in detail followed by brief reference to others that could be targeted to improve different aspects of reproduction.

Natural mutations in the BMP ligands, BMP15 and GDF9, and in the BMP receptor type 1B (BMPR1B) have been described in different breeds of high prolificacy sheep and some of them have been characterized in detail [43, 44]. Five different point mutations were identified in BMP15 (*FecX* locus) and one in each of GDF9 (*FecG*) and BMPR1B (*FecB*). These mutations led in each case to reduced production of biologically active protein and/or altered protein signaling. Homozygosis for either BMP15 or GDF9 mutations is associated with infertility due to disruption of early ovarian follicle development, whereas heterozygous animals show natural higher ovulatory rates than wild types (about 3–4 vs 2 follicles per ovulation). The BMPR1B mutation in sheep is also associated with a higher ovulation rate in heterozygous animals, but in this case homozygous animals not only are fertile but they display an even larger positive effect on ovulation rate ($\geq$5 follicles per ovulation). Expression of BMP15 and GDF9, but not BMR1B, is mostly restricted to the oocyte. For both BMP15 and GDF9 mutations, the different ovarian phenotypes observed in homozygous and heterozygous animals are thought to largely reflect the role of these growth factors in promoting cellular proliferation during early stages of follicular development and in preventing premature maturation of late stage follicles, respectively (Fig. 2) [43, 45]. In heterozygotes for either mutation, reduced growth factor levels reportedly lead to increased responsiveness to the gonadotropin, follicle-stimulating hormone, and premature acquisition of responsiveness to luteinizing hormone, by granulosa cells of developing follicles which facilitates follicle maturation leading to the recruitment of an increased number of ovulatory follicles [43]. This phenomenon could be exploited using transgenesis to increase ovulatory rate not only in other sheep breeds but also in monovular species such as cattle. Evidence from immunization studies indicates that, as in sheep, altering the levels of BMP15 and/or GDF in cattle has clear effects on follicle development and ovulation rate [46]. Based on this and other information on the biological role of BMP15 and GDF9 in the bovine ovary [47], as well as on the phenotype described in mutant sheep, it can be predicted that similar mutations in BMP15 and/or GDF9 in cattle would result in animals with increased follicular sensitivity to gonadotropins and therefore a reduced gonadotropin dependence for follicle maturation. This change would provide a clear physiological advantage during situations, such as the early lactation period, when energy demands for reproduction cannot be fully met resulting in insufficient gonadotropin stimulation that prevents normal follicle maturation and restoration of ovulatory activity [16, 48]. As a result, ovulatory activity would be predictably restored early after parturition providing an opportunity for reduced calving intervals. It is very important to consider, however, that for the increased incidence of ovulation in such transgenic cattle to effectively lead to an increase in reproductive efficiency, it would have to be associated with the implementation of measures to ensure adequate oocyte quality and uterine health, so that pregnancy could be successfully established and maintained. An increased frequency of twining would be likely in the transgenic cattle lines which, although not desirable in certain situations, could still be exploited by the cattle industry to increase productivity. In addition to the natural mutations considered here, there are other high prolificacy



Transgenic Technologies and Increased Livestock Fertility. Figure 2

Schematic of the physiological control of ovarian follicle maturation by pituitary gonadotropins and oocyte-derived BMP ligands. For simplicity, only BMP15 is shown. During normal estrous cycles, systemic gonadotropin levels stimulate follicle maturation leading to the production of estradiol which will eventually trigger a surge in pituitary LH release and ovulation followed by luteinization of follicular cells. Current evidence from ruminants indicates that BMP15 restricts the maturation-promoting effects of gonadotropins thus preventing the development of an excessive number of preovulatory follicles and early ovulation and/or luteinization. It is proposed that transgenically altering the levels of BMP15 in cattle would counteract the relative deficiency in gonadotropin levels during the postpartum period leading to early ovulation. *FSH* Follicle-stimulating hormone, *LH* Luteinizing hormone, *BMP15* Bone morphogenetic protein 15

phenotypes identified in sheep which do not seem to be associated with the BMP system [49]; identification of the specific genes involved will provide additional targets for manipulation of fertility in livestock.

Aside from the BMP system, there is evidence in livestock to indicate that the estrogen receptor (ESR) could be targeted to increase prolificacy. In pigs, there are associations between litter size and several genes including the ESR, prolactin receptor (PPLR), and retinol-binding protein (RBP) genes [50]. Rothschild and associates [51] found a *Pvull* polymorphism in intron 9 of *ESR1* in prolific Chinese Meishan pigs and commercial Large White populations. Among the Pvull genotype sows homozygous for the desired allele had larger litters at birth with more live born piglets. Introduction of a mutated or polymorphic ESR gene could therefore be used to increase litter size in several pig breeds.

The use of transgenesis has also been proposed to address gender inefficiency in livestock industries [2]. More precisely, it was suggested that genes involved in the production of functional sperm [52] or sex determination [6] and that have been successfully targeted in mice and birds could be used as targets for transgenic alteration of sex ratios in livestock. For example, the gene, *DMRT1* (doublesex and mab-3-related transcription factor 1), is implicated in male gonad development in vertebrates and its knockdown using RNA interference in early chicken embryos resulted in gonadal feminization of genetically male embryos leading to partial sex reversal [6]. An alternative approach to address gender inefficiency would be, rather than trying to alter the natural gender ratio, to transgenically enhance specific production traits in a gender-specific manner. As an example, in an attempt to produce transgenic animals with muscular hypertrophy similar to "double-muscling" which results from naturally occurring loss-of-function myostatin *(MSTN)* mutations in cattle [53], Georges and associates [54] targeted trans-inactivators of *Mstn* on the Y chromosome of mice. The resulting transgenic males showed a 5–20% increase in skeletal muscle mass. The authors suggested the use of this approach in dairy cattle to produce breeds with combined beef and dairy abilities [54].

Finally, targeting of a number of other aspects of reproductive physiology through transgenesis may be desirable to address specific causes of reduced fertility. This could be done for example to improve prenatal survival in different species by increasing the expression of known luteotrophic and antiluteolytic conceptus signals which would be expected to reduce embryo mortality and thereby improve pregnancy rate [55], or to reduce seasonal constraints on reproduction by targeting the neuroendocrine pathways involved [56] to generate transgenic small ruminants capable of year-round breeding in temperate latitudes.

Future Directions

Since first reported over 30 years ago [57], the use of transgenic technologies in mice has been extremely useful in unraveling genetic mechanisms, including reproductive processes. Although since then the application of such technologies in other species including livestock has encountered numerous roadblocks, considerable technical improvements have been made which now allow specific and precise gene targeting in vivo [38, 39]. The vast potential of transgenic technologies in livestock has been extensively demonstrated by the successful targeting of many production-related traits in ruminants, cattle and sheep [2]. Although there has been public reluctance toward the use of such technologies in food-producing animals, technical advances allowing the production of safer foods from transgenic animals together with mounting social pressures demanding an increase in the availability of animal products for a growing world population will likely change this, and recent FDA approval of a genetically modified salmon [58] is a good indication of such change already happening. Although attempts at transgenically increasing livestock fertility have not been reported, transgenic technologies hold particular promise in that regard, both in terms of reversing current decreasing fertility trends and alleviating natural fertility constraints of livestock, two aspects that have a profound impact on overall industry productivity and efficiency, and which is set to become increasingly important as demands for higher livestock productivity continue to increase. Studies in livestock species to increase understanding of their reproductive processes and to develop further refined gene targeting technologies will be key in bringing the realization of such promise much closer.

Bibliography

1. McEvoy TG, Robinson JJ, Sreenan JM (1992) A role for transgenic animals in food production? Trends Food Sci Technol 3(11):294–302
2. Fahrenkrug SC, Blake A, Carlson DF, Doran T, Van Eenennaam A, Faber D, Galli C, Gao Q, Hackett PB, Li N, Maga EA, Muir WM, Murray JD, Shi D, Stotish R, Sullivan E, Taylor JF, Walton M, Wheeler M, Whitelaw B, Glenn BP (2010) Precision genetics for complex objectives in animal agriculture. J Anim Sci 88(7):2530–2539
3. Cathomen T, Keith Joung J (2008) Zinc-finger nucleases: the next generation emerges. Mol Ther 16(7):1200–1207
4. Matzuk MM, DeMayo FJ, Hadsell LA, Kumar TR (2003) Overexpression of human chorionic gonadotropin causes multiple reproductive defects in transgenic mice. Biol Reprod 69(1):338–346
5. Rulli SB, Ahtiainen P, Makela S, Toppari J, Poutanen M, Huhtaniemi I (2003) Elevated steroidogenesis, defective reproductive organs, and infertility in transgenic male mice overexpressing human chorionic gonadotropin. Endocrinology 144(11):4980–4990
6. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH (2009) The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461(7261):267–271
7. Yan C, Elvin JA, Lin Y-N, Hadsell LA, Wang J, DeMayo FJ, Matzuk MM (2006) Regulation of growth differentiation factor 9 expression in oocytes in vivo: a key role of the E-box. Biol Reprod 74(6):999–1006
8. Huang X, Andreu-Vieyra CV, Wang M, Cooney AJ, Matzuk MM, Zhang P (2009) Preimplantation mouse embryos depend on inhibitory phosphorylation of separase to prevent chromosome missegregation. Mol Cell Biol 29(6):1498–1505
9. Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-hora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M (2007) Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer. Nat Biotechnol 25(2):233–237
10. Morioka Y, Isotani A, Oshima RG, Okabe M, Ikawa M (2009) Placenta-specific gene activation and inactivation using integrase-defective lentiviral vectors with the Cre/LoxP system. Genesis 47(12):793–798
11. Lucy MC (2001) Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 84(6):1277–1293
12. Young B, Dewey CE, Friendship RM (2010) Management factors associated with farrowing rate in commercial sow herds in Ontario. Can Vet J 51(2):185–189
13. Julian RJ (2005) Production and growth related disorders and other metabolic diseases of poultry – a review. Vet J 169(3): 350–369
14. Dobson H, Smith RF, Royal MD, Knight CH, Sheldon IM (2007) The high producing dairy cow and its reproductive performance. Reprod Domest Anim 42(Suppl 2):17–23
15. Chagas LM, Bass JJ, Blache D, Burke CR, Kay JK, Lindsay DR, Lucy MC, Martin GB, Meier S, Rhodes FM, Roche JR, Thatcher WW, Webb R (2007) Invited review: new perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. J Dairy Sci 90(9):4022–4032
16. Crowe M (2008) Resumption of ovarian cyclicity in postpartum beef and dairy cows. Reprod Domest Anim 43(s5): 20–28
17. Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth H-J (2009) Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 81(6):1025–1032
18. Yan T, Mayne CS, Keady TWJ, Agnew RE (2006) Effects of dairy cow genotype with two planes of nutrition on energy

partitioning between milk and body tissue. J Dairy Sci 89(3):1031–1042
19. Wathes DC, Fenwick M, Cheng Z, Bourne N, Llewellyn S, Morris DG, Kenny D, Murphy J, Fitzpatrick R (2007) Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. Theriogenology 68:S232–S241
20. von Borell E, Dobson H, Prunier A (2007) Stress, behaviour and reproductive performance in female cattle and pigs. Horm Behav 52(1):130–138
21. Moberg GP (1991) How behavioral stress disrupts the endocrine control of reproduction in domestic animals. J Dairy Sci 74(1):304–311
22. Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Hammer CJ, Maddock Carlin KR, Grazul-Bilska AT, Redmer DA (2010) Developmental programming: the concept, large animal models, and the key role of uteroplacental vascular development. J Anim Sci 88(13 electronic suppl):E61–E72
23. Roelofs J, López-Gatius F, Hunter RHF, van Eerdenburg FJCM, Hanzen C (2010) When is a cow in estrus? Clinical and practical aspects. Theriogenology 74(3):327–344
24. Hansen PJ, Aréchiga CF (1999) Strategies for managing reproduction in the heat-stressed dairy cow. J Anim Sci 77(E-Suppl 2): 36–50
25. Roth Z (2008) Heat stress, the follicle, and its enclosed oocyte: mechanisms and potential strategies to improve fertility in dairy cows. Reprod Domest Anim 43:238–244
26. Perera B (2008) Reproduction in domestic buffalo. Reprod Domest Anim 43:200–206
27. Presicce GA (2007) Reproduction in the water buffalo. Reprod Domest Anim 42:24–32
28. Baruselli PS, Reis EL, Marques MO, Nasser LF, Bó GA (2004) The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. Anim Reprod Sci 82:479–486
29. Renema R, Robinson F, Proudman J, Newcombe M, McKay R (1999) Effects of body weight and feed allocation during sexual maturation in broiler breeder hens. 2. Ovarian morphology and plasma hormone profiles. Poult Sci 78(5):629–639
30. Mench JA (2002) Broiler breeders: feed restriction and welfare. World Poult Sci J 58:23–29
31. Donadeu FX, Watson ED (2007) Seasonal changes in ovarian activity: lessons learnt from the horse. Anim Reprod Sci 100 (3–4):225–242
32. Thatcher WW, Moreira F, Santos JEP, Mattos RC, Lopes FL, Pancarci SM, Risco CA (2001) Effects of hormonal treatments on reproductive performance and embryo production. Theriogenology 55(1):75–89
33. Bó GA, Baruselli PS, Chesta PM, Martins CM (2006) The timing of ovulation and insemination schedules in superstimulated cattle. Theriogenology 65(1):89–101
34. De Rensis F, López-Gatius F, García-Ispierto I, Techakumpu M (2010) Clinical use of human chorionic gonadotropin in dairy cows: an update. Theriogenology 73(8):1001–1008
35. Coleman J, Pierce KM, Berry DP, Brennan A, Horan B (2009) The influence of genetic selection and feed system on the reproductive performance of spring-calving dairy cows within future pasture-based production systems. J Dairy Sci 92(10):5258–5269
36. Garnsworthy PC, Fouladi-Nashta AA, Mann GE, Sinclair KD, Webb R (2009) Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. Reproduction 137(4):759–768
37. Cui X, Ji D, Fisher DA, Wu Y, Briner DM, Weinstein EJ (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 29(1):64–67
38. Whyte JJ, Zhao J, Wells KD, Samuel MS, Whitworth KM, Walters EM, Laughlin MH, Prather RS (2011) Gene targeting with zinc finger nucleases to produce cloned eGFP knockout pigs. Mol Reprod Dev 78(1):2
39. Yang D, Yang H, Li W, Zhao B, Ouyang Z, Liu Z, Zhao Y, Fan N, Song J, Tian J, Li F, Zhang J, Chang L, Pei D, Chen YE, Lai L (2011) Generation of PPAR[gamma] mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. Cell Res 21(6):979–982
40. Roy A, Matzuk MM (2006) Deconstructing mammalian reproduction: using knockouts to define fertility pathways. Reproduction 131(2):207–219
41. Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15(6):854–866
42. Vasudevan K, Raber J, Sztein J (2010) Fertility comparison between wild type and transgenic mice by in vitro fertilization. Transgenic Res 19(4):587–594
43. McNatty KP, Smith P, Moore LG, Reader K, Lun S, Hanrahan JP, Groome NP, Laitinen M, Ritvos O, Juengel JL (2005) Oocyte-expressed genes affecting ovulation rate. Mol Cell Endocrinol 234(1–2):57–66
44. Souza CJH, GonzÃ¡lez-Bulnes A, Campbell BK, McNeilly AS, Baird DT (2004) Mechanisms of action of the principal prolific genes and their application to sheep production. Reprod Fertil Dev 16(4):395–401
45. Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004) The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25(1):72–101
46. Juengel JL, Hudson NL, Berg M, Hamel K, Smith P, Lawrence SB, Whiting L, McNatty KP (2009) Effects of active immunization against growth differentiation factor 9 and/or bone morphogenetic protein 15 on ovarian function in cattle. Reproduction 138(1):107–114
47. Knight PG, Glister C (2006) TGF-{beta} superfamily members and ovarian follicle development. Reproduction 132(2): 191–206
48. Yavas Y, Wallon JS (2000) Induction of ovulation in postpartum suckled beef cows: a review. Theriogenology 54(1):1–23
49. Juengel JL, O'Connell AR, French MC, Proctor LE, Wheeler R, Farquhar PA, Dodds KG, Galloway SM, Johnstone PD, Davis GH (2011) Identification of a line of sheep carrying a putative autosomal gene increasing ovulation rate in sheep that does

not appear to interact with mutations in the TGFB superfamily. Biol Reprod 85(1):113–120

50. Onteru SK, Ross JW, Rothschild MF (2009) The role of gene discovery, QTL analyses and gene expression in reproductive traits in the pig. Soc Reprod Fertil Suppl 66:87–102
51. Rothschild M, Jacobson C, Vaske D, Tuggle C, Wang L, Short T, Eckardt G, Sasaki S, Vincent A, McLaren D, Southwood O, van der Steen H, Mileham A, Plastow G (1996) The estrogen receptor locus is associated with a major gene influencing litter size in pigs. Proc Natl Acad Sci USA 93(1):201–205
52. Véron N, Bauer H, Weiße AY, Lüder G, Werber M, Herrmann BG (2009) Retention of gene products in syncytial spermatids promotes non-Mendelian inheritance as revealed by the t complex responder. Genes Dev 23(23):2705–2710
53. Kambadur R, Sharma M, Smith TPL, Bass JJ (1997) Mutations in myostatin (GDF8) in Double-Muscled Belgian Blue and Piedmontese Cattle. Genome Res 7(9):910–915
54. Pirottin D, Grobet L, Adamantidis A, Farnir F, Herens C, Schrøder HD, Georges M (2005) Transgenic engineering of male-specific muscular hypertrophy. Proc Natl Acad Sci USA 102(18):6413–6418
55. Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K (2010) Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Mol Hum Reprod 16(3):135–152
56. Dardente H, Wyse CA, Birnie MJ, Dupré SM, Loudon ASI, Lincoln GA, Hazlerigg DG (2010) A molecular switch for photoperiod responsiveness in mammals. Curr Biol 20(24): 2193–2198
57. Gordon J, Ruddle F (1981) Integration and stable germ line transmission of genes injected into mouse pronuclei. Science 214(4526):1244–1246
58. Fox JL (2010) Transgenic salmon inches toward finish line. Nat Biotechnol 28(11):1141–1142

Transgenics: Alternative Gene Transfer Methods

Louis-Marie Houdebine
Biologie du Développement et Reproduction, Institut National de le Recherche Agronomique, Jouy en Josas, France

Article Outline

Glossary

Animal cloning Non-sexual reproduction of an animal by transfer of a nucleus from a differentiated cell into the cytoplasm of an enucleated oocyte; SCNT (somatic cell nuclear transfer): cloning using a somatic cell as nuclear donor.

Chimeric animal Animal containing cells from two animals; chimeras are obtained by transferring embryonic cells from an animal into a recipient embryo; the chimera gametes contain the genome from one of the two embryos.

Hybrid Animal resulting from the sexual reproduction of parents from different breeds (intraspecies hybrid) or parents from different species (interspecies hybrid).

Knockdown Inhibition of the expression of a gene at the mRNA level by a siRNA.

Knockin Targeted integration of a gene by homologous recombination.

Knockout Inactivation of a gene by homologous recombination.

Lentiviral vector Gene construction able to integrate a foreign gene into a genome via an infection mechanism.

Meganuclease Endonuclease that is able to cut both strands of DNA and enhance the efficiency of homologous recombination.

Pluripotent cell Cell that is able to participate in the development of all the organs; lines of pluripotent cells can be established from early embryos (ES: embryonic stem cells) or from somatic cells (iPS: induced pluripotent cells) obtained by the transfer of genes responsible for the pluripotency of embryonic cells.

siRNA Small interfering RNA (also known as RNAi) that is able to inactivate specifically an mRNA by its degradation or by the reversible inhibition of its translation; siRNAs are generated by the degradation of long double-stranded RNAs, by the transcription of micro-RNA genes, by the transcription of gene constructions coding for shRNAs (small hairpin RNAs), or by chemical synthesis.

Transgenesis Experimental transfer of an isolated gene (or a DNA fragment of any origin) to animal cells making the transmission of the genetic modification to progeny by sexual reproduction possible. The animals harboring the foreign genes are known as transgenic animals, transgenics, GM animals (genetically modified animals), rDNA animals (recombinant DNA animals), or GE animals (genetically engineered animals).

Zinc finger nuclease (ZFN) Engineered nucleases that are able to cleave both strands of genomic DNA in specific sites. ZFNs as meganucleases are able to induce a DNA repair and a local mutation equivalent to a knockout in the absence of foreign DNA or targeted gene integration at a high efficiency in the presence of a homologous recombination vector.

Definition of the Subject

Various techniques and mainly gene cloning, genome sequencing as well as transcriptomics provide researchers with an increasing number of genes. In order to know the role of the genes and the mechanisms of their regulation, it is mandatory to reintroduce them into their natural complex environment, cells, and animals. Transgenesis has thus become a major tool for biologists, and presently, at least 90% of GM animals are generated for basic studies. Transgenesis is not only a tool for research; it is also more and more extensively used for various biotechnological projects in the traditional fields of biology applications: medicine and agriculture.

P. Christou et al. (eds.), *Sustainable Food Production*, DOI 10.1007/978-1-4614-5797-8,

Originally published in
Robert A. Meyers (ed.) Encyclopedia of Sustainability Science and Technology, © 2012, DOI 10.1007/978-1-4419-0851-3

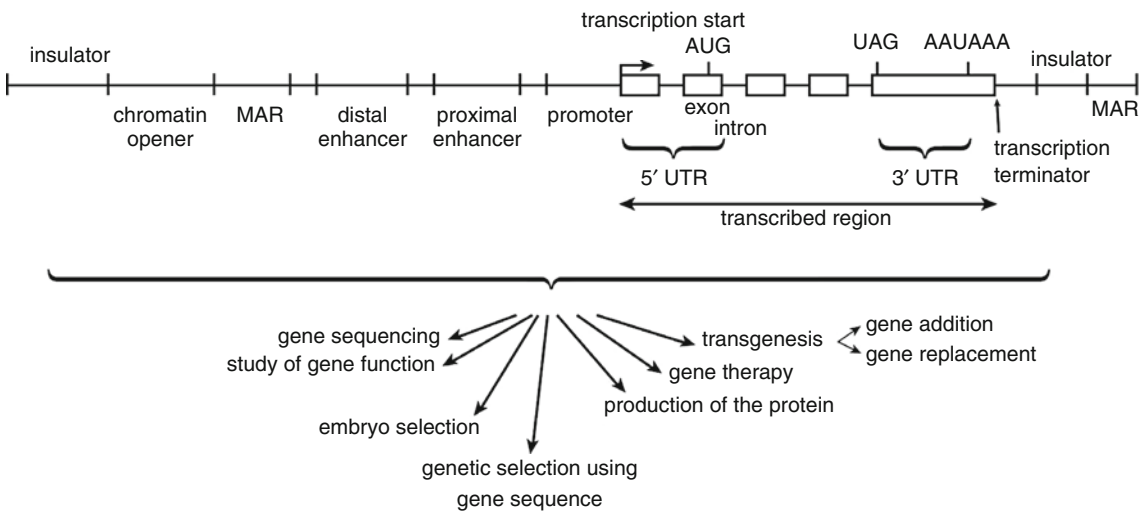
The information provided by the understanding of the biological functions of humans and animals may give clue for the design of new treatments for patients and selection of farm animals, respectively. Transgenesis has thus become a key tool for the creation of animal models for the study of human diseases. These studies may even provide researchers with genetic markers for diagnosis and genetic selection. The newly identified and studied genes may be used to produce small quantity of the corresponding recombinant proteins to establish relations between their structure and their activity. Very large quantity of recombinant proteins of pharmaceutical interest may also be produced in milk or egg white of transgenic animals. Pig cells and organs may in principle be genetically modified to become tolerated by patients. Some newly identified genes may as well be transferred to farm animal to improve production as it is already the case in plants [112](Fig. 1).

The available techniques make it possible the addition of exogenous genes and the elimination of genetic information in various animal genomes. Several more and more sophisticated tools allow also a fine-tuning of transgene expression. Although the generation of transgenic animals is no more a bottleneck for their different use, it remains laborious and costly especially in large animals. Some improvements of the methods are required and in course. The methods of gene transfer are closely linked to the reproduction techniques and to the aim of the projects. These methods, which include the construction of the genes to be transferred and which are adapted to the different animal species are described in the present chapter.

Introduction

By essence, living organisms are in permanent evolution. This phenomenon is relatively slow, and it was probably not perceived by humans until they invented agriculture and breeding. The control of plant and animal reproduction made the empirical genetic selection possible which provided to human communities all their essential food products, pets, and ornamental plants. This led to the generation of profoundly genetically modified organisms. Carrots, tomatoes, silkworm, some dogs, etc. are unable to survive without the assistance of humans.

The discovery of the Mendel laws allowed an improvement of the genetic selection. Yet, this selection remained based on spontaneous, thus random and unknown, mutations. During the first half of the last century, it appeared necessary and possible to increase the number of random mutations to enlarge the choice of the genetically modified organisms corresponding to

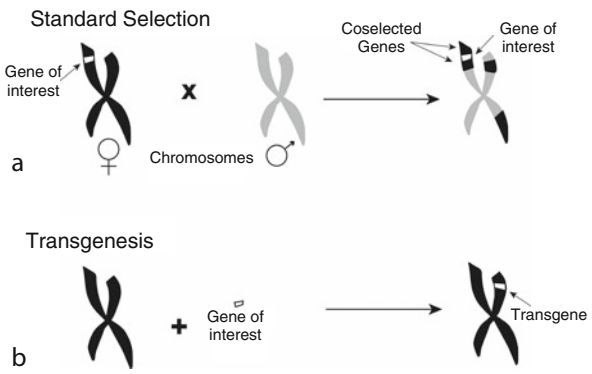


Transgenics: Alternative Gene Transfer Methods. Figure 1

The different uses of isolated genes. Transgenesis, which includes random and targeted gene addition as well as specific gene inactivation and replacement is an essential tool for gene study and for biotechnological applications

the expectations of experimenters, farmers, and breeders. This was achieved by using chemical mutagens and by generating multiple intraspecies and interspecies hybrids. One of the most impressive examples is the creation of a new cereal, triticale, which results from an artificial crossing between wheat and rye. This new plant is currently a source of feed.

All these methods are imprecise as they induce multiple unknown mutations in addition to those which are expected. One of the problems of conventional genetic selection, either to create models or to improve production, is that the selected genes are unknown and that a large number of genes also unknown are co-selected with the genes of interest (Fig. 2). Yet, these approaches were globally highly beneficial for humans. They also show that the plasticity of living organisms is high and that humans have empirically learned to manipulate them successfully with limited undesirable side effects. The discovery of DNA and genes opened wide avenues for research and biotechnological applications. Indeed, the manipulation of isolated and known genes makes it possible more diverse and better controlled genetic modifications (Fig. 2).

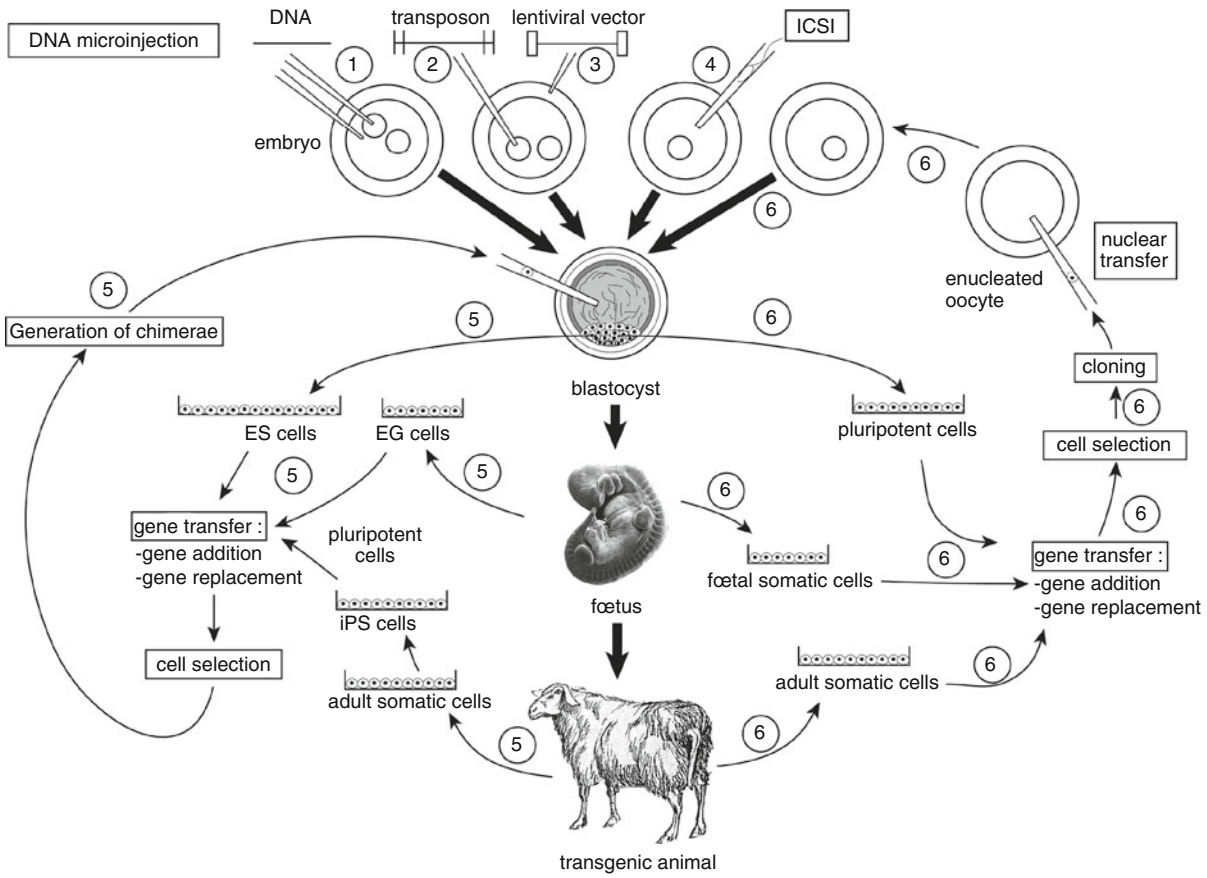


Transgenics: Alternative Gene Transfer Methods.
Figure 2
(**a**) Conventional genetic selection relies on the random chromosome rearrangement during sexual reproduction and, thus, to the random distribution of the different gene versions in offspring. The gene of interest responsible for the expression of a valuable genetic trait, and which is selected, is then unknown and the process implies the co-selection of a number of unknown potentially deleterious genes surrounding the gene of interest. (**b**) Selection by gene transfer offers the possibility to add (or delete) a single gene having a known function into a living organism. This gene may be of various origins and may be optimized before being transferred

The introduction of isolated genes into cells has become a common practise in the 1970s of the last century, soon after the emergence of the genetic engineering techniques. It represented a great progress for the understanding of gene functions and mechanisms of action. This technique is still widely used, and it started being complemented in 1980 and 1983 by gene transfer into animals and plants, respectively, to generate lines of genetically modified organisms, also known as transgenic animals and plants.

The first transgenic animals, mice, were obtained by microinjecting the genes into one of the nuclei (pronuclei) of 1-day embryos [31]. This method could be extrapolated successfully to three other mammals (rabbits, pigs, and sheep) in 1985 [32], but it soon appeared that other methods had to be implemented for some species. Transgenesis is presently carried out essentially for basic research in only a few species: a mammal (mice), an insect (drosophila), fish (medaka and zebra fish), and a worm (*Caenorhabditis elegans*). Some farm animals (rabbits, pigs, chicken, sheep, goat, and cow) are being used for specific studies, which cannot be performed for biological reasons with laboratory animals. Essentially, the products from some farm animals are expected to be improved by transgenesis in addition to classical genetic selection in the coming decades.

Several techniques to generate transgenic animals have made considerable progress in the past decade: (1) direct gene transfer into embryos either by microinjection of DNA, transposons, or lentiviral vectors or via sperm incubated with DNA and transferred to oocytes by ICSI (intracytoplasmic sperm injection), (2) via intermediate cells in which the gene modifications have been achieved and further used to generate animals harboring the foreign DNA sequence; these cells are either pluripotent cells that are able to give birth to chimeric transgenic animals ES (embryonic stem cells) in mice as well as iPS (induced pluripotent cells obtained after dedifferentiation of somatic cells) potentially in most species or somatic cells used for generating transgenic clones (Fig. 3).



Transgenics: Alternative Gene Transfer Methods. Figure 3

Different methods to generate transgenic animals: (1) DNA transfer via direct microinjection into pronucleus or cytoplasm of embryo; (2) DNA transfer via a transposon: The foreign gene is introduced into the transposon, which is injected into a pronucleus; (3) DNA transfer via a lentiviral vector: The gene of interest introduced in a lentiviral vector is injected between the zona pellucida and membrane of the oocyte or the embryo; (4) DNA transfer via sperm: Sperm is incubated with the foreign gene and injected into the oocyte cytoplasm for fertilization by ICSI (intracytoplamic sperm injection); (5) DNA transfer via pluripotent or multipotent cells: The foreign gene is introduced into pluripotent cell lines (ES, embryonic stem cells: lines established from early embryo or iPS: cells obtained after dedifferentiation of somatic cells) or into multipotent cell lines (EG, gonad cells lines established from primordial germ cells of fetal gonads); the pluripotent cells containing the foreign gene are injected into an early embryo to generate chimeric animals harboring the foreign gene DNA; the multipotent EG cells containing the foreign gene are injected into embryos to generate gametes harboring the transgene; in both cases, the transgene is transmitted to progeny; (6) DNA transfer via cloning: The foreign gene is transferred into a somatic cell, the nucleus of which is introduced into the cytoplasm of an enucleated oocyte to generate a transgenic clone. Methods 1, 2, 3, and 4 allow random gene addition whereas methods 5 and 6 allow random gene addition and targeted gene integration via homologous recombination for gene addition or gene replacement including gene knockout and knockin

Another problem emerged rapidly. The very first transgenes were expressed, and they were able, in some cases, to induce some phenotypic modifications in animals. The first example was the giant mice obtained in 1982 [71] by overexpressing growth hormone genes. It also appeared that the expression of the transgenes was often not satisfactory and not easily controlled. This was clearly due to the insufficient knowledge of the

mechanisms controlling gene expression. Thus, for years, only empirical gene constructions having sometimes limited efficiency were used. The strategy of researchers was and often still is to generate several lines of transgenic mice (or other species) and to keep only those in which the transgene is expressed as expected. This strategy appeared insufficient when costly large transgenic animals were to be used and when finely tuned transgene expression was needed. Different methods to express transgenes in a well-controlled manner were found: (1) gene targeting by homologous recombination [8] improved or not by a genomic DNA cleavage locally induced by engineered meganucleases or zinc finger nucleases, leading either to gene knockin or knockout by a gene replacement as well as by NHEJ (non-homologous end joining), (2) conditional knockout using Cre recombinase gene controlled by exogenous inducers such as doxycycline and a engineered Cre recombinase activated by 4-hydroxy tamoxifen, (3) inactivation of mRNA by knockdown with siRNA derived from transgenes coding for shRNA or miRNA, (4) overexpression of negative transdominant proteins inactivating the genes at the protein level.

The failure of transgene expression raised fundamental questions on gene mechanism of action to experimenters. This is particularly the case for the discovery of remote regulatory elements globally known as insulators. Indeed, as opposed to bacteria, yeast, and plants, essential transcription regulators are spread over long genomic DNA regions in animals and, namely, in higher vertebrates [53]. Interestingly, answers were given to some of these questions, thanks to transgenesis and transgenesis efficiency was improved, thanks to these discoveries. Despite very significant improvement of several transgenesis methods, the efficiency of gene transfer and the control of transgene expression remain limiting factors for the optimal use of transgenic animals for research as well as for biotechnological applications. Important improvements of these methods are in course. The more efficient of gene targeting via the action of meganucleases and zinc finger endonucleases is a case in point.

Methods of Gene Transfer

Gene Transfer into Cells

Spontaneous gene transfer into cells occurs only marginally. Different techniques are being used to reach this goal. A group of techniques relies on artificial physicochemical or mechanical processes whereas another group utilizes natural mechanisms of gene transfer, essentially infection. Transfection implies the association of DNA with molecules, which have the capacity to bind cell plasma membrane and to internalize the complex into the cytoplasm. The endocytosis process includes the transfer of the internalized material into lysosomes where it is processed or degraded. A proportion of non-degraded DNA is more or less randomly recombined. A part of the DNA is then transferred to the nucleus, and the genes it contains may be transiently transcribed. When cells do not divide, the DNA may stay several days in the nucleus. During cell division, most part of the foreign DNA is degraded and in a small percentage of cells, the foreign DNA becomes integrated into the host genome either randomly or in a targeted manner according to the sequence of the transferred DNA. A number of molecules are available, and new ones are regularly proposed to enhance the efficiency of cell transfection. These molecules may increase the endocytotic process according to the cell type, diminish the degradation of DNA, favor the transfer of DNA or the nucleus, or be less cytotoxic.

An alternative to transfection is electroporation. The method consists of incubating cells in the presence of DNA and to submit them to pulses of electric field, which generate transient pores in plasma membrane allowing the uptake of DNA. This technique is relatively efficient according to the cell type to obtain stable cells harboring a foreign gene, but many cells do not survive after the electric pulses. The fate of DNA in cells is essentially similar to this following transfection.

It is possible to select the cells in which the foreign DNA is stably integrated into the genome. To reach easily this goal, one popular way consists of co-transfecting a selection gene with the gene of interest. The selection gene may be independent of the gene of interest. Random recombination may then associate the two genes in the same DNA fragment, making the selection of the cells harboring the two co-integrated genes possible. Alternatively, the selection gene may have been associated to the gene of interest in a specific construction. The co-integration of the two genes is then more frequent, and the selection of the cells harboring the gene of interest is easier.

One possible drawback of this protocol is that the selection gene remains present in the cell.

The most efficient classical way to transfer gene into cells is the direct DNA microinjection in the cytoplasm or the nucleus. This method is relatively laborious, requires specific material, and a specific training. Moreover, gene transfer can then be achieved only in a limited number of cells.

Natural mechanisms of gene transfer are being implemented to transfer foreign DNA into cells. Some molecules are naturally able to transfer DNA from the outside of the cells to their nucleus. Lactoferrin was shown to have this property, but this mechanism seems insufficiently efficient to be used. The natural capacity of viruses to transfer their genes into cells by infection mechanisms has been retained to design viral vectors carrying the foreign genes. Such vectors have been studied and used during the last two decades with limited success for gene therapy. A few types of viruses have been retained to be used for gene therapy and those which transfer their genes into the cell genome are being implemented to generate transgenic animals. These vectors rely on the use of retroviral genomes, which must be integrated into the infected cells to be replicated and able to synthesize infectious viral particles.

Gene Transfer into Embryo

To generate transgenic animals, the foreign gene must be present and integrated into the genome of the embryos at the one-cell stage to allow its transmission into all the cells of the animal or at least of their gametes. The simplest theoretical way to reach this goal is to transfer the foreign gene directly into the embryo. In practice, this strategy is not always efficient. Indeed, embryos are rare and costly cells especially in large farm animals. In species in which gametes are very abundant, the direct gene transfer using biolistics has met success in fish and marine invertebrates [63] and insects [18]. In practice, this method consists of incubating DNA with gold or platinum. The DNA-coated particles are then launched through the membrane of the embryos.

Hence, only the highly efficient methods of gene transfer may be successful in these conditions even if only random gene integration is needed. The targeted integration of foreign DNA is a rare event as it is based on a homologous recombination between the host gene and the vector. In these situations, even the most efficient classical techniques of gene transfer are unable to induce a gene targeting at an acceptable rate.

The genetic modifications must then be achieved in cultured cells, which must have the capacity to participate in the development of the embryos and be able to transmit the genetic modification to progeny. Pluripotent cells (ES cells or iPS) may, in the best cases, participate in the generation of chimeric animals in which a significant proportion of the gametes harbor the foreign DNA. Particular multipotent cells, the primordial germ cells (EG cells) in birds can participate in the formation of genetically modified gametes and in the generation of transgenic animals. The animal cloning technique is another possibility. The genetic modification is then performed in somatic cells that are further used to generate transgenic clones by nuclear transfer.

These different methods to generate transgenic animals are schematically represented in Fig. 3.

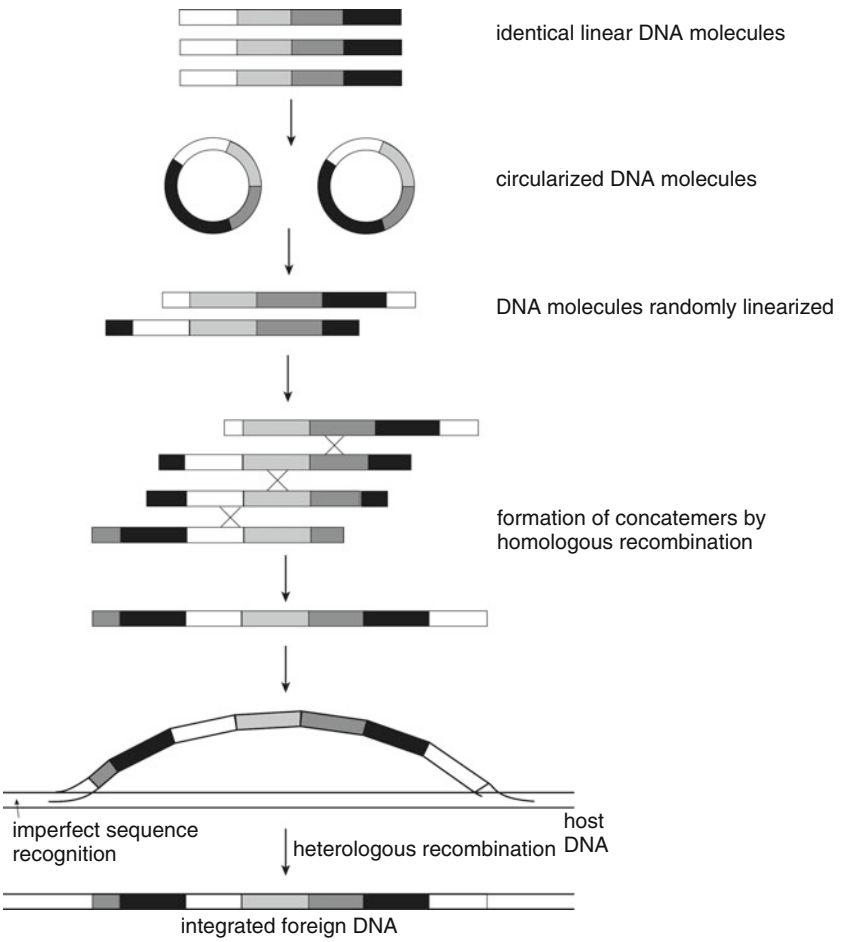
Mechanisms of Gene Integration

Random Integration The foreign DNA introduced into the cytoplasm by any method recombines according to a random process, leading to the formation of multimers known as concatemers including gene rearrangement and mutations. The different copies of the gene are then organized in head to tail or in tandem. When the foreign DNA is introduced directly into the nucleus (essentially by microinjection), a polymerization also occurs but following a process of homologous recombination generating concatemers organized essentially in tandem with limited mutations and rearrangements. The integration of foreign DNA fragment into a genome may occur by two different mechanisms. The most frequent process is considered as leading to a random integration. Targeted integration is much less frequent.

In both cases, the foreign DNA is progressively degraded by the cell. This includes the action of exonucleases, which generate randomly single-stranded sequences in both ends of the foreign DNA. These sequences can recognize complementary regions in the genome, leading to the formation of hybrids. During the cellular DNA replication, these abnormal

structures are corrected by the repair mechanisms of the cell. This leads to the elimination of the foreign DNA or to its integration in the genome [3] (Fig. 4). This process implementing a heterologous recombination is considered as random since the recognition of the host DNA site depends on the action of the exonucleases. The different lines of transgenic animals obtained in this way are thus all different from each other. They contain variable copy number of the transgene integrated each time at a different site. The integration of the foreign gene may damage locally the host DNA. Moreover, the transgene is then frequently submitted to the unpredictable and unknown effects of the transcription regulatory elements located in its vicinity. The regulatory elements of the transgene may also alter the transcription of the host genes in its vicinity.

The integration of the foreign DNA by these methods is thus not controlled but likely not completely random as it occurs more frequently in regions containing genes and thus having an open chromatin structure, favoring the access of the foreign DNA to the host DNA. The integration site may be known by sequencing the flanking regions of the transgene.



Transgenics: Alternative Gene Transfer Methods. Figure 4
Mechanism of random DNA integration. The ends of the foreign DNA are partially digested generating single-stranded sequences, which randomly recognize complementary host DNA sequences and provoke the integration of the foreign DNA

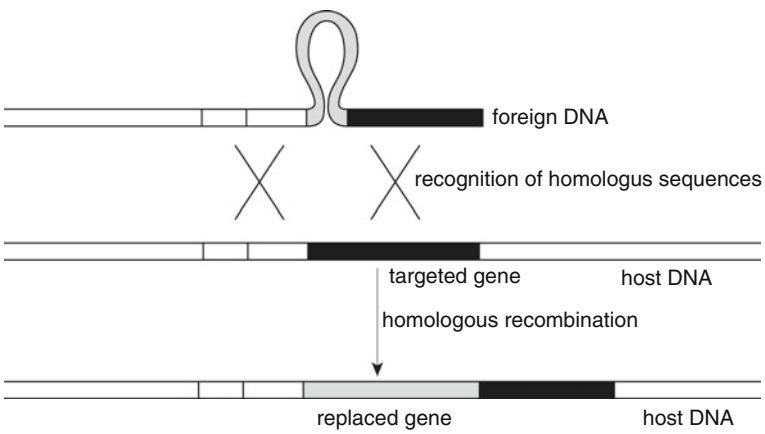
A systematic study revealed that transgenic mice heterozygous for the transgene show rarely abnormalities. On the contrary, the homozygous mice appear altered in a proportion as high as 3–10%, suggesting that the uncontrolled integration of the foreign DNA is often mutagenic [103, 104].

Targeted Integration To avoid the side effects of the random integration, it is possible to target the integration of the foreign gene using homologous recombination. This mechanism is based on the recognition between a genome sequence and the sequence of the exogenous DNA. This recognition leads to the formation of hybrid and finally by the precise replacement of the endogenous gene by the exogenous DNA (Fig. 5). Homologous recombination exists in all living organisms. It is implemented to repair mutated genes using the other allele as a matrix, to redistribute the regions of homologous chromosomes during the formation of gametes, and to generate functional antibody genes from parent genes. Homologous recombination is routinely used to genetically modify bacteria and yeast. Homologous recombination is a rare event corresponding to about 0.1–1% of the heterologous recombination. It is therefore not implemented directly in early embryos but in intermediate cells further used to generate transgenic animals.

Homologous recombination allows in practice the replacement of a given gene by an exogenous DNA fragment. Several applications of this approach are possible: (1) the replacement of a gene by a non-functional DNA sequence, leading to the inactivation of the targeted gene known as gene knockout (Fig. 5), (2) the targeting of a foreign gene into a given region of the genome or the replacement of an allele by another allele known as knockin.

DNA Microinjection

About 1,000 copies of the isolated foreign gene contained in 1–2 pl may be injected into one of the pronuclei of 1-day mammal embryos. This method implies a superovulation of the females followed by a mating with a male to obtain an optimum number of embryos. The resulting one-cell embryos are collected the next day and microinjected with DNA. The embryos are then transferred to hormonally prepared recipient females using surgery operations. The yield of this method in mice is of 1–3 of transgenics for 100 microinjected and transferred embryos. This rate is relatively low, and this is due to a large extent to the fact that only 50% of the embryos survive after the microinjection. This is clearly due to the mutagenic effect of DNA as the survival of



Transgenics: Alternative Gene Transfer Methods. Figure 5
Mechanism of targeted integration. An exogenous DNA having a sequence of several kb similar to that of a host gene may replace the host gene using a homologous recombination process. If a non-homologous sequence is surrounded by two homologous sequences, the two independent homologous recombinations direct the integration of the non-homologous sequence, leading to the knockout of the targeted gene

embryos is high when the buffer without DNA is injected. In fact, 1,000 copies of the gene correspond to a very large excess of DNA in the cell, making the multiple random deleterious integrations and genome rearrangements depicted above possible. Despite its drawback and the fact that it is laborious, this technique is still the most frequently used in mice and rabbits. The efficiency of DNA microinjection is lower in all the other mammalian species and very low in ruminants. This is not due to the difficulty to inject DNA but to the fact that the mechanism of integration is for unknown reasons much less efficient in some species.

The DNA microinjection in pronuclei gives birth to at least 30% of mice mosaic for the transgene. The transgene is then not present in all the cells of the transgenic founder. This is due to the fact that the integration of the foreign DNA occurs sometimes not in the first cell but later at the two- or four-cell stage. The transmission of the transgene from these founders appears not to respect the Mendel law. The transmission rate is due to the fact that the transgene is not present in all the gametes. At the next generation, the proportion of transgenics is Mendelian [24]. About 1% of transgenic founders do not transmit their transgene as the mosaicism is very high and the transgene is rare or non-existent in gametes.

In non-mammalian species, the pronuclei cannot be visualized as the embryo is embedded in an abundant and opaque vitellus. High amounts of DNA (millions of copies in a few nanoliters) must then be injected into the cytoplasm of the one-cell embryos. This relatively simple technique is efficient in several fish species [42, 56] but highly inefficient in chicken, in Xenopus, in some fish, and in insects. For unknown reasons, the integration of the foreign DNA thus does not occur in some species. In lower vertebrates and invertebrates, the embryo DNA and the foreign DNA are replicated very rapidly. The foreign DNA is then highly amplified leading to multiple independent integrations occurring during the first days of embryo development in the same animals. The resulting transgenic animals are often very mosaic making it difficult their use. Several reproduction cycles allow a segregation of the different transgenes until the animals contain a single integration site.

In a *C. elegans*, direct DNA injection into gonad syncytium leads to the generation of transgenics with a good yield [98].

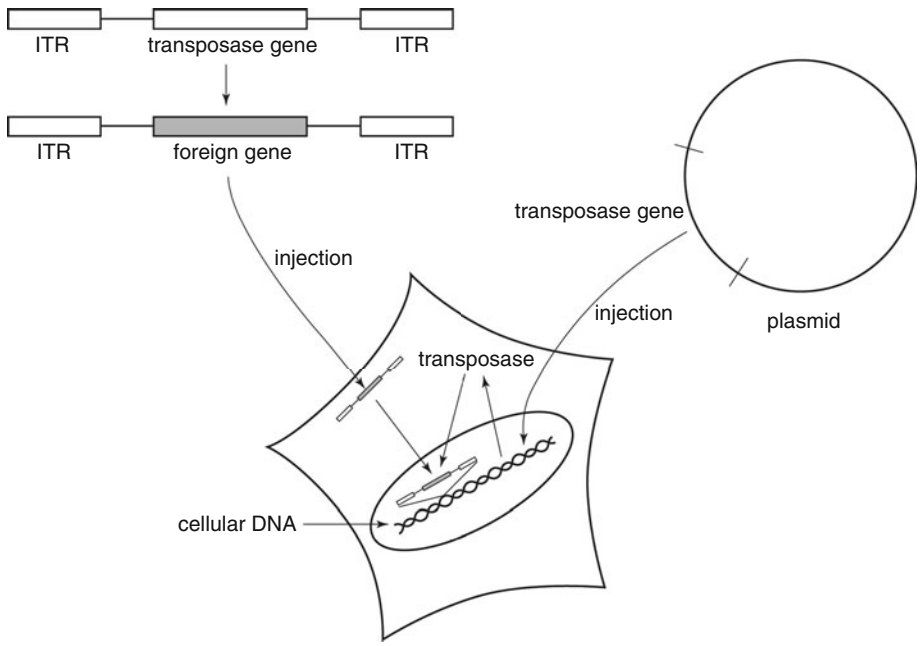
Gene constructs bordered by I-Sce1 sites have been used to generate transgenic fish. The plasmids were previously cut by the I-Sce1, and the fraction was injected without any previous DNA purification. Unexpectedly, this protocol markedly increased the yield of transgenesis. Interestingly, the same observation was made in Xenopus. The mechanism improving transgenesis is not known [97]. The I-Sce1 might protect DNA against degradation, favor DNA transfer to the nucleus, or directly stimulate the integration mechanism.

Microinjection is therefore a good technique but insufficient in some species. Additional methods have been found and are still under study.

Detection of the transgene and examination of its integrity can be achieved using Southern blotting or PCR using multiple primers corresponding to various regions of the transgene. Zygocity of the transgene may be evaluated by quantitative PCR [69] or in situ hybridization.

Use of Transposons

Transposons are short genomic DNA regions, which are replicated and randomly integrated into the same genome. The number of a given transposon is thus increasing until the cell blocks this process to protect itself from the degradation of its genome. Foreign genes can be introduced into transposons in vitro. The recombinant transposons may then be microinjected into 1-day embryos as it is for DNA. A transposon contains a gene coding for an integrase specific of this type of transposon and required for the integration of the transposon. The integrase gene is bordered by two inverted terminal repeat regions (ITR) required for the transposon integration. The addition of the foreign DNA implies the elimination of the integrase gene. This makes space for the foreign DNA, and it also prevents the recombinant transposon to disseminate in the genome. To allow the integration of the recombinant transposon, the integrase protein or a gene construction directing the synthesis of the integrase must be co-injected with the recombinant transposon (Fig. 6).



Transgenics: Alternative Gene Transfer Methods. Figure 6

Gene transfer using a transposon. The integrase gene of a transposon is replaced by the gene of interest between the two ITR (inverted terminal repeats) required with the integrase for an efficient integration. Integrase or a gene coding for this protein must be injected with the transposon to allow its integration

The foreign gene thus becomes integrated into the embryos with a yield of about 1%. Essentially, all the transgenic insects are generated by using transposons as vectors [43, 95]. Transposons also proved efficient to generate transgenic fish, chicken, and mammals [22, 23]. The transposons used for transgenesis are chosen or engineered to be unable to disseminate in the host genome under the action of the integrase of endogenous transposons. Transposons are therefore efficient and safe tools, but they can harbor no more than 2–3 kb of foreign DNA.

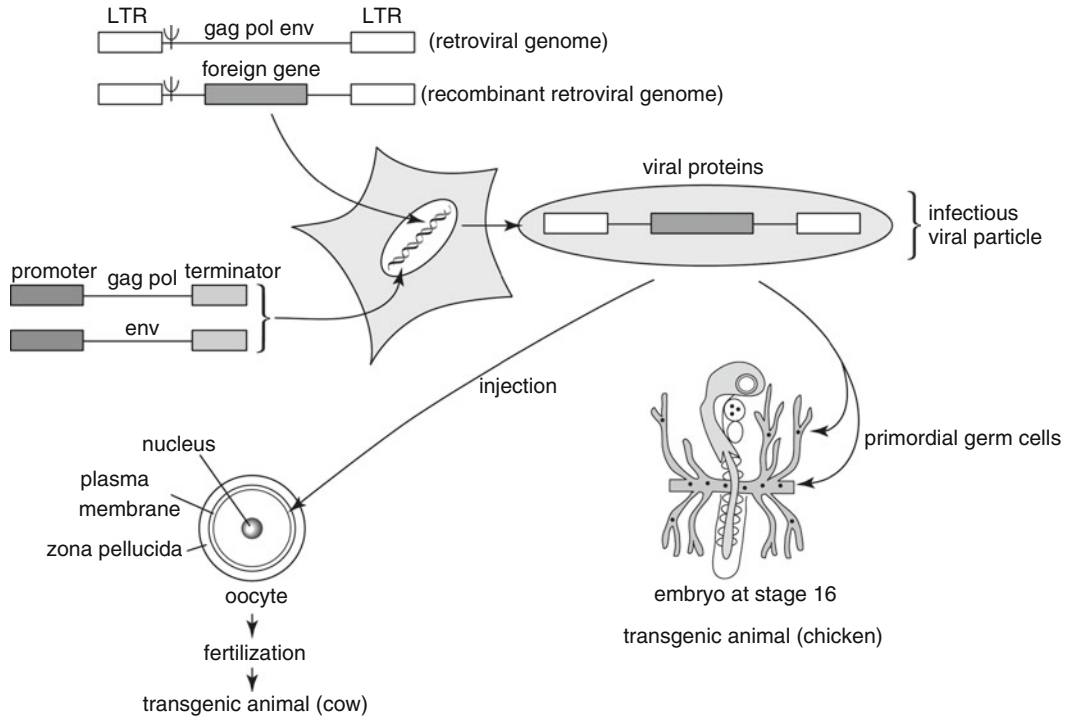
Transposons are being used with ICSI giving a high transgenensis rate [64].

Efficient transposons started being used to disseminate in genomes after the addition of the corresponding integrase into cells. This leads to multiple random integrations of the transposon providing researchers with insertional mutants. A correlation may then be established between a biological alteration of the animals and the nature of the gene inactivated by the presence of the transposon within the gene [22].

Use of Lentiviral Vectors

Retroviruses have not the capacity to autoreplicate, and they must be integrated stably into the genome of the cells they infected to replicate. This explains why up to 1% of animal genomes contain degenerated retroviral genes. This property of retroviruses is implemented to integrate foreign genes in cells, in animals, and in patient's somatic cells. For this purpose, the viral genes are removed from the genome of lentiviruses (a category of retroviruses) and replaced by the genes of interest. Viral particles are then prepared and used to transfer the foreign genes into oocytes or one-cell embryos (Fig. 7). Retroviral vectors have been studied and used during the last two decades for gene therapy. Important improvements of these vectors have markedly enhanced their efficiency. Vectors using ALV (avian leucosis virus) studied two decades ago proved poorly efficient to generate transgenic chicken [4, 83].

The lentiviruses have the advantage over the common retroviruses to be able to infect and transfer their genes in quiescent as well as in multiplying cells. The



Transgenics: Alternative Gene Transfer Methods. Figure 7

Gene transfer using lentiviral vectors. The pathogenic genes are removed from the HIV genome and replaced by the gene of interest. The gene construction is transfected into trans-complementing cells synthesizing the essential HIV proteins required for the formation of infectious recombinant particles. The particles secreted from the cells in the culture medium are injected between the zona pellucida and the membrane of the cell embryos or of oocytes or within the fetal gonad in chicken. The infection is followed by an efficient integration of the gene of interest

majority of the retrovirus genomes need a cell multiplication including a transient degradation of the nuclear membrane to reach chromatin and integrate into the cell genome. On the contrary, lentiviral viruses contain proteins carrying their genome to the nucleus in quiescent as well as in multiplying cells. EIAV (equine infectious anemia virus) proved that it is able to generate transgenic chicken [60]. Presently, the most frequently used lentiviral vector derives from the HIV (human immunodeficiency virus) genome. The natural envelope protein of the HIV, which recognizes specific receptors on the plasma membrane to induce infection has been replaced by the envelope of vesicular somatitis virus, which is not a retrovirus. This envelope recognizes the phospholipids of plasma membrane, making the infection of essentially all cell types possible. The particles containing this envelope are stable, and they can be concentrated and administered at a high concentration into embryos or oocytes. For unknown reasons, lentiviral vectors have to be injected in cow oocytes rather than in one-cell embryos to transfer their genes.

Safe experimental conditions have been defined to use the lentiviral vectors. This method proved very highly efficient in several species including mammals [74, 78] and birds [11, 52, 86]. Up to 90% of transgenic animals may be obtained. This results from the fact that with high viral particle concentration, multiple independent integrations take place in the embryo genome. These integrations may occur later than the first cell stage giving birth to mosaic animals. The selection of lines harboring a single integration is preferable, but it may take a very long time in farm animals. Reducing the concentration of viral particles reduces the yield of transgenics but also the multiple integrations.

Trangenes transferred by lentiviral vectors are reputed not to become silent on the contrary to transgenes transferred by plasmidic constructions. This may be due in part to the fact that one or several integrated copies are active whereas others have been silenced. Lentiviral proved to be efficient tools to express transgenes coding for siRNAs [99]. Interestingly, siRNA could induce specific gene knockdown in rat [19, 35]. This observation is important as the classical tools to induce gene knockout (ES cells and cloning) are not available in this species. Lentiviral harboring foreign genes driven by cell-specific promoters may direct the expression of the transgenes in the targeted cells specifically [86]. The use of lentiviral vectors is limited by the fact that they cannot harbor more than 8 kb of foreign DNA. Special constructions must also sometimes be prepared to reduce the influence of the viral enhancers on the transgene promoter.

In mammals, the lentiviral vectors are injected between the zona pellucida and the cell membrane of one-cell embryos or oocytes. The efficiency of lentiviral vectors may be enhanced by injecting envelope-free constructions into cell cytoplasm [108].

The preparation of lentiviral particles carrying foreign genes is routinely achieved by academic and private structures at a moderate price. This possibility is particularly attractive for laboratories, which do not need a frequent use of this tool.

Use of ICSI

Foreign DNA must not necessarily be introduced into one-cell embryos. In principle, it can be as well injected in gametes before fertilization. DNA microinjection into oocytes proved inefficient, and this approach was soon abandoned. Using sperm is another possibility based on the assumption that foreign DNA cannot integrate into the genome as in this cell DNA is embedded in protamines and not able to replicate. Sperm was rather postulated to bind DNA in its surface and carry it into the oocyte during fertilization.

More than a decade ago, it was shown that sperm incubated in the presence of DNA before being used for fertilization was able to transfer the foreign gene into the oocyte and generate transgenic mice. This approach proved capable of generating transgenic mice, sheep, chicken, fish, and pigs [45, 109]. However, this method appeared difficult to use as DNA was frequently degraded [91]. The seminal plasma contains high DNAse activity, which degrades the gene and only rearranged fragments of the foreign genes were generally found in the transgenic animals. An appropriate sperm washing is required to prevent DNA degradation. For no clear reasons, not all the ejaculates can lead to a success of the method. Transgenic mice and rabbits were obtained by incubating sperm with DNA in the presence of DMSO (dimethylsulfoxide) and by using conventional in vitro fertilization [87].

The method has been greatly improved by using ICSI (intracytoplasmic Sperm Injection). This technique, which consists of injecting sperm into the cytoplasm of oocytes is currently used for in vitro fertilization in humans. To transfer genes, sperms from which plasma membrane has been damaged by freezing and thawing or with a mild treatment by nonpolar detergents were incubated in the presence of the gene of interest and further used for fertilization by ICSI. The ICSI approach started being implemented in Xenopus a decade ago as all the other methods failed [59]. This method proved efficient in mice [65, 88] and pigs [109]. Transposon use and ICSI may be combined to increase the yield of transgenesis [64, 88].

ICSI is therefore an excellent method to generate transgenic animals on condition that ICSI is possible in the considered species. One advantage of ICSI is that both long and short DNA fragments of DNA may be used sucessfully. DNA rearrangements may occur during ICSI especially when long DNA fragments are used. Another advantage is that foreign DNA is integrated mostly at the first cell stage of embryos. This reduces the number of animals being mosaic for the transgene.

Use of Episomal Vectors

The methods described above to transfer foreign genes rely on the integration of the DNA into the host genome. Another theoretical possibility is the use of episomal vectors capable of autoreplicating in host cells independently of the genomic DNA and transferred to daughter cells. Fragments of chromosomes are being used for particular projects, requiring the transfer of very long DNA fragments [44]. These chromosomal vectors are not of an easy use, and they carry a number of genes in addition to the gene of interest.

These extra genes may interfere with the transgene or with the whole organism of the host.

Another possibility consists of using vectors, which derive from viruses having the capacity to replicate in animal cells and to be transferred to daughter cells. Herpes viruses are naturally stably maintained as autonomous circular minichromosome at a low copy number in animal cells. Foreign genes can be introduced into Herpes viral vectors and be maintained during cell division. This kind of vectors is generally species specific. This greatly reduces their potential use as well-known Herpes viruses are not available for all animal species.

Episomal vectors not based on the use of viral elements are available. Such a vector proved highly efficient to transfer foreign genes into pig embryo using ICSI [58]. This vector was maintained without any selection pressure in the cells of the early developing embryos but seemingly not later. These vectors are therefore excellent tools to study transgene effect during early embryo development. Hence, until now, only the integration of foreign DNA into the host genome makes it possible the generation of stable lines of transgenic animals.

A theoretical safety problem raised by the use of episomal vectors is that it could be transmitted to other cells mechanically and thus independently of any infectious process. However, this event is expected to occur very rarely. More likely, non-integrated DNA is more available to recombine with homologous sequences in the host genome, leading to mutations, chromosome rearrangement, or integration.

Use of Intermediate Cells

Use of Pluripotent Cells In some situations, the efficiency of the genetic modification is too low to be achieved by the methods described above. This is particularly the case for gene targeting. One possibility to circumvent this problem is to genetically modify pluripotent cells that are further used to participate in the development of living organisms. Pluripotent cells have the capacity to participate in the development of all the organs including gametes.

Pluripotent cells exist in the early embryos (morula and blastocysts), and they are known as ES cells (embryonic stem cells). The pluripotent cells can be cultured, genetically modified, selected and transferred into morula or blastocysts. These cells participate in the development of the embryo to give birth to chimeric transgenic animals (Fig. 3). This means that the organs of the animals, including sexual cells, derive from the genetically modified cells or from the recipient embryo. A proportion of the offsprings from these chimeric animals harbor the genetic modification if they derive from the transplanted cells.

The first ES cells implemented to genetically modify animals (mice) were used at the end of the 1980s. For unknown reasons, commonly used ES cell lines have been established only in two mouse lines and mainly in the 129/Sv strain. In other lines and species, the ES cells lose their pluripotency. They can give birth to animals, which are still chimeric but have no more the capacity to transmit the genetic modification to their offspring. In practice, this complicates sometimes the use of the knockout mice. Indeed, in a number of cases, the gene knockout should be obtained in a genetic background very different from that of the 129/Sv strain. Successive crossings allow the mutation to be transferred in the strain in which the biological function in question may be optimally studied.

After sustained efforts for two decades, authentic rat ES cell lines have been established [7, 2, 49–51]. This breakthrough relies on the use of small molecules added into the culture medium. These molecules control essential genes required for the maintenance of pluripotency. They replace proteins like LIF, which are commonly used for mouse ES cells but are not successful in other species.

Recent experiments have shown that the transfer of three genes, normally expressed in pluripotent cells, into somatic cells can dedifferentiate these organ cells into pluripotent cells known as iPS (induced pluripotent cells) and almost similar to ES cells [68, 72, 93, 105]. Interestingly, small molecules can mimic and replace Kfl4 gene, which is one of the genes required to transform somatic cells into pluripotent cells [55]. These experiments open avenues for cell therapy and gene therapy [76]. The approach known as therapeutic cloning and based on the capacity of cloning (SCNT) to dedifferentiate somatic cells into totipotent cells further differentiated in vitro into pluripotent cells has become virtually no more strictly necessary. iPS cells can potentially be obtained in different species by this

method. Similarly, iPS cells might be implemented for transgenesis in species in which ES cells are not available (Fig. 3). Cow and pig iPS cells have been obtained and are currently under study.

Use of Primordial Germ Cells and Testis Stem Cells

Experiments carried out a few years ago showed that chicken primordial germ cells (PGC) can be isolated and cultured in conditions maintaining their multipotency giving stable cell lines known as EG cells (embryonic gonad cells). Foreign genes can be transferred into EG cells, which can be implanted into recipient embryos and participate in gonad development. In practice, the EG cells, which contain the gene of interest and a selection gene are cloned, amplified, and injected into an early embryo in which the majority of the cells have been destroyed by irradiation. This gives the best chance to the EG cells to colonize the embryo and to give birth to transgenic showing a high degree of chimerism and thus transmitting their transgene to progeny with a high yield. This approach has greatly simplified the generation of transgenic chicken [33, 101].

Testicular cells, which are sperm precursors can be isolated, cultured, genetically modified, more or less differentiated in vitro, and transplanted into recipient testis to give functional sperms that are able to generate transgenic animals by fertilization. Alternatively, sperm cell precursors may be genetically modified in situ using viral vectors [33, 41, 46, 48, 94]. These methods are still under study, and they are not currently used to generate transgenic animals.

Use of Cloning

The birth of Dolly, the sheep, demonstrated that the genome of somatic cells can be reprogrammed after being introduced into an enucleated oocyte. This generates a pseudo-embryo capable, with a relatively low yield, of giving birth to clones of the cell donor. This technique was initially designed to improve transgenesis efficiency in farm animals. This approach is likely to be used to accelerate genetic selection, but its only real application is presently transgenesis [81, 85]. The principle of this method is described in Fig. 3, and this topic is the matter of another chapter. Genes are transferred into somatic cells, which are then used to generate transgenic clones. This method has become the most frequently used for big farm animals as it simplifies the task of experimenters and enhances the rate of transgenic animals. Recently published important data have shown that the cloning technique does not provoke mutations in the clones [67].

Gene Construction

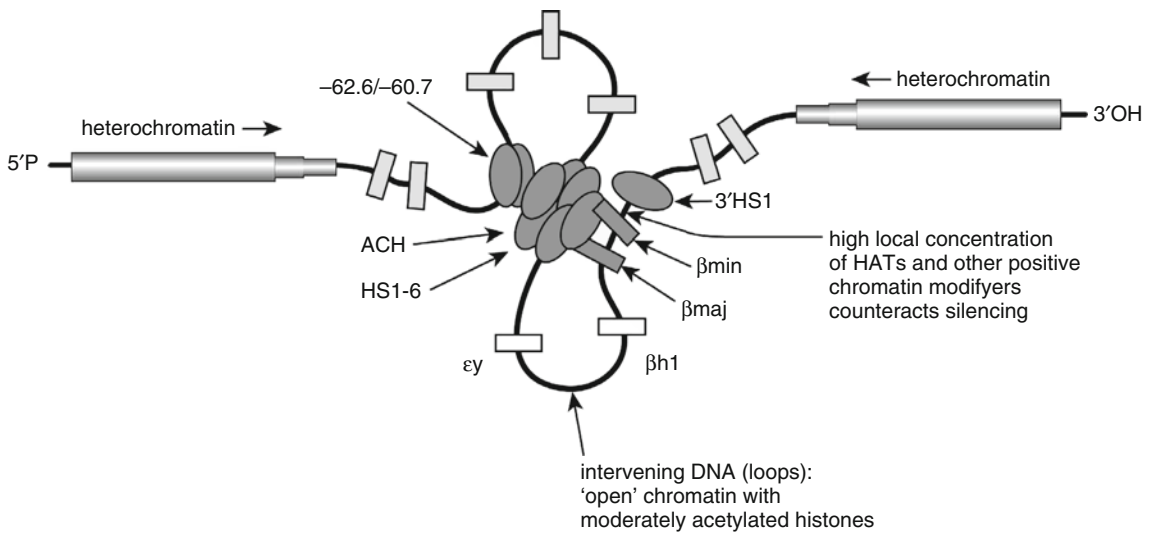
Random Integration A problem, which has not been completely solved is the reliability of transgene expression [36–38]. In the early 1980s, the first experiments to generate transgenic mice revealed that transgenes were often not working as expected. In a number of cases, the expression of the transgenes was very weak and not strictly specific to the promoter associated with the foreign gene. In a few cases, it was demonstrated that the ectopic expression of the transgenes was due to the presence of genomic enhancers in the vicinity of the integrated foreign DNA. The frequent transgene silencing was thought to be induced by the integration of the foreign genes near genomic silencers. These putative silencers were rarely identified suggesting that the ectopic transgene expression and their silencing could be not symmetrical phenomena. It was also proved that the level of transgene expression was generally not a function of the integrated copy number. In a number of cases, the expression level appeared even lower when the number of integrated copies was higher. A striking demonstration was given by the experiment in which the human β-globin gene was bordered by two LoxP sequences and integrated into mouse genome as several copies in tandem. The transgene remained silent in these mice but was reactivated in their offspring in which the copy number was reduced to one by the action of the Cre recombinase [26].

After about one decade, it appeared that this was due to chromatin position effects suggesting that the transgenes were recognized as foreign sequences by some unknown cellular mechanisms. One of the most surprising data was that a genomic DNA sequence containing the whole human β-globin gene including its promoter region allowing the gene to be expressed as expected in cultured red blood cells remained silent in transgenic mice. This discrepancy suggested that the transgene silencing occurred more in vivo than in

cultured cells and that this phenomenon could take place during the early phase of embryo development. A hypothesis was also that the genomic DNA sequence contained the whole β-globin gene and some but not all the transcription regulators. A confrontation of the very low expression level in patients suffering from β-thalassemia and the structure of their DNA in the genomic β-globin gene region revealed that, in some cases, the gene and its promoter were normal but that some remote genome regions were missing. This suggested that these regions could be the putative regulators missing in transgenic mice. An association of these regions with the β-globin gene allowed the later to be highly expressed in transgenic mice. The extensive study of the β-globin gene locus in several species revealed that remote regulatory elements are present on both side of the locus. These elements bind transcription factors specifically present in differentiated red blood cells, and they form a transcription complex known as a hub in the vicinity of the promoter through a looping process (Fig. 8) ([20, 21]). This type of mechanism seems to be common to many if not all genes in vertebrates and similarly also in invertebrates.

These observations may explain at least in part why traditionally constructed transgenes are so often poorly active and they suggest using long genomic DNA fragments contained in BAC (bacterial artificial chromosome) vectors to promote transgene expression [53]. The implementation of the long DNA fragments may be laborious as they are sensitive to mechanical degradation [100, 102]. The long DNA fragments can be transferred using microinjection, ICSI, gene targeting, or via intermediate cells. The failure of transgene activity is likely due to multiple reasons, which still complicate the construction of vectors allowing an efficient and reliable expression of transgenes. The optimized conditions to use long DNA fragments as vectors are described in a recent paper [102].

Nucleotidic Composition of the Vectors Integrated retroviral sequences and transposons are inactivated by a cytosine methylation of the CpG motifs and the local formation of condensed chromatin (heterochromatin) in which histones are deacetylated and methylated in some sites. Transgenes seem to be inactivated by similar mechanisms. Many of the vertebrate genes contain CpG islets in their regulatory regions, which contribute to their expression. Some of the CpG motifs belong to the binding site of the transcription factor Sp1, which is present not only in the promoter region of the gene but also sometimes in the first introns. An exceedingly large number CpG motif in vectors induces transgene



Transgenics: Alternative Gene Transfer Methods. Figure 8
Mechanism of action of remote transcription enhancers. The formation of loops allows the various regulatory factors to be in close vicinity and generate an active transcription complex

silencing. The replacement of some of the GC-rich regions by AT-rich regions improves transgene expression. MARs (matrix-attached region) are frequently found in the vicinity of genes, and they bind DNA to the nuclear matrix locally. MARs are generally AT rich, and they have been added into vectors to tentatively improve transgene expression. This approach met variable success. The *Escherichia coli* β-galactosidase gene is rich in CpG, and it is known to be a potent transgene silencer. This silencing potency proved to be markedly reduced as the number of CpG was diminished [17, 34]. The coding sequences of a transgene may thus be obtained by chemical synthesis to replace a part of the CpG-rich codons by others without modifying the sequence of the corresponding protein.

Use of Insulators In order to improve the expression of transgenes, it is possible to use large genomic DNA fragments (50–250 kb) expected to contain all the regulatory elements of the gene of interest [53]. It implies only the isolation and characterization of BACs (bacterial artificial chromosome) from a bank. Linearized or circular BACs may be used as such if the expression of the gene(s) they contain is wanted [102]. One drawback of using BACs is that they often contain several genes. The BACs thus transfer all these genes potentially generating unknown and unwanted interactions with the animals. If needed, these genes may be inactivated in BACs by performing short deletions, for example, of the cap region, using homologous recombination in bacteria.

An attractive approach consists of using BACs as vectors harboring the foreign genes. The foreign DNA sequence must be introduced into the BAC using homologous recombination in bacteria. It is important to note that the transgenes driven by BACs rarely work in an ideal fashion, if only this concept has a real meaning. Long genomic DNA fragments are expected to suppress the position effects, which is not often fully the case. Indeed, it is clear that the variegated expression, which characterizes the conventional transgenes is less or even much less frequent in animals harboring BAC vectors. A higher proportion of animals expressing the transgenes is generally found with BAC than with plasmid vectors. Yet, the different lines harboring BACs vectors usually do not express the transgenes at an identical level for a given number of integrated copies. Similarly, it has been rarely reported that the expression of the transgenes was strictly a function of the copy number of the integrated BACs. This means that the BACs provide transgenes with essential elements for their expression but they remain often not fully able to suppress the position effects. This disappointing observation may not be surprising from a theoretical point of view. Indeed, a locus has been constructed during evolution to express in an appropriate manner the genes it contains. This implies that these genes are protected against deleterious positions effects in their natural chromatin environment. They have no theoretical reasons to be independent of the position effect in all their integration sites. Some BACs may contain all the elements providing transgenes with a complete independence of the integration site. If not, a BAC vector may still contain enough regulatory elements improving significantly transgene expression to justify its use.

A more sophisticated approach could consist of using as vectors containing not all the DNA sequence of BACs but only the major elements involved in the control of gene and transgene expression. This implies that these regulatory elements have been identified, characterized, and introduced into mini-BACs or even plasmids. An example of this is a major regulatory region of the genes present in mammalian β-globin locus, which is located upstream within the locus of olfactory receptor genes [21]. The study of the remote genomic regulatory elements is still in infancy. Apart from the technical difficulty to study them is the fact that each gene or gene cluster seems to have used available genomic sequences to design specific mechanisms allowing a satisfactory expression. Some of these regulatory elements have an unexpected structure. Examples are SINE B2 and Alu sequences or some active tRNA genes, which are essential regulators for neighbor genes [54].

The notion of boundary elements and insulators is essential to understand how unrelated genes can be expressed in a specific manner without being under the dependency of the neighbor gene regulators. This situation is clearly often not encountered for transgenes suggesting that the constructs commonly used do not contains the natural insulators. Insulator activity has been found in the LCR (locus control region) of the chicken β-globin locus. This activity was identified in

a 300 bp fragment, which proved its ability to block in a specific sense the action of an enhancer when added between this enhancer and a promoter directing the expression of a reporter gene. The enhancer blocker was mediated by the binding of the regulatory protein CTCF to a specific DNA sequence. The CTCF element has now been found in the boundary region of several other genes. This type of elements, known as enhancer-blocking insulators, cannot be assimilated to silencers as the former act only when they are located between an enhancer and a promoter. Moreover, the enhancer-blocking insulators often show unidirectional action.

This 300-bp region of the chicken β-globin locus was found later to contain another sequence known as a chromatin opener. Chromatin openers are regulators capable of maintaining a local euchromatin configuration favoring the expression of the neighbor gene by preventing the local formation of condensed chromatin (heterochromatin) [27]. The elements having this function are known as barrier insulators [27]. The barrier insulators cannot be assimilated to enhancers as their effect does not occur during transient foreign gene expression in transfected cells. The region locus known as 5'HS4 and containing the 300-bp sequence can improve the expression of a number of unrelated transgenes in mammals [29, 92]. However, the potency of the 5'HS4 element remains generally insufficient to express transgenes in a fully satisfactory manner.

In the mean time, the presence of AT-rich MARs within the genomic region required for the expression of a gene suggested that these sequences were essential remote regulators. This hypothesis was not confirmed [90].

Optimization of the Transcribed Region The 5′UTR (untranslated region) must have less GC sequences stabilizing double-stranded hairpin structures, which do not favor ribosome migration to the initiation codon. The AUG initiation codon must preferably be in the Kozak consensus sequence GCCA/GCCAUGG to optimize translation initiation. The natural 5′UTR of the gene of interest may contain sequences regulating translation. It may be then useful not to keep this region and replace it by a short (not less than 80 nucleotides) AT-rich 5′UTR region from gene known to be efficiently translated in many cell types or in the targeted cells of the animals. Some mRNAs encode proteins, which are not naturally secreted. Peptide signals may be added to their cDNA.

A transgene must contain at least one intron, which is required to favor the transfer of the mRNA to the cytoplasm. The first intron of many genes contains sites, which bind transcription factors and they may favor transgene expression. The intron excision is dependent upon several signals comprising consensus sequences in both splicing sites (CAG GUA/GAGUA/UGGG in 5′ and CAG G. . .GAA/G. . .GAA/G. . .in 3′), namely, a CU-rich region immediately upstream of the 3′splicing site and a BPS site (branched point sequence) U/CNCUGAC at about 30 nucleotides upstream of the 3′splicing site and upon splicing enhancers [61]. The second intron of the rabbit β-globin gene is considered as efficient to express transgenes in mammals. The intron(s) must preferably be put before the coding region. If an intron is added after the translated region, the 5′splicing site must be located not more than 50 nucleotides from the termination codon to avoid the activation of the NMD (nonsense-mediated decay), which degrades the mRNA [10].

The cDNA and other regions of the vectors may preferably be chemically synthesized. This allows reducing the number of CpG motifs, to choose the best codons, to eliminate cryptic 3′or 5′splicing sites and sequences known to prevent transcription or translation.

The 3′UTR region of a number of mRNA contains signals for mRNA translation and stability. A number of mRNAs have an AU-rich region with the AUUUA motif in their 3′UTR. These mRNAs have a short half-life controlled by the cell cycle (Beelman and Parker 1995). The fortuitous presence of such sequences must be searched and eliminated to prevent a poor transgene expression. Some mRNAs contain translation regulators acting by the binding of proteins favoring the recycling of ribosomes by binding to the 5′UTR. CU-rich regions in the 3′UTR enhance the stability of the mRNAs, and they may be added in the vectors downstream of the cDNAs. Stabilizing sequence can be taken in the 3′UTR of the human or bovine genes and of the α-globin gene, which also contain efficient transcription terminators [13]. Some proteins are anchored to the plasma membrane by a GPI structure (glycophosphatidylinositol). A protein normally not anchored in this way acquires

this property by adding in the 3′end of the cDNA the peptide, allowing the addition of GPI. Micro-RNAs (miRNA), the role of which was recently discovered, inhibit specifically the translation of an mRNA after forming a hybrid with its 3′UTR. The presence of target sequence for a miRNA may unduly inhibit the expression of a transgene. This target sequence should then be deleted.

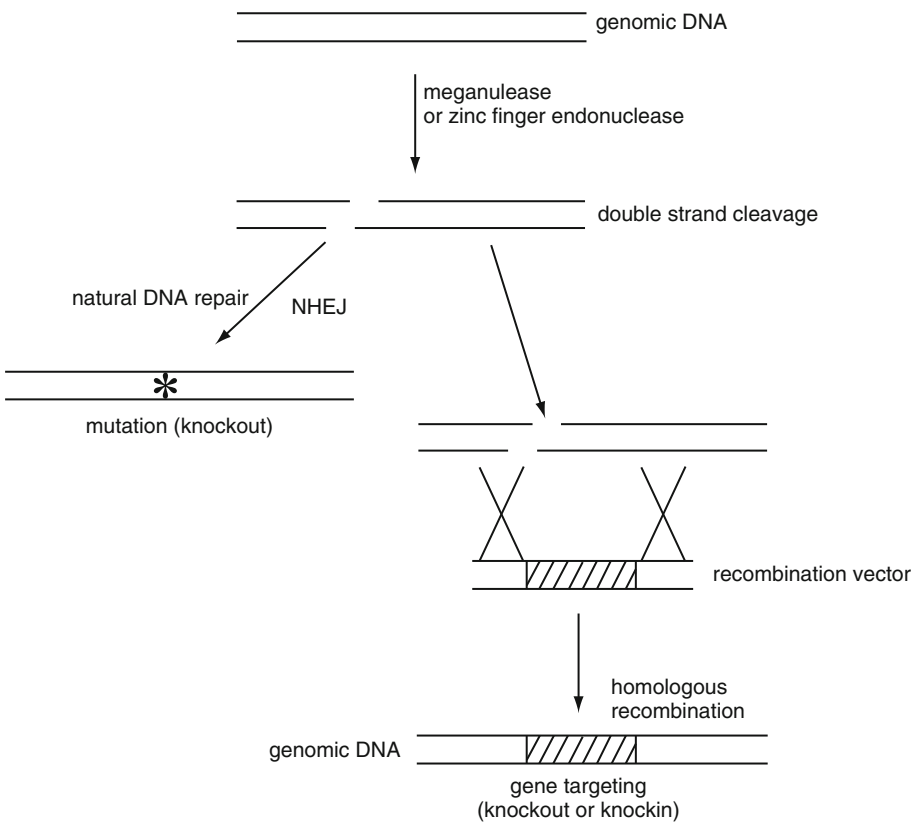
Targeted Integration The techniques described above lead to uncontrolled but not strictly random gene integration. Foreign DNA is preferentially integrated into gene-rich genome regions, and its location can be precisely identified. A foreign DNA fragment can recombine very precisely with a genomic DNA region containing a similar sequence. This natural mechanism known as homologous recombination makes the precise replacement of a gene by another possible. An active gene may thus be replaced by an inactive version, leading precisely to an inactivation of the targeted gene (gene knockout).

The targeted gene may as well be replaced by an active gene (gene knockin). This technique allows therefore a better controlled transgenesis reducing possible damage of the genomic DNA at the integration site and frequent side effects of the genes located in the vicinity of the transgene on the expression of the transgene. Two mouse genomic loci are currently being used as foreign gene knockin. One is the HPRT locus [6] and the other is Rosa 26 locus [110]. The genes of these loci are known to be expressed constitutively, and they were supposed to be bordered by regulators able to drive the expression of transgenes in a reliable manner. In practice, a number of transgenes appeared to be expressed as expected when integrated in these loci. Interestingly, also the expression of the transgenes added in these loci remained specific to the promoter linked to the foreign genes. The two loci thus appear to maintain an open chromatin configuration, favoring the expression of the transgenes irrespective of their composition.

Yet, this approach remains limited by the fact that the homologous recombination required for gene targeting is a rare event. The targeted integrations by homologous recombination of a foreign DNA represent 0.1–1% of the total integrations. The cells in which targeted integration occurred must be selected and used to generate a transgenic animal. The formation of chimeric embryos using pluripotent cells, multipotent cells, or the cloning technique is presently required to obtain a targeted integration.

Meganucleases were discovered in yeast about two decades ago, and it was shown that they participate in intron generation. These enzymes recognize sites as long as 18 nucleotides leaving negligible chance to cleave mammalian genomic DNA. About 80 natural meganucleases have been identified so far. It was observed later than one of these meganucleases I-Sce1 induces chromosome recombination in mammalian cells and this was attributed to the fact that meganucleases cleave both DNA strands [14]. Such breaks stimulate DNA repair mechanism. It was also shown that I-Sce1 amplifies the rate of homologous recombination [15] and consequently gene replacement in mammalian cells [16]. The DNA sequences recognized by natural meganucleases must then be added to the genome of animals either at targeted sites by homologous recombination or at random sites. To circumvent this problem, hundreds of engineered meganucleases each recognizing a specific genomic DNA sites have been obtained making a local cleavage of DNA and gene targeting possible [25].

Multiple engineered zinc finger nucleases (ZFN) have also been obtained. Each of these enzymes has the capacity to cut one DNA strand at a unique genomic DNA site. To mimic natural meganucleases and cut locally both DNA strands, two ZFN each recognizing a DNA strand at neighbor sites are needed [75]. Engineered meganucleases and engineered zinc finger nucleases, thus, make it possible gene targeting at multiple sites of the genome. This method, which is being developed to improve the efficiency and the precision of gene therapy for humans can be applied to target the integration of foreign DNA into experimental animals. Interestingly, when the recombination vector is not added with the meganuclease or the ZFN, the genomic DNA repair takes place spontaneously but often with alteration of the sequence. This process known as NHEJ (non-homologous end joining) corresponds to a knockout (Fig. 9) [84, 106]. This mechanism is efficient, and it allowed a knockout in one cell fish and rat embryos after the injection of engineered meganucleases [28, 107]. This method can be considered as a transgenesis without transgene. This inclines to think that gene targeting might be achieved directly



Transgenics: Alternative Gene Transfer Methods. Figure 9
Engineered meganucleases or ZFN (zinc finger nucleases) induce a targeted local cleavage of both genomic DNA strands. In the absence of recombination vector, the DNA is repaired leading to frequent local mutations and to a knockout. In the presence of a recombination vector, the foreign gene is integrated into the targeted site with a high efficiency

in mammal embryos by injecting an engineered meganuclease or ZFN with or without a homologous recombination vector.

In the same line, the bacterial enzyme phiC31, which is an integrase, recognizes several sites in various animal genomes and allows the efficient integration of foreign genes at the targeted sites. Several other recombination systems rely on the use of integrases such as Cre and Flp, which recognize specific sites of about 30 nucleotides (LoxP and FRT, respectively), which must be added to the animal genome. In practice, deletion of the integrated foreign DNA has more chance to occur than integration. Mutated LoxP and FRT sequences capable of promoting integration but not deletion must be used. This approach is known as RMCE (Recombinase-mediated Cassette Exchange) [1]. These systems are more often used to delete a DNA region previously bordered by the LoxP or the FRT sequences.

The Co-expression of Several Cistrons It is sometimes necessary to express two or even three genes in the same transgenic animals. The co-injection of several independent vectors makes the generation of up to 80% of the animals harboring the two or three genes, which are co-integrated at the same site possible. An alternative consists of using IRES (internal ribosome entry site). Such sequences exist in the 5′UTR of many mRNAs, the translation of which is controlled by these sequences, which bind specific cellular inducible proteins. Such sequences may be added between two cistrons and allow their simultaneous translation from a single vector. The addition of the IRES 80 nucleotides after the termination codon of the first cistron may

contribute to favor the expression of the second cistron [39]. It should be kept in mind that IRES represents a family of sequences acting by different mechanisms, which are only partly known.

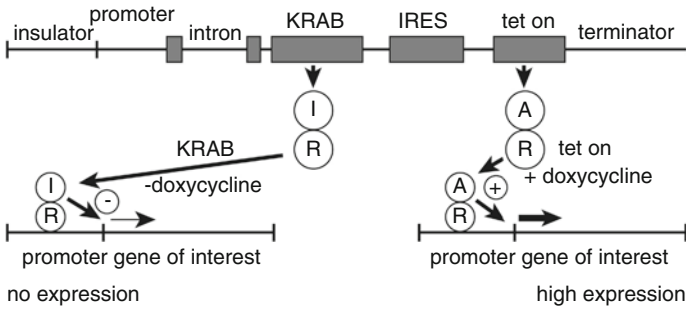
Gene Inactivation (Knockdown) with Interfering RNAs Long double-stranded RNAs are randomly cut into 19–21 nucleotide fragments known as siRNA (small interfering RNA) or RNAi. One of the two strands of the siRNA is kept and targeted to an mRNA having a complementary sequence. This induces the degradation of the mRNA or the reversible inhibition of its translation. In practise, a synthetic gene containing the targeted 19–21 nucleotide sequence followed a short random sequence and by the targeted sequence in the opposite orientation is linked to a promoter acting with RNA polymerase III (usually U6 or H1 gene promoters). The RNAs synthesized by such vectors form a 19–21 nucleotide double-stranded RNA known as shRNAs (short hairpin RNA) and are processed in cells to generate active siRNAs. An appropriate expression of siRNA genes in transgenic animals can be obtained when they are introduced into lentiviral vectors [99].

The recent discovery of the role of micro-RNAs has increased the possibility to use interfering RNAs. Micro-RNAs are encoded by short genes expressed under the control of RNA polymerase II promoters. Their primary products are transformed into siRNAs. Alternatively, the sequence coding for a micro-RNA and even several micro-RNAs in tandem may be inserted into introns without any promoter. The promoter of the gene or transgene thus drives transcription of the micro-RNA genes and the intron degradation releases micro-RNA precursors, which are processed in the nucleus and the cytoplasm, generating active siRNAs (Ripoll et al. unpublished data). The mature miRNAs, which are fully complementary to the targeted mRNA induce degradation of this mRNA. The miRNAs, which are only partially complementary to the targeted mRNA and which recognize a sequence located in the 3′UTR (3′untranslated region) of the mRNA inhibit translation of this mRNA without inducing its degradation. The possibility known as knockdown to generate transgenic animals expressing siRNAs preventing specifically the expression of a gene by degrading the corresponding mRNA or inhibiting its translation has opened avenues for the control of gene expression in vivo. The application of the siRNA approach raises specific problems in animals. Long double-stranded RNAs induce interferons and some unspecific immune reactions [89]. On the other hand, siRNAs are not autoamplified in higher animals and this reduces their potency. Vectors to express miRNA gene are available, but simple shRNA genes are not easily expressed in transgenic animals using conventional vectors. Moreover, siRNAs may off-target mRNAs and generate deleterious side effects.

Several programs based on empirical data indicate the putative optimal shRNA sequences that are able to generate siRNA strands complementary to the mRNAs. A very important point is to choose a target region of the mRNA, which is not in double-stranded structure and thus accessible to the siRNA. Banks of shRNA genes in lentiviral vectors are available for the mRNAs of different species. It remains that most of the siRNAs do not inhibit the targeted gene to more than 70–80%, which may be insufficient to obtain some relevant animal models. It is tempting to use vectors expressing the shRNA genes at a relatively high level. This may not lead to any significant increase of the inhibition and to an off-targeting, which may be detrimental or even lethal for the animals [89]. In fact, it seems that a well-targeted siRNA can be highly active even at a low concentration. It appears, therefore, to be of paramount importance to select the shRNA capable of inhibiting strongly the targeted mRNA even at a low concentration in cell systems before generating transgenic animals [79].

Use of Transdominant Negative Proteins The action of a gene can be blocked at the protein level by expressing specific inhibitors such as antibodies recognizing the protein of interest [66]. Alternatively, transdominant negative proteins acting as decoys may be used. Transgenic mice mimicking type II diabetes were obtained by overexpressing a mutated insulin receptor still capable of binding the hormone but not of transducing its message [9]. Similarly, overexpression of pseudorabies virus receptor in transgenic mice protects these animals against Aujeszky disease [70].

Genetic Ablation Destroying specifically given cell types in animals may reveal their role in organogenesis.

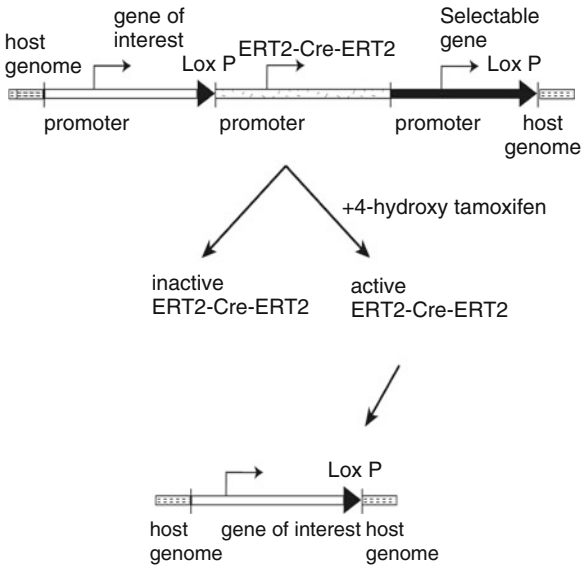


Transgenics: Alternative Gene Transfer Methods. Figure 10
The control of transgene expression may be controlled by an exogenous inducer. In the absence of the inducer (doxycycline), the transcription enhancer (Tet on) is not bound to DNA and it does not stimulate transcription whereas the transcription inhibitor (KRAB) is bound to DNA and reduces the background expression of the gene of interest. In the presence of doxycycline, the reverse is true and the gene of interest is activated

This can be achieved by expressing genes coding for toxins. The challenge is then to express quite specifically the transgenes. Different systems are implemented for this purpose [12, 82]. They rely on two-step mechanisms, which reduce the risk of ectopic expression of the toxin genes.

Control of Transgenes by Exogenous Inducers All the vectors described above and used to express transgenes contain promoters, which are naturally active in the cells of the transgenic animals. This implies that the transgenes are regulated by the natural inducers of the host genes. The induction of a transgene may then coincide with the unwanted stimulation of a number of host genes. Artificial promoters containing regulatory elements from both animal genes and bacterial genes have been designed. The resulting promoters are active in animal cells but controlled by substances active in bacteria but not in animals. The most popular system is based on the use the bacterial tetracycline repressor gene. In practise, the transgene becomes reversibly activated only when tetracycline or doxycycline is administered to the animals. Various mutants are available making it possible either the induction of the transgene or its inhibition by the addition of the exogenous inducer. A number of similar systems are available and currently used in transgenic animals with a good success [30, 57]. In order to reduce the background expression of the transgene in the absence of the exogenous inducers, the alternative expression of a transcription repressor as KRAB and of an inducer as Tet-on system may be implemented (Fig. 10). These tools, which require the transfer of several genes offer virtually the possibility to express a transgene precisely in a given cell type and at a given moment.

Gene Deletion Conventional homologous recombination makes it possible gene deletion known as knockout. Another possibility consists of using the Cre-LoxP or Flp-FRT systems. A LoxP sequence must first be added to both ends of the fragment to delete. The presence of the Cre recombinase will then recombine the two LoxP sites, leading to a deletion of the DNA fragment located between the LoxP regions. This makes it possible the elimination of a selection gene. The same approach allows the specific and controlled deletion of an inhibitory DNA region, leading to the activation of the gene located in its vicinity. The Cre recombinase may be synthesized by the corresponding gene under the direction of a cell-specific promoter including promoters under the control of doxycycline. Another level of control can be obtained by using an engineered Cre recombinase, which becomes reversibly active in the presence of an estrogen analogue, 4-hydroxy tamoxifen (Fig. 11) [62]. This offers the advantage of having the active Cre recombinase for short periods of time. This prevents the non-specific action of the Cre recombinase, which can recognize cryptic sites in the host genome and induce illegitimate recombination damaging the host DNA. This tool is appropriate to delete genes for resistance to antibiotics.



Transgenics: Alternative Gene Transfer Methods. Figure 11

Activation of Cre recombinase and selectable gene elimination by 4-hydroxy tamoxifen. The Cre recombinase gene expression may be under the control of the Tet-on system itself under the control of a cell-specific promoter. The fusion protein Cre recombinase–mutated estrogen receptor is active only in the presence 4-hydroxy tamoxifen. The elimination of the DNA region bordered by LoxP sequences is thus sharply controlled. The selection gene and the Cre recombinase may thus be eliminated from transgenic animals at any stage of their life

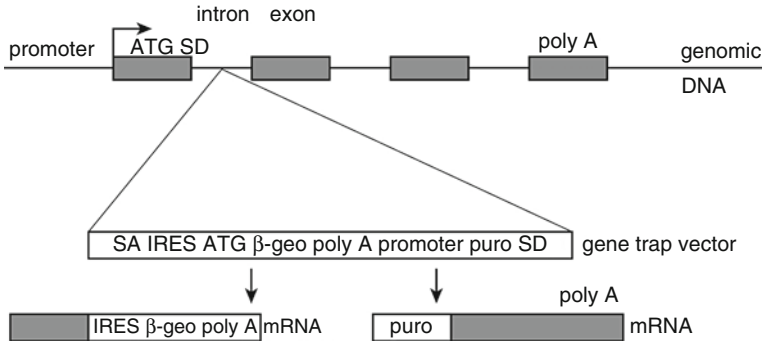
Vectors for Gene Trapping The identification of genes involved in a given biological function is an essential step to understand the role of the genes particularly those responsible for human diseases. One possibility is to use vectors for the trapping of active genes. One such vector is described in Fig. 12. Other vectors are shown in the chapter of a book [36]. New tools make it possible random gene knockout in genomes by inserting foreign DNA sequences using transposons [22] or lentiviral vectors. Banks of siRNA genes in lentiviral vectors can also be used to knockdown genes. The inhibited genes can thus be identified and correlations with altered biological functions in the animals become possible.

Future Directions

During the coming decade, transgenesis in animals is expected to be as intensively or even more used than presently. One of the trends is to refine the different tools for basic research as for the different applications. The time when transgenic mice were prepared in a blind manner by microinjecting simple gene constructions is getting over. The recent and very significant improvement of the gene transfer techniques should make several animal species less marginal in the transgenesis field. The number of transgenic models for basic research is being extended to a larger number of species, namely to rats, chicken, Xenopus, and even cow [47, 80, 96]. The sequencing of an increasing number of genomes, including different mouse strains and even different individuals, will contribute to extend the use of animal transgenesis.

The proportion of transgenic animals used for basic research, including the models for the study of human diseases, is currently very high. This proportion should change but moderately in favor of applications in the food domain and also in the pharmaceutical field including the production of pig cells and organs for patients and the production of recombinant pharmaceutical proteins. Indeed, the production of pig cells and organs is making significant progress but it remains unpredictable when and if the transplantation of pig cells and organs will become a tangible reality as many problems remain to be solved [73].

The production of pharmaceutical proteins in milk and egg white has become a realistic approach as the techniques have reached sufficient maturity even if the production of each protein is a challenge. Yet, this application of transgenesis is coming more slowly than anticipated. For complex reasons not clearly related to the efficiency of the technique or to biosafety guidelines, the pharmaceutical companies remain more or less reluctant to start using animals to obtain pharmaceutical proteins. Companies may consider that their profit is presently higher with the production of recombinant proteins in conventional fermentors harboring animal cell lines, even or because this system generates a shortage of medicaments for patients. A recent multiauthor book is a survey of all the aspects of this technique including the ethical considerations [77].



Transgenics: Alternative Gene Transfer Methods. Figure 12

Schematic representation of a gene trapping vector. The vector is randomly integrated into the genome of cells that are able to generate transgenic animals (Fig. 3). The β-geo gene is a fusion of the β-galactosidase gene, giving a blue color to cells, and neomycin resistance gene, giving a resistance to geneticin. The vector contains also the puromycin resistance gene, splicing acceptor sites, no promoter, and no transcription terminator. The β-geo selection gene is expressed only when it was integrated into a functional gene. The inactivated gene may be identified by sequencing the genomic DNA flanking the integrated vector. A correlation between the inactivated gene and an altered biological function of the animal may be established

Whatever happens, the application of transgenic animals for food production should become a reality in the coming decade. The increasing knowledge of alleles in farm animals and of a variety of other possible transgenes should give more space to the use of transgenesis to improve animal production. The recent achievement of the cow genome sequencing will contribute to using cattle alleles more extensively. It remains that the generation of relevant transgenic founders and their extensive use in breeding will be slower than in plants due to the high cost of transgenesis and the time required for the dissemination of their genomes in herds. The poor acceptability of biotechnology particularly in the EU is another hurdle.

The recent progress of several techniques of gene transfer are becoming more popular in laboratories and companies and therefore should be pursued. DNA microinjection into embryos will still be used in the species in which it is efficient. Lentiviral vectors are more and more frequently used, and this trend should become still stronger as the preparation of efficient and safe lentiviral particles has become a standard technique. Other viral vectors could be implemented in future. One candidate is AAV (adeno-associated virus). This virus is spontaneously integrated into cell genomes as lentiviruses. AAV vectors are used with some success for gene therapy in humans [111]. One of its advantages over lentiviruses is that they can harbor longer DNA fragments. Viral vectors might prove attractive for some applications in breeding. It is indeed conceivable to introduce massively foreign genes only into somatic cells. Examples are fish or more generally the animals, which swim or fly and can disseminate in environment. Growth hormone genes transferred into a sufficient number of salmon somatic cells might have the same biological effect on growth than the corresponding transgene without any risk of its dissemination in oceans.

More and more transposons are found and engineered to be able to generate efficiently and in a safe manner a variety of transgenic animals. One of the challenges of modern biology is to decipher the mechanisms involved between given genetic information and the expression of a physiological function. The systematic random integration of transposons and also of lentiviral vectors into the genome of cells able to participate in the development of transgenic animals is expected to generate banks of cells and animals in which physiological disorders can be connected to the genes interrupted by the vectors. This approach is similar to that of generating banks of animals having

genes inactivated by knockout or knockdown. Several animal and data banks are available: EMMA in EU, Jackson Laboratory in USA, IMSR (International Mouse Strain Resource), ISTT (International Society for Transgenic Technologies) www.transtechsociety.org, [40], private companies (namely Charles River) as well as academic and private structures for the generation of transgenic mice, rats, and rabbits.

Gene transfer using ICSI has met a great success in mice and a few other species. It is simpler and more efficient than conventional DNA injection. A broader implementation of this technique is presently limited by the fact that ICSI independently of transgenesis is still possible in only a small number of species. This situation is essentially due to the fact that researchers have not yet acquired this knowledge. The principle of ICSI is common to all species, but the manipulation of oocytes and sperm needs specific training. ICSI should thus be used more intensively in the near future in mice and extended to other species. The possible combination of ICSI and transposons as well as of envelope-free lentiviral particles might contribute to enhance gene transfer efficiency.

A trend is clearly to perform the genetic modifications not directly in embryos but rather in intermediate cells further used to generate transgenic animals. The long-term success of mouse ES cells to target gene transfer via the generation of chimeric animals is expected to be rapidly extended to the use of rat ES cells and iPS cells in different species, which are able to generate chimeric animals as ES cells. Progress must be made before iPS cells are used for transgenesis. This is being achieved as iPS cells are extensively studied since they are expected to be a major tool for cell therapy and gene therapy in humans. The techniques to generate iPS cells from somatic cells must be adapted to each species. An important recent progress is the possibility of dedifferentiating somatic cells no more by transferring the three identified key genes but by introducing the corresponding mRNAs, which are unstable by essence and thus leave no genetic information in the iPS cells. It is conceivable to transfer the three genes able to generate iPS cells into embryonic cells to obtain more easily ES cells. Indeed, cultured ES cells are known to quickly lose their pluripotency in most mouse lines and essentially in all other species but rats. ES cells from a number of species might become a reality in this way. The preliminary success, which made it possible the use of small molecules in culture medium instead of proteins to generate pluripotent cells from embryos or somatic cells is very encouraging. It suggests that the generation and the use of pluripotent cells in multiple species might become a relatively easy task. This would facilitate markedly the genetic modification of animal genomes. A more speculative reasoning is to postulate that the complete dedifferentiation of somatic cells into totipotent cells will be possible some day by the transfer of genes or chemical inducers, which remain to be found. Cloning and transgenesis as well as their coupling and related ethical problems would be considerably simplified.

Cloning by SCNT proved that it is possible to make the random and targeted gene transfer. The popularization of this technique is likely, but it depends on improvement of the technique. Indeed, clones are often if not always epigenetically modified leading to a limited efficiency, to a reduced welfare of the animals, and potentially to health problems. Significant improvement has been achieved in 10 years, and a better control of cloning should occur in the coming years.

The construction of vectors making it possible a reliable and well-controlled expression of the transgenes will be progressively improved by the empirical use of BAC containing long genomic DNA fragments with the majority of the remote transcription regulators. The use of long genomic DNA fragments as vectors is expected to become more and more frequent but to remain empirical for some time. Most likely, an increasing number of BAC vectors containing regulatory elements for the expression of transgenes in the different cells types will become available. Despite the higher difficulty to manipulate BAC rather than plasmids constructs, researchers will prefer in a number of cases to invest a part of their time in the construction of BAC vectors to tentatively obtain better transgenic models. Rapid progress is being made on the description of chromatin structure and activity. Gene study is more and more performed at the level of a locus and not only of a given gene. This will provide researchers with well-identified chromatin regulators, which will be used to design compact vectors containing these regulators capable of optimizing transgene expression.

Gene targeting is essential to obtain relevant transgenic models. The demonstration that a local cleavage of DNA by natural or engineered meganucleases or by engineered zinc finger nuclease considerably increases the efficiency of homologous recombination is of major importance for basic and applied research particularly for projects involving transgenesis. Gene knockin and knockout are expected to become much more efficient, up to the point perhaps to be possible directly in one-cell embryos and not only via intermediate cells. The demonstration that DNA cleavage by zinc finger nuclease in the absence of recombination vectors is followed by an imperfect DNA repair (NHEJ), which in a significant proportion of cases leads to a mutation, thus to a gene knockout. The fact that gene knockout by NHEJ could be obtained directly in fish embryos opens new avenues as this protocol does not require any more the laborious gene knockout by homologous recombination. Hundreds of engineered zinc fingers have been designed to recognize specific sites in different genomes. This number should increase rapidly in the coming years. A particularly fascinating point is that NHEJ as well as conventional knockout and knockin might be applied not only to genes proper but also to their genomic regulators. This would allow the study of the regulators of various loci without implementing the laborious manipulation of BACs.

The discovery of siRNA has rapidly been followed by the generation of gene knockdown in plants. The same did not occur in animals. The use of long double-stranded RNA induces deleterious side effects in animals. The availability of lentiviral vector banks harboring genes coding for shRNAs makes gene knockdown easier in animals. The efficiency of this approach remains limited by the insufficient knowledge on the mechanisms of RNA interference. Progress has been made to empirically design siRNA having a high and specific inhibitory effect on gene expression. An important parameter is now taken into consideration. It is known that the sequence of the siRNA determines the choice of the strand, which will target the mRNA of interest and its capacity to inactivate this mRNA. It now clear that the local structure of the mRNA is essential to make the access of the siRNA possible. Additional progress is expected in this field, and it should facilitate gene knockdown in transgenic animals.

Using constructs in which the transgene is under the control of exogenous inducers like doxycycline is a reality. Sophisticated systems reducing the background expression of the transgene expression in the absence of the inducers are currently used. The possibility of using these systems to create relevant transgenic models is expected to be more and more frequently used. Improvement of these techniques is likely by finding additional inducers.

It is essential to be able to knock out genes in chosen cells and at defined period of the animal life. This conditional knockout implies the used of the Cre/LoxP or similar systems. The conditional expression of the Cre recombinase and its conditional activation by 4-hydroxy tamoxifen offer a great flexibility. Improvement of these systems is possible. A more extensive use of these systems, which give satisfaction to researchers will be done in the coming years.

Transgenesis techniques are thus making important progress for the generation of transgenic animals as for the fine control of transgene expression. The available tools and those in development are expected to be adapted to the systematic study of multiple genes required for the development of integrative biology.

Bibliography

Primary Literature

1. Baer A, Bode J (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. Curr Opin Biotechnol 12:473–448
2. Baker M (2009) Authentic rat embryonic stem cells. Nat Rep Stem Cells. doi:10.1038/stemcells 2009.11
3. Bishop JO (1997) Chromosomal insertion of foreign DNA. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 219–224
4. Bosselman RA, Hsu RY, Boggs T, Hu S, Bruszewski J, Ou S, Kozar L, Martin F, Green C, Jacobsen F, Nicolson M, Schultz JA, Semon KM, Rishell W, Stewart RG (1989) Germline transmission of exogenous genes in the chicken. Science 243:533–535
5. Bronson SK, Smithies O (1994) Altering mice by homologous recombination using embryonic stem cells. J Biol Chem 269:27155–27158
6. Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Maeda N, Smithies O (1996) Single-copy transgenic mice with chosen-site integration. Proc Natl Acad Sci USA 93:9067–9072
7. Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A (2008) Capture of authentic embryonic stem cells from rat blastocysts. Cell 135:1287–1298

8. Capecchi MR (1989) Altering the genome by homologous recombination. Science 244:1288–1292
9. Chang PY, Benecke H, Le Marchand-Brustel Y, Lawitts J, Moller DE (1994) Expression of a dominant-negative mutant human insulin receptor in the muscle of transgenic mice. J Biol Chem 269:16034–16040
10. Chang YF, Imam JS, Wilkinson M (2007) The nonsense-mediated decay RNA surveillance pathway. Annu Rev Biochem 76:51–74
11. Chapman SC, Lawson A, Macarthur WC, Wiese RJ, Loechel RH, Burgos-Trinidad M, Wakefield JK, Ramabhadran R, Mauch TJ, Schoenwolf GC (2005) Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. Development 132:935–940
12. Chen YT, Levasseur R, Vaishnav S, Karsenty G, Bradley A (2004) Bigenic Cre/loxP, puDeltatk conditional genetic ablation. Nucleic Acids Res 32:e161
13. Chkheidze AN, Lyakhov DL, Makeyev AV, Morales J, Kong J, Liebhaber SA (1999) Assembly of the α-globin mRNA stability complex reflects binary interaction between the pyrimidine-rich 3′ untranslated region determinant and poly(C) binding protein α CP. Mol Cell Biol 19:4572–4581
14. Choulika A, Perrin A, Dujon B, Nicolas JF (1994) The yeast I-Sce I meganuclease induces site-directed chromosomal recombination in mammalian cells. C R Acad Sci 317:1013–1019
15. Choulika A, Perrin A, Dujon B, Nicolas JF (1995) Induction of homologous recombination in mammalian cells using the I-Sce I system of Saccharomyces cerevisiae. Mol Cell Biol 15:1968–1973
16. Cohen-Tannoudji M, Robine S, Choulika A, Babinet C, Louvard D, Jaisser F (1998) I-Sce I-induced gene replacement at a natural locus in embryonic stem cells. Mol Cell Biol 18:1444–1448
17. Cohen-Tannoudji M, Babinet C, Morello D (2000) Lac Z and ubiquitously expressed genes: should divorce be pronounced? Transgenic Res 9:233–235
18. Couble P (1997) The biolistic. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 209–213
19. Dann CT (2007) New technology for an old favorite: lentiviral transgenesis and RNAi in rats. Transgenic Res 16:571–580
20. de Laat W, Grosveld F (2003) Spatial organization of gene expression: the active chromatin hub. Chromosome Res 11:447–459
21. de Laat W, Klous P, Kooren J, Noordermeer D, Palstra RJ, Simonis M, Splinter E, Grosveld F (2008) Three-dimensional organization of gene expression in erythroid cells. Curr Top Dev Biol 82:117–139
22. Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122:473–483
23. Dupuy AJ, Clark K, Carlson CM, Fritz S, Davidson AE, Markley KM, Finley K, Fletcher CF, Ekker SC, Hackett PB, Horn S, Largaespada DA (2002) Mammalian germ-line transgenesis by transposition. Proc Natl Acad Sci USA 99:4495–4499
24. Echelard Y (1997) Genetic mosaicism in the generation of transgenic mice. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 233–225
25. Epinat JC, Arnould S, Chames P, Rochaix P, Desfontaines D, Puzin C, Patin A, Zanghellini A, Paques F, Lacroix E (2003) A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. Nucleic Acids Res 31:2952–2962
26. Garrick D, Fiering S, Martin DI, Whitelaw E (1998) Repeat-induced gene silencing in mammals. Nat Genet 18:56–59
27. Gaszner M, Felsenfeld G (2006) Insulators: exploiting transcriptional and epigenetic mechanisms. Nat Rev Genet 7:703–713
28. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325:433
29. Giraldo P, Rival-Gervier S, Houdebine LM, Montoliu L (2003) The potential benefits of insulators on heterogonous constructs in transgenic. Transgenic Res 12:751–755
30. Gitzinger M, Kemmer C, El-Baba MD, Weber W, Fussenegger M (2009) Controlling transgene expression in subcutaneous implants using a skin lotion containing the apple metabolite phloretin. Proc Natl Acad Sci USA 106:10638–10643
31. Gordon JW, Scangos GA, Plotkin DJ et al (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 77:7380–7384
32. Hammer RE, Pursel VG, Rexroad CE et al (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680–683
33. Han JY (2009) Germ cells and transgenic chicken. Comp Immunol Microbiol Infect Dis 32:61–80
34. Henry I, Forlani S, Vaillant S, Muschler J, Choulika A, Nicolas JF (1999) LagoZ and LagZ, 2 genes depleted of CpG dinucleotides, derived from the LacZ gene for the study of epigenetic control. C R Acad Sci III 322:1061–1070
35. Herold MJ, van den Brandt J, Seibler J, Reichardt HM (2008) Inducible and reversible gene silencing by stable integration of an shRNA-encoding lentivirus in transgenic rats. Proc Natl Acad Sci USA 105:18507–18512
36. Houdebine LM (2003) Animal transgenesis and cloning. Wiley, Chichester, 250 pp
37. Houdebine LM (2007) Transgenic animal models and target validation. Methods Mol Biol 360:163–202
38. Houdebine LM (2009) Methods to generate transgenic animals in ethics of science and technology assessment. Springer-Verlag, Heildelberg/Berlin/New York, pp 31–48
39. Houdebine LM, Attal J (1999) Internal ribosome entry sites (IRESs): reality and use. Transgenic Res 8:157–177
40. Jerchow B (2009) Transgenic technology meeting 2008 in Toronto, Canada: a meeting report. Transgenic Res. doi:10.1007/s11248-008-9238-8

41. Kalina J, Senigl F, Micáková A, Mucksová J, Blazkova J, Yan H, Poplstein M, Hejnar J, Trefil P (2007) Retrovirus-mediated in vitro gene transfer into chicken male germ line cells. Reproduction 134:445–453
42. Kapuscinski AR, Hayes KR, Li S, Dana G (2007) Environmental risk assessment of genetically modified organisms, vol. 3 Methodologies for transgenic fish. Series eds. Hallerman EM (Guest) and Schei PJ. CAB International, Oxford, pp 304
43. Kayser K (1997) Gene transfer in Drosophila melanogaster. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 133–137
44. Kuroiwa Y, Kasinathan P, Choi YJ, Naeem R, Tomizuka K, Sullivan EJ, Knott JG, Duteau A, Goldsby RA, Osborne BA, Ishida I, Robl JM (2002) Cloned transchromosomic calves producing human immunoglobulin. Nat Biotechnol 20: 889–894
45. Lavitrano M, Bacci ML, Forni M, Lazzereschi D, Di Stefano C, Fioretti D, Giancotti P, Marfe G, Pucci L, Renzi L, Wang H, Stoppacciaro A, Stassi G, Sargiacomo M, Sinibaldi P, Turchi V, Giovannoni R, Della Casa G, Seren E, Rossi G (2002) Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proc Natl Acad Sci USA 99:14230–14235
46. Lee YM, Jung JG, Kim JN, Park TS, Kim TM, Shin SS, Kang DK, Lim JM, Han JY (2006) A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. Biol Reprod 75:380–386
47. Lewin HA (2009) It's a Bull's Market. Research on domesticated livestock heralds new advances in evolutionary biology, animal breeding, and animal models for human diseases. Science 324:478–479
48. Li BC, Sun GB, Sun HC, Xu Q, Gao B, Zhou GY, Zhao WM, Wu XS, Bao WB, Yu F, Wang KH, Chen GH (2007) Efficient generation of transgenic chickens using the spermatogonial stem cells in vivo and ex vivo transfection. Reproduction 134: 445–453
49. Li P, Tong C, Mehrian-Shai R, Jia L, Wu N, Yan Y, Maxson RE, Schulze EN, Song H, Hsieh CL, Pera MF, Ying QL (2008) Germline competent embryonic stem cells derived from rat blastocysts. Cell 135:1299–1310
50. Li W, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S (2009) Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell 4:16–19
51. Liao J, Cui C, Chen S, Ren J, Chen J, Gao Y, Li H, Jia N, Cheng L, Xiao H, Xiao L (2008) Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell 4:11–15
52. Lillico SG, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous A, Elliot EA, Sang HM (2007) Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc Natl Acad Sci USA 104:1771–1776
53. Long X, Miano JM (2007) Remote control of gene expression. J Biol Chem 282:15941–15945
54. Lunyak VV, Prefontaine GG, Núñez E, Cramer T, Ju BG, Ohgi KA, Hutt K, Roy R, García-Díaz A, Zhu X, Yung Y, Montoliu L, Glass CK, Rosenfeld MG (2007) Developmentally regulated activation of a SINE B2 repeat as a domain boundary in organogenesis. Science 317:248–251
55. Lyssiotis CA, Foreman RK, Staerk J, Garcia M, Mathur D, Markoulaki S, Hanna J, Lairson LL, Charette BD, Bouchez LC, Bollong M, Kunick C, Brinker A, Cho CY, Schultz PG, Jaenisch R (2009) Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. Proc Natl Acad Sci USA 106:8912–8917
56. Maclean N (2003) Genetically modified fish and their effects on food quality and human health and nutrition. Trends Food Sci Technol 14:242–252
57. Malphettes L, Fussenegger M (2006) Improved transgene expression fine-tuning in mammalian cells using a novel transcription-translation network. J Biotechnol 124:732–746
58. Manzini S, Vargiolu A, Stehle IM, Bacci ML, Cerrito MG, Giovannoni R, Zannoni A, Bianco MR, Forni M, Donini P, Papa M, Lipps HJ, Lavitrano M (2006) Genetically modified pigs produced with a nonviral episomal vector. Proc Natl Acad Sci USA 103:17672–17677
59. Marsh-Armstrong N, Huang H, Berry DL, Brown DD (1999) Germ-line transmission of transgenes in Xenopus laevis. Proc Natl Acad Sci USA 96:14389–14393
60. McGrew MJ, Sherman A, Ellard FM, Lillico SG, Gilhooley HJ, Kingsman AJ, Mitrophanous KA, Sang H (2004) Efficient production of transgenic chickens using lentiviral vectors. EMBO Rep 5:728–733
61. Mersch B, Gepperth A, Suhai Sand Hotz-Wagenblatt A (2008) Automatic detection of exonic splicing enhancers (ESEs) using SVMs. BMC Bioinform 9:369. doi:10.1186/1471-2105-9-369
62. Metzger D, Chambon P (2001) Site- and time-specific gene targeting in the mouse. Methods 24:71–80
63. Mialhe E, Boulo V, Cadoret JP, Cedeno V, Rousseau C, Motte E, Gendreau S, Bachère E (1997) Gene transfer technology in marine invertebrates. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 151–155
64. Moisyadi S, Kaminski JM, Yanagimachi R (2009) Use of intracytoplasmic sperm injection (ICSI) to generate transgenic animals. Comp Immunol Microbiol Infect Dis 32:47–60
65. Moreira PN, Pozueta J, Pérez-Crespo M, Valdivieso F, Gutiérrez-Adán A, Montoliu L (2007) Improving the generation of genomic-type transgenic mice by ICSI. Transgenic Res 16:163–168
66. Müller M (2000) Increasing disease resistance in transgenic domestic. In: Toutant JP, Balazs E (eds) Molecular farming. Institute National de la Recherche Agronomique, Paris, pp 87–98
67. Murphey P, Yamazaki Y, McMahan CA, Walter CA, Yanagimachi R, McCarrey JR (2009) Epigenetic regulation of genetic integrity is reprogrammed during cloning. Proc Natl Acad Sci USA 106:4731–4735
68. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008)

Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26: 101–110
69. Notini AJ, Li R, Western PS, Sinclair AH, White SJ (2009) Rapid and reliable determination of transgene zygosity in mice by multiplex ligation-dependent probe amplification. Transgenic Res. 18:987–991
70. Ono E, Amagai K, Taharaguchi S, Tomioka Y, Yoshino S, Watanabe Y, Cherel P, Houdebine LM, Adam M, Eloit M, Inobe M, Uede T (2004) Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock. Proc Natl Acad Sci USA 101:16150–16155
71. Palmiter RD, Brinster RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611–615
72. Pera MF, Hasegawa K (2008) Simpler and safer cell reprogramming. Nat Biotechnol 26:59–60
73. Petersen B, Carnwath W, Niemann H (2009) The perspectives for porcine-to-human xenografts. Comp Immunol Microbiol Infect Dis 32:61–80
74. Pfeifer A (2006) Lentiviral transgenesis – a versatile tool for basic research and gene therapy. Curr Gene Ther 6:535–542
75. Porteus MF, Carroll D (2005) Gene targeting using zinc finger nucleases. Nat Biotechnol 23:967–973
76. Rao M (2007) Scalable human ES culture for therapeutic use: propagation, differentiation, genetic modification and regulatory issues. Gene Ther 15:82–88
77. Rehbinder E, Engelhard M, Hagen K, Jorgensen RB, Pardo-Avellaneda R, Schnieke A, Thiele F (2009) Pharming. Promises and risks of biopharmaceuticals derived from genetically modified plants and animals. Ethics of Science and Technology Assessment Vol 35. Springer-Verlag, Berlin/Heidelberg
78. Ritchie WA, Neil C, King T, Whitelaw CB (2007) Transgenic embryos and mice produced from low titre lentiviral vectors. Transgenic Res 16:661–664
79. RNAi: Multi-author review (2009) Nature 457:7228
80. Roberts RM, Smith GW, Bazer FW, Cibelli J, Seidel GE Jr, Bauman DE, Reynolds LP, Ireland JJ (2009) Farm animal research in crisis. Science 324:468–469
81. Robl JM, Wang Z, Kasinathan P, Kuroiwa Y (2007) Transgenic animal production and animal biotechnology. Theriogenology 67:127–133
82. Saito M, Iwawaki T, Taya C, Yonekawa H, Noda M, Inui Y, Mekada E, Kimata Y, Tsuru A, Kohno K (2001) Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. Nat Biotechnol 19:746–750
83. Salter DW, Smith EJ, Hughes SH, Wright SE, Crittenden LB (1987) Transgenic chickens: insertion of retroviral genes into the chicken germ line. Virology 157:236–240
84. Santiago Y, Chan E, Liu PQ et al (2008) Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. Proc Natl Acad Sci USA 105:5809–5814
85. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130–2133
86. Scott BB, Lois C (2005) Generation of tissue-specific transgenic birds with lentiviral vectors. Proc Natl Acad Sci USA 102:16443–16447
87. Shen W, Li L, Pan Q, Min L, Dong H, Deng J (2006) Efficient and simple production of transgenic mice and rabbits using the new DMSO-sperm mediated exogenous DNA transfer method. Mol Reprod Dev 73:589–594
88. Shinohara ET, Kaminski JM, Segal DJ, Pelczar P, Kolhe R, Ryan T, Coates CJ, Fraser MJ, Handler AM, Yanagimachi R, Moisyadi S (2007) Active integration: new strategies for transgenesis. Transgenic Res 16:333–339
89. Sioud M (2006) Innate sensing of self and non-self RNAs by toll-like receptors. Trends Mol Med 12:167–176
90. Sippel AE, Saueressig H, Hubler MC, Faust N, Bonifer C (1997) Insulation of transgenes from chromosomal position effects. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 257–266
91. Smith K, Spadafora C (2005) Sperm mediated gene transfer: applications and implications. BioEssays 27:551–562
92. Taboit-Dameron F, Malassagne B, Viglietta C, Puissant C, Leroux-Coyau M, Chéreau C, Attal J, Weill B, Houdebine LM (1999) Association of the 5'HS4 sequence of the chicken beta-globin locus control region with human EF1-alpha gene promoter induces ubiquitous and high expression of human CD55 and CD59 cDNAs in transgenic rabbits. Transgenic Res 8:223–235
93. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
94. Takehashi M, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Ogura A, Shinohara T (2007) Adenovirus-mediated gene delivery into mouse spermatogonial stem cells. Proc Natl Acad Sci USA 104:2596–2601
95. Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P (2000) Germline transformation of the silkworm Bombyx mori L. using a piggyBac transposon-derived vector. Nat Biotechnol 1:81–84
96. The Bovine Genome Sequencing and Analysis Consortium, Elsik CG, Tellam RL, Worley KC (2009) The genome sequence of taurine cattle: a window to ruminant biology and evolution. Science 324:522–528
97. Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly JS (2002) I-SceI meganuclease mediates highly efficient transgenesis in fish. Mech Dev 118:91–98
98. Thierry-Mieg D, Naert K, Bonnerot C (1997) Genetic transformation of *Caenorhabditis elegans*. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 137–151

99. Tiscornia G, Singer O, Ikawa M, Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci USA 100:1844–1848
100. Umland T, Montoliu L, Schütz G (1997) The use of yeast artificial chromosomes for transgenesis. In: Houdebine LM (ed) Transgenic animal generation and use. Harwood Academic Publishers, Amsterdam, pp 289–298
101. Van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessara TM, Swanberg SE, Delany ME, Etches RJ (2006) Germline transmission of genetically modified primordial germ cells. Nature 441:766–769
102. Van Keuren ML, Gavrilina GB, Filipiak WE, Zeidler MG, Saunders TL (2009) Generating transgenic mice from bacterial artificial chromosomes: transgenesis efficiency, integration and expression outcomes. Transgenic Res. doi 10.1007/s11248-009-9271-2
103. Van Reenen CG (2009) Assessing the welfare of transgenic farm animals. In Ethics of science and technology assessment. Springer-Verlag, Heildelberg/Berlin/New York, pp 119–143
104. Van Reenen CG, Meuwissen THE, Hopster H, Oldenbroek K, Kruip TH, Blokhuis HJ (2001) Transgenesis may affect farm animal welfare: a case for systemic risk assessment. J Anim Sci 79:1763–1769
105. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448:318–324
106. Wilson JH (2008) Knockout punches with a fistful of zinc fingers. Proc Natl Acad Sci USA 105:5653–5654
107. Woods IG, Schier AF (2008) Targeted mutagenesis in zebrafish. Nat Biotechnol 26:650–651
108. Yang SH, Agca Y, Cheng PH, Yang JJ, Agca C, Wing Sang Chan A (2007) Enhanced transgenesis by intracytoplasmic injection of envelope-free lentivirus. Genesis 45:177–183
109. Yong HY, Hao Y, Lai L, Li R, Murphy CN, Rieke A, Wax D, Samuel M, Prather RS (2006) Production of a transgenic piglet by a sperm injection technique in which no chemical or physical treatments were used for oocytes or sperm. Mol Reprod Dev 73:595–599
110. Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci USA 94:3789–3794

Books and Reviews

Honaramooz A, Megee S, Zeng W, Destrempes MM, Overton SA, Luo J, Galantino-Homer H, Modelski M, Chen F, Blash S, Melican DT, Gavin WG, Ayres S, Yang F, Wang PJ, Echelard Y, Dobrinski I (2008) Adeno-associated virus (AAV)–mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation. FASEB J 22:374–382

Niemann H, Kues W, Carnwath JW (2009) Methods to generate transgenic animals in ethics of science and technology assessment, vol. 34. Springer-Verlag, Heildelberg/Berlin/New York, pp 1–30

Glossary Terms

Index

B

D

E

H

J

K

L

M

N

Q

R

U

V

W

X

Y

Z